Review

Hepatitis C virus infection, cryoglobulinaemia, and beyond

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Hepatitis C virus (HCV) infection is the major cause of mixed cryoglobulinaemia (MC), an immune complex (IC)-mediated systemic vasculitis mainly involving the small blood vessels. The precise mechanism of cryoprotein production is currently unknown. HCV virions and non-enveloped core protein participate in the formation of cold-insoluble ICs. Cryoglobulinaemic patients represent a distinct HCV-infected population, in which significant HCV enrichment of lymphoid cells is accompanied by evidence of productive virus infection and increased frequency of B cells. Liver, the major target organ of HCV, is the site of accumulation of inflammatory infiltrates that shares many architectural features with lymphoid tissue and reflects a distorted homeostatic balance between factors that enhance cellular recruitment, proliferation and retention, and those that decrease cellularity (cell death and emigration). There is now overwhelming evidence of a direct contribution to B-cell growth and survival through production of a variety of cytokines and chemokines. Liver tissue over-expression and abnormal circulating levels of B-cell activating factor belonging to the TNF family can provide effective costimulatory mechanisms to sustain the B-cell clonal expansion, which constitutes molecular stigmata of MC. Indolent lymphoproliferation might act as the starting point of chronic, multistage lymphomagenesis. An innovative therapeutic strategy is directed to ‘eradication of the virus’ and deletion of B-cell clonalties.

Introduction

Hepatitis C virus (HCV) is a member of the genus Hepacivirus in the Flaviviridae family [1]. It is an enveloped RNA virus that causes chronic infection in ~200 million people worldwide [2]. Almost 80% of the infected patients develop chronic hepatitis, followed by cirrhosis in 10–20% and hepatocellular carcinoma in 1–5% [3]. Its complete replication in cell culture has recently been achieved [4]. Even so, its characterization and that of its life cycle are still difficult questions.

A surprising feature of HCV is that its association with some B-cell-related disorders is becoming increasingly evident. It is a major causal factor of mixed cryoglobulinaemia (MC), a chronic immune complex (IC)-mediated systemic vasculitis with underlying B-cell proliferation [5, 6], and contributes to the development of monoclonal gammapathy of undetermined significance (MGUS) [7] and post-transplant proliferative disorders [8]. B-cell malignant conversion may be the consequence of additional genetic accidents in the latently infected cells, or abnormal conditions resulting from modifications of host cell genes involved in the control of oncogenes and oncoproteins.

In this review we will discuss the mechanisms underlying the production of cryoglobulins in chronically infected MC patients; how signalling from B-cell receptor (BCR) and cytokines contribute to sustain B-cell clonal expansions; the role of ICs in the production of tissue damage and the therapeutic prospects offered by combining selective B-cell depletion with antiviral treatment. Little will be said about the clinical picture since this has been the subject of a recent comprehensive review [9].

Hepatitis C virus

The HCV genome carries a single positive-strand RNA containing a single open reading frame encoding for a long polyprotein of 3010–3040 amino acids, flanked at either end by a highly conserved untranslated region (UTR) [10]. The 5’ UTR harbours an internal ribosome entry site that directs a cap-independent translation of the viral gene and synthesis of the viral polyprotein, which is cleaved into structural (C, E1, E2 and p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins. RNA replication takes place in distinct cytoplasmic compartments and requires both viral (NS3 to NS5) and host proteins. During replication, HCV genomic RNA is transcribed into a complementary RNA strand, which subsequently constitutes a template for synthesis of a new genome [10].

Helicase activity of NS3 specifically recognizes the 3’ ends of both the minus and the plus virus strands [12]. It has been speculated that the NS3/NS4A complex may associate with NS5B, which subsequently recognizes the 3’ UTR of the plus strand, where initiation of minus strand synthesis occurs.

