Mixed cryoglobulinemia (MC) and glomerulonephritis are the most important extrahepatic manifestations of chronic hepatitis C virus (HCV) infection. MC is a non-neoplastic B cell lymphoproliferative process induced by HCV in an antigen-driven mechanism. The clinical expression of cryoglobulinemia varies from an indolent course to the development of systemic vasculitis. Glomerulonephritis is predominantly associated with MC, and almost always takes the form of membranoproliferative glomerulonephritis. The renal manifestations may range from isolated proteinuria to overt nephritic or nephrotic syndrome with variable progression towards chronic renal insufficiency. The treatment of these virus-related diseases must be individualized on the basis of the severity of clinical symptoms. Antiviral therapy with interferon alpha and ribavirin (the currently recommended treatment of HCV infection) may be successful in patients with mild-to-moderate disease, but sustained responses are uncommon. In case of severe and rapidly progressive disease, although it is capable of suppressing viremia and cryoglobulinemia, antiviral therapy is not fully effective in controlling the inflammatory and self-perpetuating reaction consequent to the deposition of cryoglobulins in the glomeruli and vessel walls. In such cases, a short course of steroids and cytotoxic drugs (with or without plasmapheresis) may be needed to improve the vascular manifestations and decrease the production of cryoglobulins. Once the acute disease flare has been controlled, antiviral therapy may be administered to eradicate HCV, the causative agent of the cryoglobulinemic syndrome. In patients in whom antiviral therapy is ineffective, contraindicated or not tolerated, rituximab, a monoclonal anti-CD20 antibody, may be an alternative to standard immunosuppression.

Key words: Hepatitis C virus; Interferon-α; Membranoproliferative glomerulonephritis; Mixed cryoglobulinemia; Ribavirin; Rituximab.

Introduction

Hepatitis C virus (HCV) is the most common cause of chronic viral hepatitis in Western countries. Persistent HCV infection may lead to the development of chronic liver disease, cirrhosis and hepatocellular carcinoma. In addition to liver disease, HCV infection is associated with several extrahepatic manifestations (Table I), the most important of which are mixed cryoglobulinemia (MC) and glomerulonephritis. Recent evidence of the direct role of HCV in the etiology of these diseases has cast new light on their pathogenesis and treatment. Antiviral therapy is now regarded as an etiological treatment, and attempts to eradicate the virus should be made in almost all cases of HCV-related cryoglobulinemia and glomerulonephritis. Unfortunately, the current therapy for HCV infection is only partially effective and sometimes associated with significant side effects. Furthermore, it may be inadequate or even harmful in patients with severe acute systemic vasculitis. These patients are usually treated with corticosteroids and immunosuppressive agents to decrease cryoglobulin production and improve vascular manifestations, but immunosuppressive therapy increases HCV viremia and may worsen the underlying hepatic disease.

The treatment of cryoglobulinemia and glomerulonephritis associated with HCV infection is challenging because of the multifactorial origin of the syndrome and the broad spectrum of clinical manifestations. This review summarizes the available information on hepatitis C and focuses on major advances in the understanding, management and therapy of HCV-related cryoglobulinemia and glomerulonephritis.

Hepatitis C virus infection

HCV is a worldwide infection that affects between 0.5 and 2% of the Western population. The main sources of infection are blood transfusions, intravenous drug use and inadequately sterilized medical equipment. In 40 to 50% of infected subjects the mode of viral transmission remains unknown.
HCV is a single-stranded RNA virus that encodes for a capsid protein, two envelope proteins (E1 and E2) and seven non-structural proteins involved in the viral life cycle. The region of the envelope protein E2 contains the binding site for CD81, a tetraspanin expressed on hepatocytes and B lymphocytes that is thought to function as a cell receptor or co-receptor of the virus. As the natural targets of HCV are hepatocytes and B lymphocytes, the identification of CD81 provides a mechanism by which these cells are infected by HCV.

Viral replication, which occurs through a direct RNA-RNA mechanism without a DNA intermediate and genomic integration, is highly error-prone, resulting in the generation of different but related variants known as quasispecies. This genetic variability confers to the virus an important survival advantage against host immune surveillance, and may contribute to the persistence of HCV infection.

On the basis of the nucleic acid sequences, at least six major genotypes and 90 or more subtypes of HCV have been identified worldwide. In the United States and Western Europe genotypes 1a and 1b are the most common, followed by genotypes 2 and 3.

