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Autoimmune thrombocytopenia related to chronic hepatitis C virus infection

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Since the identification of hepatitis C virus (HCV) in 1989 as a causative agent for a number of the extrahepatic alterations related to HCV infection an underlying immune mediated pathogenetic mechanism has been postulated. HCV-associated thrombocytopenia may be considered complex and multifactorial in origin, since different mechanisms have been implicated in its pathophysiology. With respect to autoimmune thrombocytopenia in chronic HCV infection, the detection of specific antibodies against platelet glycoproteins have been reported only in a few studies, but no systematic study has been carried out. We examined the clinical, laboratory, and virological characteristics of a case series of 10 patients with autoimmune thrombocytopenia (platelet count $<150.0 \times 10^9/L$) related to chronic HCV infection. Cases, six males and four females, aged 57.1 ± 12.6 years, presented high titers of antibodies against platelet glycoprotein (GP) IIb/IIIa, GP Ia/IIa, and/or GP Ib/IX, and no other mechanism involved in the pathogenesis of HCV-associated thrombocytopenia was identified. Furthermore, cases were not associated with particular HCV genotype. Complete platelet response was observed in two patients treated with pegylated interferon plus ribavirin, and partial platelet response was seen in two patients treated with anti-D Ig and one patient treated with corticosteroids. These findings indicate that an autoimmune mechanism may play a role in the pathogenesis of HCV-associated thrombocytopenia in a proportion of these patients.

Keywords: autoimmune thrombocytopenia, chronic hepatitis C, HCV, platelets

Introduction

Since the identification of hepatitis C virus (HCV) in 1989,¹ a wide variety of extrahepatic alterations have been reported to be related to HCV infection, and for

a number of these conditions the underlying pathogenetic mechanism seems to be immune mediated.²⁻⁴ Among all the HCV-associated extrahepatic manifestations there appears to be a stronger degree of association of mixed cryoglobulinemia with HCV infection. Other extrahepatic diseases such as type 2 diabetes mellitus, malignant lymphoproliferative disorders and thrombocytopenia appear to have a milder degree of association with HCV infection.⁵⁻⁷

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HCV-associated thrombocytopenia may be considered complex and multifactorial in origin, since different mechanisms were reported to be implicated in its pathophysiology.⁸ Among the proposed major mechanisms are: sequestration of platelets in the enlarged spleen secondary to portal hypertension (hypersplenism), dysregulation of the host immune system triggering the production of autoantibodies against platelet glycoproteins (GP), inadequate production of thrombopoietin in advanced stage liver disease, and interaction of HCV with platelets and megakaryocytes.⁹

With respect to autoimmune thrombocytopenia in chronic HCV infection, most reports have demonstrated elevated titers of platelet-associated immunoglobulin G (PAIgG) in platelet samples separated from whole blood of patients with HCV infection.¹⁰⁻¹³ Nevertheless, these antibodies appear to represent non-specific IgG adsorbed to platelets and are commonly detected in patients with thrombocytopenia related to non-autoimmune process.¹⁴ There are several types of assay to detect antibodies against various autoantigens located on platelet GP complexes such as GPIIb/IIIa, GPIb/IX, and GPIa/IIa, but these specific assays are done primarily for research interest and are unavailable in most clinical laboratories. Detection of specific antibodies against platelet GP were reported only in few studies,¹⁵⁻¹⁷ however, no concomitant investigation of additional pathogenic mechanisms of thrombocytopenia was carried out. The aim of this study was to examine the clinical, laboratory and virological characteristics as well as the response to treatment and subsequent clinical course of a series of cases with autoimmune thrombocytopenia related to chronic HCV infection.

Patients and methods

Setting and study design

This prospective study was carried out at the Hematology and Liver Diseases Clinics of the Gaffrée & Guinle University Hospital, attending to the population living in the State of Rio de Janeiro, Brazil. This research was approved by the Ethical Committee of the institution on August 30, 2004 and conforms to the ethical guidelines of the Declaration of Helsinki. All patients gave informed consent before entry into the study.

Patients

Patients were referred for hematological evaluation between August 2004 and August 2007 because of chronic thrombocytopenia (platelet count $<150 \times 10^9/L$ of more than 6 months' duration) detected

during evaluation for HCV-associated chronic hepatic disease. The diagnosis of chronic HCV infection was made on the basis of the presence of antibodies to hepatitis C virus (anti-HCV) and HCV-RNA in serum samples of patients with infection of more than 6 months' duration. Anti-HCV antibodies were detected by using a commercial kit (ORTHO HCV 3-0 ELISA Test System with Enhanced SAVE, Ortho Clinical Diagnostics, Raritan, NJ, USA) with estimated sensitivity and specificity in patients with chronic liver diseases of 97.2 and 100%, respectively,¹⁸ and viremia was detected by reverse transcriptase-nested polymerase chain reaction (RT-nested PCR) for the 5' non-coding region (5'NCR) by using an in-house assay.