HCV forms a membrane-associated replication complex composed of viral proteins, replicating RNA, and altered cellular membranes [13]. Viral proteins are generated by co- and post-translational cleavage of the precursor polyprotein, while host peptidases located in the endoplasmic reticulum catalyse the cleavage of structural proteins. After genome amplification and protein expression, progeny virions are assembled and released, probably through a constitutive pathway [14].

Cryoglobulins and related clinical features

Cryoglobulins are immunoglobulins (Igs) that become insoluble below 37°C and give rise to high-molecular-weight aggregates [15]. They are found in small quantities in normal serum [16], but in variable concentrations in many pathological conditions, including tumours of the lymphoid system, autoimmune disorders and several infectious diseases.

Conventionally, cryoglobulins are classified on the basis of their Ig composition as type I, consisting of a monoclonal Ig alone, type II as a mixture of monoclonal and polycolonal Igs and type III consisting of polycolonal Igs. In MC, which comprises types II and III, IgM typically has rheumatoid factor (RF) activity [17].

Type I cryoglobulinaemia accounts for 10–15% of people with cryoprecipitates. IgM cryoglobulins (the most common type I variety) occur in almost 6% of malignant IgM paraproteinaemia. IgG (usually IgG2 or IgG3) cryoglobulins usually occur in almost
2% of all myelomas. IgA cryoglobulins are rare and cryo-Bence Jones proteins have been occasionally described [18].

MC contains Ig/anti-Ig complexes and frequently includes other proteins such as β-lipoproteins [19]. Type II accounts for 50–60% of cryoglobulins. Ig components, usually IgM and IgG, are not cold-precipitable on their own. IgG is always polyclonal, whereas IgM is monoclonal and frequently mounts kappa chains. Most IgM react with both intact IgG and its F(\text{ab\textprime})2 fragment, and also with the Fc fragment of autologous IgG. Type III accounts for the remaining 30–40% of cryoglobulins. MC is found in connective tissue and autoimmune disorders, and chronic bacterial, mycotic, and viral infections [20, 21].

Cryoglobulinaemic patients without identifiable underlying disease are considered to have ‘essential’ MC. It is now clear that most of them are chronically infected with HCV [22]. Cryoproteins are the result of a specific process in these patients in that, compared with supernatants, they are HCV RNA enriched [23].

Incidence of HCV infection in MC ranges from 40 to 90% and varies geographically [9]. MC can be found in up to 60% of HCV-infected patients; however, overt cryoglobulinaemic syndrome develops in 5–20% [24–26]. This striking association rate emphasizes the causative role of HCV in MC [27]. HCV-negative MCs, account for almost 5% in our geographical area and may represent true ‘essential’ MC [9]. Meticulous collection and processing of samples is essential for the detection of cryoprecipitable substances in the serum [28].

The most frequent signs and symptoms are much the same as those originally described by Melzett et al. [29]. Skin reactions are very frequent and many patients are first seen by dermatologists for purpura, leg ulcers, Raynaud’s phenomenon, oedema and urticaria.

The purpura does not usually cause itching, and appears intermittently on the exposed part of the body, especially the legs, fingers, ears and nose. It occurs most frequently in cold weather, lasts for 1–2 weeks, and leaves a darkened and diffusely hyperpigmented area. Chronic ulcers are especially frequent in the supramalleolar regions. They often appear in the absence of severe stasis dermatitis.

Raynaud’s phenomenon is sometime the first manifestation at diagnosis. It affects the distal part of the limbs, ears and nose. Arthralgia is present in most patients. It is intermittent, symmetrical and not migrating, and especially affects the hands and knees. Profound weakness is almost unifying. Many organs may be affected at the same time as the skin, or even earlier.

Renal damage leads to hypertension, microhaematuria and, less frequently, to proteinuria [30].