**Diagnostic tests**

Enzyme-linked immunoassays (ELISA) are the best tests for initial screening. The currently used second- and third-generation enzyme immunoassays may detect antibodies in over 97% of infected persons within 4 to 10 weeks after infection. Supplemental testing with recombinant immunoblot assays is used to validate ELISA positive results. Direct detection of HCV RNA, based on the reverse transcription-polymerase chain reaction or transcription-mediated amplification, allows diagnosis in the early stages of acute infection and in patients who are unable to mount an antibody response. Qualitative HCV RNA tests are the gold standard for confirming the viremia and assessing the treatment response. Quantitative HCV RNA assays are less sensitive than qualitative assays, and should be used to monitor the viral load in patients with confirmed HCV infection. HCV genotyping based on polymerase chain reaction amplification is clinically relevant because genotypes 2 and 3 have better response rates to interferon-ribavirin therapy than genotype 1.

When a serological diagnosis of chronic HCV infection has been made, the histological activity of HCV-related hepatic disease may be assessed by determining the degree of necro-inflammation and stage of fibrosis in a liver biopsy. There is a poor correlation between the biochemical activity measured by serum alanine aminotransferase (ALT) levels and histological activity. ALT levels typically fluctuate and may even be normal during the course of HCV infection.

**Natural history**

Chronic infection, defined as the persistence of HCV RNA for 6 months after the (estimated) onset, occurs in up to 70% of HCV-infected individuals. However, the outcome of patients with chronic HCV infection is highly variable. Among those with biochemical evidence of chronic hepatitis the majority have only mild-to-moderate necro-inflammatory lesions and minimal fibrosis, which may be accompanied by relatively non-specific symptoms. About 20% of HCV-infected patients develop cirrhosis within 10-20 years of onset; once cirrhosis is established, the risk of hepatocellular carcinoma is 1-4% per year.

**Hepatitis C virus-associated mixed cryoglobulinemia**

Mixed cryoglobulins (MCs) are proteins that reversibly precipitate at ≤ 37°C and consist of a mixture of monoclonal or polyclonal IgM that have antiglobulin (rheumatoid factor-RF) activity and bind to polyclonal IgG. MCs are categorized as type II if the IgM RF is monoclonal, and as type III if polyclonal IgM RF is present. Unusual MCs consisting of oligoclonal IgM RF and trace amounts of polyclonal IgG have been detected and termed type II-type III variant. This particular subset of MCs has been considered as indirect evidence of a possible transition from type III to type II.

MCs may be found in association with infections, systemic autoimmune diseases and lymphoproliferative dis-
orders ("secondary MCs")\textsuperscript{21,24} or without any identifiable underlying disease. The latter condition, occurring in 30-50\% of MCs, has been referred to in the past as "essential MC"\textsuperscript{25}. It is now known that HCV is involved in the pathogenesis of the majority (> 90\%) of essential MC\textsuperscript{3,4}. The percentage of HCV-infected patients with MC has been reported as being between 40 and 56\%\textsuperscript{26,27}. The circumstances that predispose HCV-infected patients to the development of MC remain unclear.

**Clinical manifestations and laboratory detection**

The majority of cryoglobulinemic HCV-infected patients are either asymptomatic or have non-specific findings (e.g., weakness, arthralgias)\textsuperscript{24-27}. The triad of purpura, asthenia, and arthralgia first described by Meltzer et al.\textsuperscript{25} is evident at disease onset in a variable percentage of cases (27.5\% of patients in one large multicenter study)\textsuperscript{27}. Cryoglobulinemic vasculitis, predominantly involving the small vessels, is observed in less than 10\% of patients\textsuperscript{28}. The most frequently affected organs are the skin (purpura, leg ulcers) (Fig. 1), nerves (peripheral neuropathy) and kidneys (glomerulonephritis). The vessel wall deposition of circulating cryoglobulins leads to complement activation, and is responsible for the vasculitic lesions and organ damage.

Cryoglobulins are quantified either as the cryocrit or as the protein concentration. For immunochemical typing, electrophoresis on cellulose acetate and immunoelectrophoresis or immunofixation are used (Fig. 2). High levels of RF and cryoglobulins, and low levels of complement (C4, C3, and CH50) are characteristic findings in MC. The cryoglobulin level does not generally correlate with the severity/activity of the disease\textsuperscript{29}.