Autoimmune thrombocytopenia associated with chronic HCV infection was defined as follows: (i) isolated thrombocytopenia, (ii) HCV infection diagnosed before or concurrent with the development of thrombocytopenia, (iii) a positive test for the detection of antibodies to platelet GP, and (iv) failure to document other mechanism involved in the process leading to thrombocytopenia.

Patients were excluded from the study if they met one or more of the following criteria: (i) patients who had received or were under antiviral therapy, (ii) positivity for hepatitis B surface antigen, (iii) positivity for antibodies to human immunodeficiency virus (HIV-1/2), (iv) presence of any endoscopic and/or echographic signs of portal hypertension syndrome, such as esophageal varices, ascites and/or splenomegaly, (v) presence of other hematologic cytopenias, (vi) presence of myelodysplastic features on bone marrow, (vii) evidence of consumption coagulopathy and (viii) pregnancy. None of the enrolled patients had received therapy with drugs implicated with the development of thrombocytopenia such as non-steroidal anti-inflammatory drugs, immunosuppressive and myelotoxic agents, for at least one year before the development of thrombocytopenia. No other known causes of thrombocytopenia such as systemic lupus erythematosus and rheumatoid arthritis, or disorders associated with thrombocytopenia, hemolysis, and fragmented red blood cells were found in the patients enrolled in this study.

All patients had at least hematological follow-up for 1 year after any therapy was stopped. To assess platelet count responses in patients receiving therapy, we followed the criteria previously described for adult patients with immune thrombocytopenic purpura (ITP). All measurements obtained 1 month or longer

after the completion of therapy were considered to define the kind of platelet response:¹⁹ (i) complete response was regarded as achievement and maintenance of a platelet count equal to or greater than $150 \times 10^9/L$, (ii) partial response was regarded as an increase in platelet count to a minimum of $50 \times 10^9/L$ but less than $150 \times 10^9/L$, including patients who relapsed after initially achieving a platelet count equal to or greater than $150 \times 10^9/L$, and (iii) no response was defined as a failure to achieve an increase in platelet count to a minimum of $50 \times 10^9/L$.

Methods

Detection of specific antiplatelet antibodies

A commercially available solid phase enzyme-linked immunosorbent assay (ELISA) was used for the detection of serum antibodies directed against epitopes on various platelet GP (Pak 12, GTI, Waukesha, WI, USA). In accordance with the manufacturer's directions, the test results were considered valid when the mean OD value for the positive control was equal to or greater than eight times the mean value of the negative controls. A test result was considered to be positive when showing OD values equal to or greater than twice the value obtained for the mean value of the negative controls for the corresponding antigen.

Quantitative determination of thrombopoietin concentrations

Thrombopoietin (TPO) levels were measured in serum samples by using a commercial quantitative sandwich enzyme immunoassay (Quantikine, Human TPO Immunoassay, R & D Systems, Minneapolis, MN, USA). According to the manufacturer's instructions, this assay has typically a minimum detectable dose (MDD) of less than 18.5 pg/ml (mean MDD = 7.45 pg/ml), recognizes recombinant and natural human TPO, and presents no significant cross-reactivity with a variety of other cytokines. In our laboratory the intra-assay and inter-assay coefficient of variation were 10.79 and 9.54%, respectively. A total of 10 healthy subjects formed the control group, four males and six females, aged 51.2 ± 12.1 years (mean \pm standard deviation).

Detection of HCV-RNA

Paired samples of serum and peripheral blood platelets were collected from all patients on an outpatient basis. Viral RNA was extracted from paired serum and platelets with QIAamp Viral RNA and RNeasy Mini Kit (QIAGEN, Hilden, Germany), respectively, according to the manufacturer's protocols. Platelet pellets were separated from 500 μ l of

platelet-rich plasma obtained after appropriate centrifugation of whole blood collected into acid-citrate-dextrose 3.2% tubes. Platelet pellets were washed seven times with Tyrode's solution (137 mM NaCl, 0.42 mM NaH₂PO₄, 5 mM glucose, 0.35% albumin, pH 7.35) before viral RNA extraction. The solution used for the final wash was also analyzed for the presence of HCV-RNA and used as negative control. The purity of platelet pellet samples was ensured by cytomorphological analysis (May-Grünwald-Giemsa staining) and counting (Neubauer chamber and automated cell counter) assays. For the detection of HCV genomic sequences in serum, peripheral blood platelet and final platelet washing solution samples, RT-nested PCR using primers for the 5'NCR of the HCV genome were performed, as described previously.²⁰ Briefly, synthesis of complementary DNA and PCR amplification were performed by using SuperScriptTM One-Step RT-PCR with Platinum[®] Taq (Life Technologies, Invitrogen, Carlsbad, CA, USA). A second step of PCR amplification was performed and PCR products (10 μ l) were fractionated on 1.5% agarose gel electrophoresis and stained by ethidium bromide to visualize a fragment of expected length of 278 base pairs.