Participation of the nervous system in HCV-related MC patients is very high and its incidence may exceed 60%. Involvement of the peripheral system presents as a sensory-motor neuropathy, especially in the lower limbs often as painful paresthesias with loss of strength [31]. CNS involvement with transient dysarthria is very high and its incidence may exceed 60%. Involvement of the nervous system in HCV-related MC patients is very high and its incidence may exceed 60%. Liver abnormalities occur in almost 70% of patients. The histology of pre-treatment biopsy material is consistent with chronic active hepatitis, with or without cirrhosis [33, 34].

Significant impact on survival rate has been estimated in MC patients as compared with the general population. Cryoglobulinaemic vasculitis may involve multiple organs leading to significant negative impact on the quality and expectancy of life [35].

Why are cryoglobulins produced in only a subgroup of HCV-infected individuals?

Interaction between HCV and lymphocytes directly modulates B [6] and T cell function [36] and results in in vivo polyclonal activation and expansion of CD5+ cells [37], regarded as the major source of IgM RF molecules in type III MC [38]. It can be postulated that initial activation is followed by the emergence of a single dominant clone that produces a monoclonal IgM RF and provides support for type II MC. In addition, clonal heterogeneity of IgM defines a type II–type III variant, which can be considered a transitional stage in the switch from type III to type II [39, 40].

B-cell oligo/monoclonal expansions can be viewed as the result of the ability of HCV to persist chronically in the host and trigger sustained lymphoid proliferation, giving selective advantage to lymphoid clones that still remain dependent upon antigen stimulation [6]. In addition, direct infection of B cells promotes favourable conditions for lymphocyte proliferation [41]. In this respect, presence of the HCV minus strand RNA is the key proof of active viral replication in tissues and cells, whereas plus strand RNA merely implies contamination of circulating virions.

Application of a highly specific and sensitive method for HCV minus strand RNA detection indicated that HCV replicates in lymphocytes [42]. We extended these results and found the viral replicative intermediary in lymphoid cells from MC patients, whereas no trace of productive infection was found in acutely or chronically HCV-infected individuals without cryoglobulin production [43]. These results point to a direct relation between HCV productive infection in lymphoid cells and cryoglobulinaemia. Indeed, lymphocytes from MC patients have the unique property to concentrate higher amounts of HCV particles on their surface [44].

HCV may be primed for cell entry by receptor-binding molecules such as CD81 [45], scavenger receptor class B type I [46], low density lipoprotein [47] or as yet unidentified molecules [48]. Factors responsible for HCV enrichment on lymphoid cells from MC patients are unknown, but may be related to higher density [49] and/or polymorphism of receptor genes for binding [50, 51].

Evidence of productive infection in the mononuclear cells of a distinct cohort of individuals enables HCV-infected patients to be divided into two groups: the first referring to one compartment model, where it is assumed that viral replication occurs only in the liver, and the other compatible with a second replication compartment model where extrahepatic replication also exists [52]. In this context, occurrence of cryoglobulins may predict lymphoid compartmentalization of HCV productive infection.

Even if infected lymphocytes carry only ~3% of the circulating viral load [53–55], they are of high pathogenetic significance as an effective reservoir of HCV infection. Up-regulation of HCV genotype expression and virus replication in lymphoid cells require host cell factors that concentrate viral particles on their surface. Occurrence of a subset of HCV-infected patients carrying phenotypically distinct lymphoid cells implies the existence of high-affinity receptor molecules capable of mediating trafficking, fusion and cell entry [48].

HCV infects lymphoid cells of cryoglobulinaemic patients and these cells are capable of producing cryoglobulins

The intrinsic mechanism by which HCV promotes cryoglobulin production is unclear. However, expansion of RF-synthesizing B cells is a biological hallmark of cryoglobulinaemic patients [56, 57]. Analysis of the Ig variable (IgV) genes supports the concept of an antigen-driven expansion. The IgV heavy and IgV light chain genes are heavily mutated, compatible with a germinal centre or post-germinal centre derivation [58, 59].