**Pathogenesis**

MC is a non-neoplastic B cell lymphoproliferative process induced by HCV in an antigen-driven mechanism\textsuperscript{3,4,30,31}. Lymphoid cells are involved in the early stages of HCV infection, but the mechanisms leading to B cell expansion are still unknown. The interaction between HCV envelope protein E2 and the CD81 receptor on B cells\textsuperscript{9,10} may lower the activation threshold of these cells, thus facilitating the production of various autoantibodies, including IgM RF. By itself, this process could lead to type III (polyclonal) cryoglobulinemia as monoclonal RF rarely arises in chronic human immune complex diseases\textsuperscript{32}. Transformation of the IgM RF fraction from polyclonal (type III) to the oligoclonal (type II-type III variant) and then to the monoclonal (type II) may occur over a decade or more of chronic disease\textsuperscript{33}, and is probably the consequence of a genetic mutation. The high prevalence of Bcl-2 rearrangement in patients with HCV-related MC is consistent with this hypothesis. T(14;18) translocation of the Bcl-2 oncogene results in overexpression of the gene, which may prolong the lifespan of B lymphocytes, favoring the emergence of a dominant clone capable of producing monoclonal IgM-κ RF\textsuperscript{34}.

In a limited number of subjects (< 10\%), monoclonal B cell expansion leading to type II MC may evolve into frank B cell non-Hodgkin lymphoma\textsuperscript{35}. Given that the viral RNA sequences cannot be integrated in the host genome, and that a direct transforming role of HCV appears unlikely, the emergence of a malignant B cell clone may be triggered by further mutagenic events (Fig. 3).

**Hepatitis C virus-associated glomerulonephritis**

The most common renal disease in patients with HCV infection is a form of membranoproliferative glomerulonephritis (MPGN), usually in the context of cryoglobulinemia\textsuperscript{36-38}. Renal involvement is reported in one third of cryoglobulinemic patients and almost exclusively occurs in association with type II MC\textsuperscript{38}. In some cases, the
glomerular damage may be mediated by HCV infection regardless of the concomitant existence of MC (non-cryoglobulinemic MPGN)36, 37.

Clinical features, laboratory testing, and disease course

The renal disease typically occurs in patients with long-standing HCV infection, often in association with subclinical liver disease and systemic symptoms of cryoglobulinemia (palpable purpura, arthralgia, weakness, peripheral neuropathy). The most common clinical presentation includes a non-nephrotic proteinuria and microscopic hematuria, with or without functional renal impairment36-38. About 20-30% of patients present with nephritic syndrome, and another 20% with nephrotic syn-
glomerular involvement may develop acutely and may be associated with oliguric acute renal failure (5% of cases)\textsuperscript{37,38}. The majority of patients have arterial hypertension, which is often severe and difficult to control.

Laboratory testing establishes the diagnosis of HCV-related MPGN. Most patients have HCV antibodies and HCV RNA in serum. ALT levels are elevated in 70% of patients, and the majority have RF and low C1q, C4 and C3 concentrations\textsuperscript{40}.

The disease course is generally characterized by periods of remission and exacerbation. Although end-stage renal failure requiring dialysis is rare (about 10% of cases)\textsuperscript{39}, patients with cryoglobulinemic nephritis have a poor prognosis because of an unusually high incidence of infection and cardiovascular disease\textsuperscript{41}.

Renal pathology and pathogenesis

The classic renal pathology associated with HCV-related cryoglobulinemia is a form of type I MPGN\textsuperscript{36-38}. Renal histology (Fig. 4) shows a widespread endocapillary proliferative or mesangiocapillary lesion with crescents in a few glomeruli and numerous subendothelial and, to a lesser extent, mesangial deposits; large, eosinophilic intracapillary thrombi may be seen in the acute phases (flares). Interstitial infiltration by mononuclear cells is usually found, whereas interstitial fibrosis is not usually prominent. Necrotizing
vасculitis of the small- and medium-sized arteries is observed in one third of renal biopsy specimens. Immunofluorescence microscopy reveals granular capillary wall and mesangial deposits and intraluminal masses of C3, IgM and IgG that are immunologically similar to the circulating cryoglobulins. On electron microscopy the intraluminal and subendothelial deposits may show a finely fibrillar pattern suggestive of cryoglobulin deposition.

Cryoglobulinemic glomerulonephritis is an immune complex-mediated disease. The glomerular damage might be initiated by the in situ or in-circulation binding of both anti-HCV IgG and non-specific IgG to the IgM RF of MC, with subsequent complement activation and cytokine production. The pathogenesis of a few cases of non-cryoglobulinemic type I MPGN is probably related to the glomerular deposition of immune complexes containing HCV antigens and IgG anti-HCV antibodies.

**Treatment of chronic hepatitis C virus infection**

Antiviral therapy is indicated in patients with HCV viremia who have persistently high ALT levels, and liver biopsy findings of moderate/severe necro-inflammation and/or fibrosis. These patients are at high risk of disease progression, and treatment is strongly recommended in the absence of contraindications. For other groups of patients, the indications for treatment are less clear and decisions should be made on an individual basis.