The lower limit of HCV-RNA detection of this RT-PCR assay was estimated as 50 IU/ml by testing serum dilutions in DNase/RNase free distilled water at serial ten- and two-fold of a sample obtained from an HCV-infected patient with a viral load previously determined as 20,000 IU/ml. All PCR assays were carried out according to the recommendations of Kwok and Higuchi.²¹ In addition, negative controls (anti-HCV/HCV-RNA negative human serum and diethyl pyrocarbonate-treated distilled water) and positive controls (human HCV-infected sera) were used in each PCR step to assure the specificity of the assay.

Quantification of HCV-RNA load

HCV-RNA was quantified in plasma samples according to the manufacturer's recommendations by using the Amplicor HCV Monitor[®] Test, version 2.0 (Roche Diagnostics, Mannheim, Germany). This assay has a range of detection between 600 and 850,000 IU/ml, as described elsewhere.²² Samples with HCV-RNA over the limit of 850,000 IU/L were diluted to determine its appropriate levels.

Genotyping

Determination of HCV genotypes was performed in serum samples by using direct nucleotide sequencing

of the PCR products from part of HCV core gene, as described previously.²³ All analyzed products were sequenced directly in both directions by using automatic sequencer (ABI Prism 3100 Genetic Analyser, Applied Biosystems, CA, USA). The genotypes of obtained sequences were determined by phylogenetic analysis together with the main subtype reference sequences obtained from GenBank database²⁴ by using MEGA 3.1 software.²⁵

Liver biopsy

A liver biopsy was performed before starting therapy and closest to the time of collection of blood samples. The liver histology was assessed using the METAVIR scoring system which describes well-defined categories of histological activity (A0 = no activity, A1 = mild activity, A2 = moderate activity, and A3 = severe activity) and fibrosis (F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis with rare septa, and F4 = cirrhosis).²⁶

Bone marrow examination

Sternum bone marrow aspiration was carried out in all HCV-associated thrombocytopenia patients using an appropriate needle. Marrow films were stained by May-Grünwald-Giemsa stain before cytomorphological examination.

Other laboratory tests

Blood samples were collected in different tubes containing either ethylenediaminetetraacetate (EDTA) or sodium citrate using two or more determinations of platelet count over a period of 6 months. Platelet counts were carried out with an automated cell counter (ABX PENTRA 120, ABX Diagnostics, Montpellier, France). Prothrombin time (PT) was obtained in citrated plasma and was expressed as international normalized ratio (INR). Serum alanine aminotransferase (ALT), and immunoglobulins (IgG, IgA, and IgM) were measured with an automatic analyzer (TARGA BT-3000 Plus, Biotecnica Instruments SpA, Rome, Italy). Adults reference values for these parameters were as follows (Wiener lab, Rosario, Argentina): ALT, male up to 41 U/L and female up to 31 U/L; IgG, 650 to 1600 mg/dl; IgA, 70 to 400 mg/dl; and IgM, 60 to 350 mg/dl. For the detection of cryoglobulins, venous blood was collected, allowed to clot, and centrifuged at 37°C. Serum samples were then incubated at 4°C for 72 h, and inspected daily for the presence of cryoprecipitation. Rheumatoid factor (RF) was measured by nephelometry using the BN2 Nephelometer (Dade Behring, Marburg, Germany). RF titers greater than 20 IU/ml were considered positive according to the

manufacturer's instructions (N Latex RF System, Dade Behring, Marburg, Germany). Quantitative determination of anti-cardiolipin antibodies (ACA) were carried out by using ETI-Cardiolipin ELISA assays, according to the manufacturer's recommendations (DiaSorin, Saluggia, Italy). The results were expressed as GPL/ml, APL/ml, and MPL/ml units for IgG, IgA, and IgM isotypes of ACA antibodies, respectively. Values above 14 GPL/ml, 15 APL/ml and 10 MPL/ml were regarded as positive. Anti-mitochondrial, anti-smooth muscle (ASMA) and anti-liver-kidney microsomal (LKM) antibodies were evaluated by indirect immunofluorescence assay on rat liver-kidney-stomach substrate slides (Autoantibody Screen, DTS, Johannesburg, South Africa). Antinuclear antibodies were detected in serum by indirect immunofluorescence assay on Hep-2 cell line as substrate, and several different patterns of nuclear fluorescence were reported as appropriate (ANA Hep-2, Bion, Des Plaines, IL, USA). Any fluorescence observed at a 1:40 dilution (or greater) based on a 1+ (weak reaction) to 4+ (strong reaction) scale was regarded as a positive test. The following investigations were also performed before any therapy: abdominal ultrasonography and esophagogastroduodenoscopy with biopsy specimens taken from antral mucosa to be subjected to rapid urease test and Giemsa staining in order to exclude *Helicobacter pylori* infection.