Little is known about the role of HCV-related antigens on the ligands these B-cell expansions may recognize. No specific viral proteins, however, seem to be implicated as BCR ligands [60]. B cell expanded clones, all carrying significantly hypermutated IgV genes, seem to recognize a single epitope, suggesting that they arise randomly out of the pool of B cells selected for non-self-antigens, most likely during germinal centre reaction [59].

Most B cell expansions display a complementarity determining region-3 (CDR-3) resembling those present in the normal B cell
Cryoglobulinaemic vasculitis

Cryoglobulinaemic vasculitis (CV) is a pathological process characterized by inflammation and damage to blood vessels. This process is usually associated with impairment of the lumen and ischemic changes in tissues supplied by the vessels involved [66]. Though any organ may be affected, CV is most frequently evident in the skin. The major role of HCV in the pathogenesis of CV is demonstrated by the presence of HCV-related proteins in the skin blood vessels of cryoglobulinaemic patients. Immune deposits sometimes filling the microvascular spaces alternate with deposition of HCV-encoded proteins in vessel walls and perivascular spaces with no lumen alteration [67]. Immunodeposits are rarely substantiated by in situ demonstration of HCV RNA sequences [68].

Role of cold-insoluble ICs is sustained by the presence of IgM and/or IgG molecules at the site of vascular damage, in addition to complement fractions [69].

Involvement of non-enveloped HCV core protein as a substantial ligand has been illustrated [70]. Experimental data concluded that cold-insoluble ICs are formed primarily by IgM RF linked to IgG, which in turn is bound to HCV core protein. IgM RF acts as incomplete cryoglobulin, in that it precipitates at low temperature in the presence of IgG with anti-core reactivity. The dynamics of cold-dependent insolubility demonstrates a peculiar feature, in that addition of an irrelevant IgG to an IgM RF/HCV core protein mixture was unable to precipitate the protein. This implies that the functional RF repertoire may be selected positively by IgG anti-HCV molecules. It can be argued that IgM RFs are distinct high-affinity molecules directed against preferential self-antigen [71].

Following their binding and when exposed to cold, RF molecules are subjected to a conformational change that is probably responsible for their cryoprecipitation [72]. Nevertheless, ICs deposition depends greatly on haemodynamic factors and the anatomy of particular sites. The glomerulus, choroid plexus, synovium, uveal tract and skin all receive a high blood flow per unit mass of tissue and are thus exposed to and can trap large quantities of ICs in their vascular walls [73].

C1q protein and C1q binding activity are substantially enriched in the cryoprecipitates [70]. The efficient engagement of C1q protein by cryoprecipitating ICs may represent an important pathogenetic mechanism in the cryoglobulin-related pathway. C1q, indeed, governs deposition of circulating ICs [74] and is required for ICs binding to endothelial cells in vitro [75]. These findings are particularly noteworthy in HCV pathogenesis, since HCV core protein/C1q receptor through its globular domain (gC1qR) [76] interaction may favour specific binding of ICs to endothelial and blood cells [9]. Interestingly, difference in gC1qR expression levels may explain susceptibility to HCV-core-induced biological effects [77].

Mechanisms leading to deposition of ICs in the vascular bed have not been fully elucidated. Occupancy of the Fc portion of IgG by IgM molecules is probably a major factor in the functional properties of ICs. They are large ICs known to be poor acceptors of C3 and C4 [78], and deplete complement rather than fix it. Nevertheless, they are good acceptors of C1q [79] that can favour indirect binding of HCV core protein to the cell surface (Fig. 1). Recently, a role for C1q autoantibodies has been reported in these patients [80].

HCV sustains indolent lymphoproliferation

In the course of B-cell proliferation, somatic mutations arising in IgV genes generate different types of mutants. Polymerase chain reaction (PCR) directed against the variable-determining-joining (VDJ) region defines the unique combination of N-regions along with variations in the DH and JH regions, and can be used as a clonal marker of the cell progeny. Applying this technique to characterize B lymphocytes from liver tissue samples of HCV-infected cryoglobulinaemic patients, we have demonstrated that B-cell clonal expansions occur in the liver of almost 90% of these patients and less frequently in bone and blood marrow [56, 81]. Isolated intrahepatic B lymphocytes are capable of spontaneous production of RFs that most frequently display the WA cross-reactive idioype [56].