The aim of HCV therapy is the long-term eradication of detectable virus in serum. A sustained virological response (SVR), the usual standard of successful therapy, is defined as the absence of detectable HCV RNA 24 weeks after the discontinuation of therapy. Interferon alpha has been the mainstay of HCV therapy since 1989. The SVR rate for monotherapy with interferon alpha (3 million units or 9 µg subcutaneously thrice weekly) is 10-12% after a 6-month course and 20-25% after 12-24 months of treatment. The addition of ribavirin improves SVR rates to approximately 40%, with higher rates (64-66%) in patients with genotypes 2 and 3, and lower rates (28-31%) in patients with genotype 1. A further advance in the therapy of HCV has been the introduction of peginterferon, a long-acting formulation of interferon. Treatment with peginterferon alone leads to a significantly higher SVR rate than treatment with standard interferon alone.

A 48-week course of therapy with once-weekly peginterferon alpha-2a plus ribavirin leads to significant improvements in the SVR rate in comparison with interferon alpha-2b plus ribavirin (56 vs 44%, p < 0.001) or peginterferon alpha-2a plus placebo (56 vs 29%, p < 0.001). The response rates to peginterferon alpha-2a plus ribavirin were 46% in patients with genotype 1 and 76% in those with genotypes 2 or 3.

The duration of therapy depends on the HCV genotype. The currently recommended treatment consists of a 48-week course of once-weekly peginterferon alpha-2a (180 µg) or peginterferon alpha-2b (1.5 µg/kg) combined with daily ribavirin (1000 mg for body weights < 75 kg or 1200 mg for patients weighing > 75 kg) for patients with genotype 1, and a 24-week course of peginterferon combined with 800 mg/day of ribavirin for patients with genotypes 2 or 3.

Despite the recent advances in interferon and combination therapy, approximately 50% of patients fail to respond. For this expanding group of patients there are no recommended treatments.

**Treatment of hepatitis C virus-related cryoglobulinemia**

Patients with HCV-related MC may be managed by means of etiological, pathogenetic or symptomatic therapeutic modalities. The choice of the more appropriate treatment is strictly related to the assessment of disease activity, and to the extent and severity of organ involvement.

Patients with minor clinical manifestations, such as purpura, arthralgias and peripheral sensory neuropathy, should be treated with symptomatic and supportive measures, which include low steroid doses (methylprednisolone 0.1-0.3 mg/kg/day) and/or a low-antigen-content diet. This particular dietary regimen may improve the serum clearance of immune complexes by restoring the activity of the reticulo-endothelial system, overloaded with a large amount of circulating cryoglobulins.

The benefits of antiviral treatment in patients with mild disease are uncertain. However, antiviral therapy is indicated in the presence of persistently high ALT levels and histological findings of significant liver injury.

In patients with major clinical manifestations (such as peripheral motor neuropathy, active skin ulcers and severe organ involvement), there is clinical evidence that antiviral therapy (interferon with or without ribavirin), though capable of suppressing viremia and cryoglobulinemia, is not fully effective in controlling the inflammatory and self-perpetuating immune-mediated reaction that arises from the deposition of cryoglobulins in the vascular system and tissue. Furthermore, antiviral therapy is often ineffective or not tolerated, and, in some patients may even be associated with worsening of cryoglobulinemic manifestations.

In patients with clinically severe vasculitis, the primary goal is to suppress the vessel wall inflammation using immunosuppressive therapy. A short course (8-12 weeks) of corticosteroids (methylprednisolone pulses of 0.5-1 g/day for 3 days, followed by 0.5-1.0 mg/kg/day...
of prednisone) and cytotoxic drugs (usually oral cyclophosphamide at the dose of 2 mg/kg/day) may improve the vascular manifestations and decrease the production of cryoglobulins. In the most severe cases, plasmapheresis (exchanges of 3 L of plasma 3–4 times/week for 2–3 weeks) is used to remove cryoglobulins, inflammation mediators and toxins. There is concern that immunosuppressive therapy may increase viral replication and viremia, but no consistent evidence of acute liver damage has been reported during short treatment courses with corticosteroids and cytotoxic agents. When the acute disease flare has been controlled, antiviral therapy should be administered to eradicate HCV, the causative agent of the cryoglobulinemic syndrome.

**Treatment of hepatitis C virus-related cryoglobulinemic glomerulonephritis**

The currently available knowledge of the relationship between cryoglobulinemic glomerulonephritis and HCV infection has prompted a growing use of antiviral therapy in this condition. Results from large numbers of patients treated with interferon alpha in monotherapy or in combination with ribavirin are lacking, but positive results have been reported during short treatment courses with corticosteroids and cytotoxic agents. When the acute disease flare has been controlled, antiviral therapy should be administered to eradicate HCV, the causative agent of the cryoglobulinemic syndrome.