Statistical analysis

Data are expressed as mean \pm standard deviation, and frequencies. For continuous variables with normal distribution, homoscedasticity was tested by the Levene's test and we used the unpaired Student's *t* test to compare means, and Pearson's *r* coefficient for correlation analysis. A *p* value less than 0.05 was considered to be statistically significant. Statistical analysis was performed using MedCalc for Windows, version 7.6.0.0 (MedCalc Software, Mariakerke, Belgium).

Results

Demographic, clinical and laboratory data

A total of 40 patients with chronic thrombocytopenia related to chronic HCV infection, 16 males and 24 females, aged 55.9 ± 9.1 years, were studied. Ten of them (six males and four females, aged 57.1 ± 12.6 years) were diagnosed as having autoimmune thrombocytopenia associated with chronic HCV infection. The demographic, clinical and laboratory features of our case series of ten patients with HCV-related autoimmune thrombocytopenia are summarized in Table 1. Exposure to blood product transfusion in the early 1980s for unrelated conditions and multiple

Table 1 Patients demographics, baseline clinical and laboratory features

Cases	Sex	Age (years)	Risk factor	Hemorrhagic manifestations	Platelet count ($\times 10^9/L$)	Thrombocytopenia duration (months)	INR	ALT* (U/ml)	Viral genotype [†]	Viral load [‡] (IU/ml)	Thrombopoietin [§] (pg/ml)	Liver histology	Antiplatelet antibodies positivity [¶]
1	M	36	Transfusion	Epistaxis	17	96	1.06	80.9	1b	9658	110.76	Not done	Anti-GPIIb/IIIa Anti-GPIa/IIa
2	F	65	Multiple surgical procedures	Absent	85	18	1.35	80.5	1b	767,682	60.60	A1, F2	Anti-GPIIb/IIIa Anti-GPIa/IIa
3	F	69	Transfusion	Uterine bleeding	67	12	1.60	275.0	1b	162,564	95.25	Not done	Anti-GPIIb/IIIa Anti-GPIa/IIa
4	F	60	Transfusion	Ecchymoses, gingival bleeding	59	72	1.36	84.0	3b	3970	82.41	A1, F2	Anti-GPIb/IX Anti-GPIIb/IIIa Anti-GPIa/IIa
5	M	67	Multiple surgical procedures	Absent	135	7	1.00	97.9	1b	1,960,710	69.39	Not done	Anti-GPIb/IX Anti-GPIIb/IIIa Anti-GPIa/IIa
6	M	48	IV drug abuse; Multiple surgical procedures	Absent	129	5	1.13	211.0	1a	973,135	232.43	A2, F2	Anti-GPIb/IX Anti-GPIIb/IIIa Anti-GPIa/IIa
7	M	44	Multiple surgical procedures	Absent	75	11	1.06	70.9	1a	32,792	116.35	Not done	Anti-GPIb/IX Anti-GPIa/IIa
8	M	60	Multiple surgical procedures	Absent	127	12	1.07	43	1b	159,821	239.27	Not done	Anti-GPIb/IX Anti-GPIa/IIa
9	M	75	Multiple surgical procedures	Absent	118	16	1.00	18	2b	117,800	129.27	A1, F2	Anti-GPIa/IIa
10	F	47	Transfusion; Multiple surgical procedures	Absent	66	4	1.38	233	1b	398,512	98.12	A2, F2	Anti-GPIIb/IIIa Anti-GPIa/IIa

*Normal values: male up to 41 U/L and female up to 31 U/L.

[†]Direct nucleotide sequencing of the PCR products from part of HCV core gene.[‡]HCV-Amplicor Monitor 2.0 (Roche Diagnostics, Mannheim, Germany). Lower detection limit: 600 IU/ml.[§]Quantitative sandwich enzyme immunoassay technique (Quantikine Human Tpo Immunoassay, R&D Systems, Minneapolis, MN, USA). Minimum detectable dose less than 18.5 pg/ml.^{||}According to the METAVIR Cooperative Study Group (Bedossa & Poinard, 1996).[¶]Solid phase enzyme-linked immunosorbent assay (Pak 12, GTI, Waukesha, WI, USA).