In patients with severe renal disease (e.g. nephritic syndrome with renal function impairment, nephrotic syndrome with active urine sediment, and severe histological lesions at renal biopsy), the therapeutic approach should be similar to that used for patients with cryoglobulinemic vasculitis (see above). An initial short-term course of corticosteroids and cytotoxic agents in combination with plasmapheresis may be administered to control the acute manifestations of renal disease. Once a clinical remission (indicated by stable or falling creatinine levels, the absence of urinary cell casts and a reduction in proteinuria) is achieved, the immunosuppressive drugs are stopped and replaced by antiviral treatment.

The sequential administration of immunosuppressive and antiviral agents is a reasonable therapeutic approach to HCV-positive patients with severe cryoglobulinemic glomerulonephritis. However, long-term prospective studies are needed to establish the efficacy and safety of this therapeutic modality.

**Conclusions**

MC and glomerulonephritis, the most distinctive extrahepatic manifestations of chronic HCV infection, may be treated by means of etiological, pathogenetic and symptomatic therapies, which must be individualized according to the activity/severity of the underlying disease. Symptomatic and supportive measures are generally sufficient for patients with minor clinical manifestations. Antiviral therapy alone may be successful in patients with mild-to-moderate disease activity, but relapses are frequent after therapy discontinuation. The therapeutic strategy for patients with severe and rapidly progressive disease should include, at different stages, both immunosuppressive and antiviral treatment. With regard to patients in whom antiviral therapy is ineffective, contraindicated or not tolerated, rituximab (a monoclonal antibody against the CD20 antigen expressed on pre-B lymphocytes and mature lymphocytes) has recently been proposed as an alternative to standard immunosuppressive treatment. Monoclonal anti-CD20 antibodies effectively reduce the B cell clonal expansion that sustains the production of pathogenetic cryoglobulins, but further investigations are needed to confirm the initially promising results of rituximab in type II MC.

Innovative treatments may be expected in the near future for patients who do not respond to combined interferon and ribavirin therapy. In addition to the three most widely investigated anti-HCV strategies (protease inhibitors, vaccines and immunomodulating substances),
La crioglobulinemia mista e la glomerulonefrite sono le più importanti manifestazioni extrapatiche dell’infezione cronica da virus dell’epatite C (HCV). La crioglobulinemia mista è sostenuta da una linfoproliferazione B cellulare non neoplastica, stimolata da HCV. Il quadro sintomatologico della crioglobulinemia è variabile, potendo palesarsi con fenomeni attenuati (scarce lesioni purpuri- che, saltuarie artralgie) o con manifestazioni cliniche severe (glomerulonefrite rapidamente progressiva, vasculi- te sistemica). La glomerulonefrite compare in un terzo dei pazienti crioglobulinemicici HCV-positivi e presenta solitamente il quadro istologico della glomerulonefrite membrano-proliferativa. Clinicamente la glomerulonefrite crioglobulinemica associata a HCV può manifestarsi con alterazioni urinarie isolate, sindrome nefritica (20-30%) o sindrome nefrosica (20%) con variabile progressione ver- so l’insufficienza renale cronica. Il trattamento di queste affezioni virus-correlate deve essere commisurato alla gravità del quadro clinico. Nelle forme lievi-moderate la terapia antivirale con interferone e ribavirina (il trattamento attualmente raccomandato per l’infezione cronica da HCV), pur determinando una risposta virologica duratura in meno del 50% dei pazienti, rappresenta la terapia di scelta. Nelle forme cliniche gravi e rapidamente progres- sive la terapia antivirale, anche quando riesce ad abbattere i livelli viremici e crioglobulinemici, non risulta in grado di controllare la reazione infiammatoria prodotta dalla deposizione delle crioglobuline nelle pareti vascolari e nei glomeruli. In questi casi la terapia antivirale dovrebbe essere preceduta da un trattamento immunosoppressivo di breve durata (8-12 settimane) costituito dalla combinazione di steroidi e alchilanti (con o senza plasmaderesi). Nei pa- zienti, in cui la terapia antivirale è inefficace, controindicata o non tollerata, l’anticorpo monoclonale anti-CD20 (rituximab) può rappresentare un’alternativa all’immuno- soppressione convenzionale.

Parole chiave: Crioglobulinemia mista; Glomerulonefrite membranoproliferativa; Interferone-α; Ribavirina; Rituximab; Virus dell’epatite C.

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