M, male; F, female; INR, international normalized ratio; ALT, alanine aminotransferase.

surgical procedures were reported to be the probable sources of HCV infection in most cases of the series. At presentation, only two patients (cases 1 and 4) had mild hemorrhagic manifestations such as epistaxis, gingival bleeding or ecchymoses, and patient 3 had experienced two recent episodes of abnormal uterine bleeding.

At diagnosis, laboratory data showed a mean platelet count $85.1 \pm 36.2 \times 10^9/L$ (range 17.0 – $135.0 \times 10^9/L$). The mean duration of thrombocytopenia was 25.3 ± 31.7 months (range 4.0 – 96.0 months). PT was mildly (cases 2, 4 and 10) to moderately (case 3) prolonged, the remaining six patients having normal PT as expressed as INR. All patients, with the exception of case 9, had elevated ALT levels (119.4 ± 87.3 U/L, range 18.0 – 275.0 U/L), and three of them had at least ALT levels 1.5 times the upper limit of normal range. Mean serum IgG, IgA, and IgM levels were 1717.7 ± 452.9 mg/dl (range 1029.0 – 2260.0 mg/dl), 339.1 ± 113.5 mg/dl (range 189.0 – 603.0 mg/dl), and 222.9 ± 131.4 mg/dl (range 85.6 – 470.0 mg/dl), respectively. Serum IgG, IgA and IgM levels were slightly increased in 7/70% (cases 1, 3, 4, 5, 7, 8 and 10), 2/20% (cases 5 and 6), and 3/30% patients (cases 2, 4 and 6), respectively. There was no significant correlation between platelet counts and serum IgG ($r = -0.3610$, $p = 0.3054$), IgA ($r = 0.5527$, $p = 0.1171$), and IgM ($r = -0.1642$, $p = 0.6503$) levels. Mean TPO concentrations obtained in healthy subjects who served as control group were 211.2 ± 57.5 pg/ml (range 126.5 – 334.6 pg/ml). Serum TPO levels were significantly lower in our series of cases (123.8 ± 62.8 pg/ml, range 60.6 – 239.2 pg/ml) than in the controls ($t = 3.260$, $p = 0.0044$). Among these cases correlation analysis showed that platelet counts and TPO concentrations were not related ($r = 0.4666$, $p = 0.1741$). No HCV-RNA was detected in peripheral blood platelets isolated from patients of our series. The mean viral load measured was $458,664.4 \pm 623,861.0$ IU/ml (range 3970.0 – $1,960,710.0$ IU/ml). Mean log HCV viral load found was 5.17 ± 0.83 (range 4.00 – 6.89). No correlation between platelet count and viral load were found ($r = 0.5886$, $p = 0.0734$). Genotype determination demonstrated that HCV genotype 1b was the most prevalent among patients of our series (cases 1, 2, 3, 5, 8 and 10), followed by genotype 1a (cases 6 and 7). All patients with available data on liver biopsy (cases 2, 4, 6, 9 and 10) had F2 score according to METAVIR scoring system. Except for cases 2, 4 and 5, who presented an increased number of mature megakaryocytes, bone marrow aspiration revealed a normal

number of mature megakaryocytes without myelodysplastic features in the remaining patients of our series.

All 10 patients with autoimmune thrombocytopenia related to chronic HCV infection had high titers of antibodies against GP IIb/IIIa, GP Ia/IIa and/or GP Ib/IX. The remaining patients ($n = 30$) with HCV-associated thrombocytopenia of the present study, who had HCV-RNA detected in platelets, showed normal titers of antiplatelet antibodies. In addition, a total of 11 patients with other conditions were also studied for the detection of specific antiplatelet antibodies and presented normal titers of antiplatelet antibodies: patients with HBV-associated chronic hepatic disease ($n = 4$) with a normal platelet count (equal to or greater than $150 \times 10^9/L$); and patients with HCV-associated chronic hepatic disease ($n = 7$) with a normal platelet count. Patients with ITP ($n = 3$), a condition known to be associated with antiplatelet antibodies positivity²⁷ also presented high titers of antibodies against GP IIb/IIIa, GP Ia/IIa and/or GP Ib/IX.

All ten patients of the series had evidence of other immunological abnormalities in serum samples. Cryoglobulins (cryocrit 2%) were detected only in one patient (case 1). Slightly high titers of RF were found in two patients (case 6 and 7). ACA (IgM) were detected in three patients: case 2 (14.7 MPL/ml), case 4 (16.7 MPL/ml), and case 7 (13.5 MPL/ml). Slightly elevated titers of ASMA (1:40) were detected in case 1. Speckled pattern ANA were detected in patients 1 (1:80), 3 (1:320), 5 (1:80), 8 (1:80), and 13 (1:80). Case 9 had nucleolar pattern ANA (1:80).

Platelet count response

With the exception of cases 5, 7, 8 and 9, all patients of the series received some kind of therapeutic intervention. Regarding platelet count response, case 1 was considered to have no response after receiving therapy with two courses of corticosteroids (dexamethasone $20 \text{ mg/m}^2/\text{day}$ for 4 days, repeated every 28 days) and further two courses of intravenous immunoglobulins (IVIg) (400 mg/kg/day for 3 days, repeated every 28 days). Subsequently, he achieved a partial response (platelet count $99.0 \times 10^9/L$) after two courses of anti-D immune globulin ($50 \text{ } \mu\text{g/kg/day}$ for 2 days, repeated every 28 days), which persisted for 6 months when his platelet count decreased to $24.0 \times 10^9/L$. This patient was lost to follow-up after this time (June 2006) because of depression. Cases 2 and 6 were treated with pegylated interferon (IFN)-alfa 2b, administered subcutaneously at a dose of 1.0 – $1.5 \text{ } \mu\text{g/kg}$ weekly plus full-dose ribavirin,

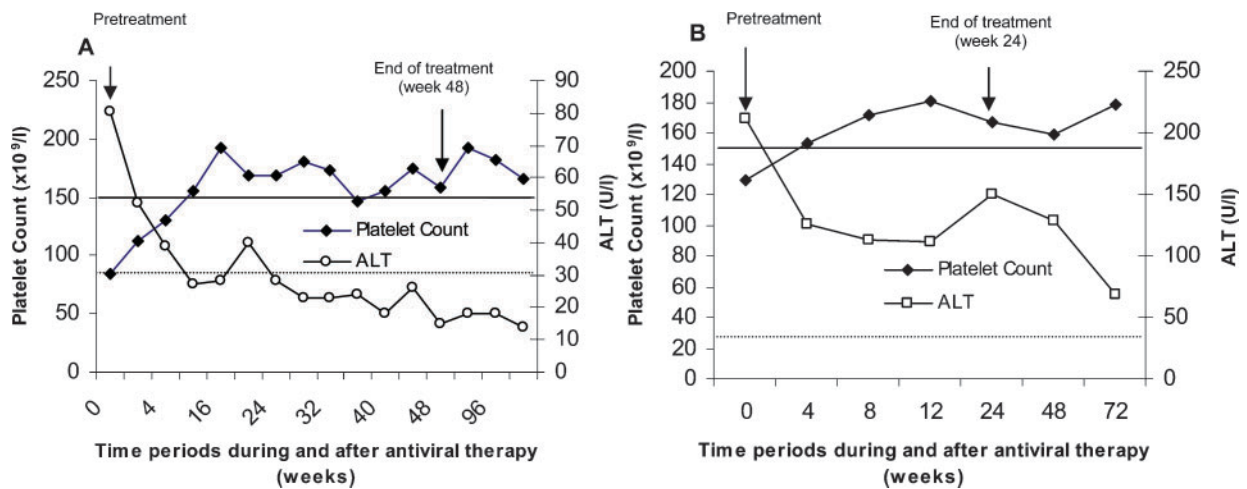


Figure 1 Platelet and biochemical responses to antiviral therapy (pegylated interferon- α 2b plus ribavirin) of two cases of autoimmune thrombocytopenia associated with chronic hepatitis C virus infection. (A) Case 2 achieved sustained platelet, biochemical, and virological responses (24 weeks after the end of antiviral therapy). (B) Case 6 achieved sustained platelet response, in spite of lack of biochemical and virological responses. In both cases, platelet counts remained in normal range (greater than $150 \times 10^9/L$) for the period of analysis after the end of treatment. Platelet counts greater than $150 \times 10^9/L$ are indicated by a solid line. The upper limit of normal levels (31 U/L) of ALT is indicated by a dotted line. ALT = alanine aminotransferase

administered orally at a dose of 1000 mg/day in divided doses (500 mg twice daily) during 48 weeks. Both patients had complete platelet response (platelet counts equal to or greater than $150.0 \times 10^9/L$), but only patient 2 achieved biochemical and sustained virological response. Twenty four weeks after the end of antiviral therapy both patients tested negative for antiplatelet antibodies, and case 6 had a significant decrease in viral load (1600.00 IU/ml; log 3.2). Figure 1A and B shows the platelet responses of case 2 and case 6 respectively during and after antiviral therapy. Cases 3 and 4 were treated with two courses of anti-D immune globulin at a dose of $50 \mu\text{g/kg/day}$ for 2 days, repeated every 28 days: case 3 had no response, and case 4 achieved partial response (platelet count $100.0 \times 10^9/L$) which lasted 6 months when her platelet count decreased to $64.0 \times 10^9/L$. Case 5 did not require any therapy because he was without hemorrhagic manifestations and had mild thrombocytopenia. In addition, case 5 is currently subjected to diagnostic evaluation of an intrathoracic solid mass detected after carrying out a chest radiography ordered for another reason. Case 7 had no signs of bleeding and is lost to follow-up since November 2005 when reported psychiatric symptoms. His platelet count at that time was $52.0 \times 10^9/L$. Case 8 was asymptomatic and is lost to follow-up since January 2005 (platelet count $100.0 \times 10^9/L$) while being on a waiting list for receiving antiviral therapy. Case 9 was newly diagnosed as having prostatic adenocarcinoma and is undergoing staging

evaluation before starting any therapy. His last platelet count (January 2008) was $135.0 \times 10^9/L$. Case 10 had received therapy with three courses of dexamethasone $20 \text{ mg/m}^2/\text{day}$ for 4 days, repeated every 28 days (from August 8, 2007 to December 21, 2007), and her platelet count rapidly increased to $123.0 \times 10^9/L$ after the first course of corticosteroid. During her last visit with the hematologist at our clinic (March 2008), her platelet count was $104.0 \times 10^9/L$ (partial response) and specific antiplatelet antibodies were not detected at this time. No significant changes in quantitative determination of HCV-RNA and ALT levels were observed by comparing values before and after therapy with corticosteroids. Currently, she is also on a waiting list to receive antiviral therapy. None of the patients of our series required platelet transfusion for controlling bleeding secondary to thrombocytopenia.

Discussion

Thrombocytopenia is one of the most frequent hematological alteration in patients with chronic liver disease.²⁸ According to population-based studies, there appears to be a strong association of HCV infection with thrombocytopenia.^{29,30} In the present study, 10 out of 40 patients with HCV-associated thrombocytopenia showed evidence of immune-mediated platelet destruction, based on the presence of detectable circulating specific antiplatelet antibodies. Moreover, among features supporting the concept that an autoantibody-mediated process was

involved in these cases are: (i) the HCV infection was diagnosed before or concurrent with the development of thrombocytopenia; (ii) no other mechanism involved in the process leading to HCV-associated thrombocytopenia was identified; and (iii) a proportion of these patients showed platelet response after receiving conventional ITP treatment such as corticosteroids and anti-D Ig.

The assay used in our work is capable of detecting serum antibodies directed against epitopes on various platelet GP (GPIIb/IIIa, GPIb/IX, and GPIa/IIa). However, according to the manufacturer's directions, the assay has some limitations: excess immune complexes or other immunoglobulin aggregates in the patient's serum samples may cause an increased non-specific binding; on the other hand, low titers or low avidity antibodies may not be detected. In spite of the frequent finding of thrombocytopenia and antiplatelet autoantibodies in patients with chronic HCV infection, it has been postulated that detection of these autoantibodies per se is not sufficient to establish the diagnosis of autoimmune thrombocytopenia since these autoantibodies may be detected in non-thrombocytopenic patients with HCV infection.¹⁷ A way to discriminate impaired platelet production from accelerated platelet destruction is to quantify reticulated platelets in peripheral blood by flow cytometry,³¹ but this test is not available at our laboratory. For these reasons, the results obtained herein were analyzed in conjunction with other clinical and serological findings to reach some conclusions.

In several instances multiple mechanisms involved in the thrombocytopenia related to chronic HCV infection appear to be present simultaneously in the same patient, representing a diagnostic and therapeutic challenge to practising haematologists. Historically, thrombocytopenia was mainly attributed to an increased sequestration and subsequent destruction of platelets in a pathologically enlarged spleen.³² This condition was excluded in our case series by appropriate clinical and echographic investigations. Advanced hepatic fibrosis causing reduced production of thrombopoietin, which is mainly produced by the liver, was found to play a central role in the pathogenesis of thrombocytopenia in patients with chronic viral hepatitis.³³ This mechanism seems not to be the cause of the thrombocytopenia since five patients of our series with available data on liver biopsy did not have advanced fibrosis or cirrhosis. In addition, our data showed no correlation between platelet counts and TPO concentrations.

Previous studies have indicated the presence of HCV in cells other than hepatocytes, such as peripheral blood platelets.³⁴⁻³⁶ Recently, we have demonstrated a statistically significant association between the detection of HCV in platelets and thrombocytopenia, suggesting that the binding of HCV to platelets is involved in the process that, at least in part, leads to thrombocytopenia.⁹ However, no HCV-RNA was detected in platelets from our ten cases, so that this possibility was also ruled out. Finally, another study suggested that HCV might cause direct bone marrow suppression and consequent decreased thrombopoiesis,³⁷ but this mechanism was considered as unlikely alternative since a normal or increased number of mature megakaryocytes, without myelodysplastic features on bone marrow examination, was observed in our patients. No other mechanism involved in the process leading to HCV-associated thrombocytopenia was identified in our cases.

Chronic HCV infection has been implicated in the development of various extrahepatic hematologic and immunologic alterations. The coincidental association between chronic HCV infection and immune thrombocytopenia was considered to be extremely unlikely by Pockros and colleagues.¹⁵ A recent study on the potential role of platelet destruction mediated by autoantibodies in a cohort of 72 thrombocytopenic patients with liver cirrhosis, including 37 patients with HCV infection, suggested that in those patients with liver cirrhosis the autoantibody-mediated platelet destruction was found to contribute, at least in part, to thrombocytopenia.¹⁶ Both studies, however, did not exclude the possibility of other proposed mechanisms such as splenic sequestration, insufficient production of thrombopoietin, and interaction of HCV with peripheral blood platelets.

The mechanisms whereby HCV would trigger autoimmune diseases are uncertain. The direct infection of B-cells and chronic stimulus on the immune system by HCV appear to play a role in the pathophysiology of the proliferation of B-cells in patients with HCV infection.^{38,39} A prospective case-control study aimed at determining the prevalence of immunological abnormalities in a series of 61 treatment-naïve patients with chronic HCV infection reported an overall detection rate of anti-tissue autoantibodies (FAN, ASMA, LKM, antithyroglobulin, antithyroperoxidase) of 41%.⁴⁰ Recently, Ramos-Casals and colleagues retrospectively analyzed the clinical characteristics of 35 patients with chronic HCV infection presenting severe autoimmune cytopenias not related to antiviral therapy. In 19

(68%) of the cases, including six patients with severe HCV-related thrombocytopenia, the investigators detected immunological markers such as hypocomplementemia, cryoglobulins, ANA and RF.⁴¹ In agreement with these studies, we found evidence of other immunological abnormalities such as cryoglobulins, RF, ACA, and ANA in almost all patients of our series.

Treatment of patients with HCV-associated thrombocytopenia still remains undefined. Several reports described transient partial or complete platelet response of variable duration after receiving conventional therapy for ITP such as prednisone and/or IVIg.^{15,42,43} In agreement with these studies, we observed variable platelet response to corticosteroids and/or IVIg in our series: case 1 presented no response and case 10 presented a good partial response. In addition, we also assessed the platelet responses in three patients treated with anti-D immune globulin: cases 1 and 4 had partial responses, and case 3 had no response. A study reported higher mean HCV-RNA load among patients with HCV-associated chronic liver disease treated with long-term (>3 months) course of corticosteroid.⁴⁴ On the other hand, no evidence of liver damage among patients with autoimmune thrombocytopenia associated with hepatitis C virus infection treated with short-term course of prednisone was found.⁴² We also observed no significant changes in ALT levels and HCV-RNA load in a patient (case 10) treated with short-term course of corticosteroids.

Currently, standard antiviral therapy for chronic HCV infection is based on combination of IFN- α or pegylated IFN with ribavirin, and a myriad of side effects of both interferons have been described, including the induction of autoantibodies and autoimmune disorders.^{45,46} A number of studies have demonstrated the development of immune thrombocytopenia in patients receiving antiviral therapy.^{47–51} On the other hand, previous reports have demonstrated that IFN therapy could be an option to treat patients with thrombocytopenia associated with chronic HCV infection. Platelet response to antiviral therapy was considered variable, although cases with long-term (>1 year) platelet responses have been reported.^{52–56} A significant increase of the platelet count was reported among patients with chronic hepatitis C who showed HCV eradication in serum samples 6 months after the cessation of therapy with IFN- α .⁵⁷ Our data on cases 2 and 6, taken together with those aforementioned, appear to support the idea that IFN therapy is not necessarily

contraindicated in patients with HCV-associated thrombocytopenia, including cases with an autoimmune underlying mechanism.

In conclusion, we described in the present series 10 patients with autoimmune-mediated thrombocytopenia, in whom no other proposed mechanism involved in the process leading to HCV-associated thrombocytopenia was identified. Taken together, these findings indicate that an autoimmune mechanism may play a role in the pathogenesis of HCV-associated thrombocytopenia in a proportion of the patients. A routine investigation concerning the possible pathogenic mechanisms of thrombocytopenia should be recommended in all patients with chronic HCV infection for the therapeutic approach may differ. The exact treatment option remains to be better defined in larger, controlled studies before practice guidelines or consensus recommendations can be provided.

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