VDJ PCR products displayed oligoclonal to monoclonal patterns, indicating that B-cell expansions in the liver originate from very few or single cells. Each focus may derive from a different B cell of the polyclonal repertoire, with the result that different foci contain unrelated B-cell clones.

Occurrence of HCV enrichment in intrahepatic inflammatory infiltrates supports the notion that HCV is directly involved in the emergence and maintenance of these B-cell expansions [82]. Intrahepatic B-cell clonality is invariably associated with extrahepatic manifestations of HCV infection, including high serum levels of RF activity, cryoglobulins, MGUS and frank B-cell non-Hodgkin’s lymphoma (NHL), indicating that they have a direct impact on clinical features [83].

The liver is the main target of HCV infection and the major site of inflammatory events, including recruitment of inflammatory cells. An emerging area of research is directed to the definition of effective signals that enhance the survival of immunocompetent cells [84]. Uncontrolled and inappropriate survival signals are known to underlie many autoimmune disorders. The B-cell activating factor of the TNF family (BAFF), in particular, is a fundamental survival factor [85, 86].

BAFF mRNA is mainly present in lymphoid tissues. It is expressed by monocytes, macrophages and dendritic cells, and by growth-factor-stimulated neutrophils. Low expression is observed in T cells, while BAFF is absent in B cells [87]. Dendritic cells and neutrophils release a soluble form of BAFF more potently than monocytes and macrophages. Signals mediated by BAFF cooperate with those mediated by BCR to up-regulate expression of specific targets and favour cell proliferation [88]. The prime role of BAFF is to protect B cells from apoptosis [89].

We noted intense expression in inflammatory cell-containing portal tracts in liver biopsy tissues and the skin of cryoglobulinaemic patients in addition to very high amounts of mRNA (Fig. 2). Inappropriate higher circulating BAFF levels were also demonstrated (manuscript in preparation). It can be inferred that
inflamed liver and skin are the sites for the production of BAFF which distributes to the blood [89].

Overproduction of BAFF in these patients can overcome death signals triggered by autoantigen binding to the BCR and allow these cells to survive and mature. Survival signals are mediated by either up-regulation of the expression of intracellular proteins, such as pro-survival oncogenes that protect cells from apoptotic signals, or down-regulation of expression of pro-apoptotic proteins.

As a pro-survival oncogene, up-regulation of Bcl2 protein in cryoglobulinaemic patients has been reported to be pathogenetically linked to t(14;18) chromosomal translocation [90]. However, in a recent report it has been emphasized that acquisition of particular chromosomal translocation makes little or no contribution to B-cell expansions in cryoglobulinaemia [91].

BAFF appears to be a key factor explaining the role of BCR-mediated signals in the fate of B cells [87]. Mounting evidence supports the view that B-cell maturation in HCV-related cryoglobulinaemia relies on a signal through the BCR complemented by a survival signal provided by BAFF [92, 93].

**HCV infection and B-cell non-Hodgkin's lymphomas (NHL)**

HCV may also determine the development of some types of B-cell NHL, though this association seems to be limited to geographic areas where the presence of HCV is more substantial or endemic [94].

Overall, the clinical outcome of HCV-positive NHL does not seem to be different from that of NHL patients without HCV infection. However, HCV-positive NHL often displays some distinctive features, such as older age, liver damage, presence of monoclonal gammopathy, increased rate of autoimmune disorders, extranodal localizations and restricted histological subtypes. Comparison of the primary sites of B-cell lymphoma at onset in primary extranodal lymphoma patients with and without HCV infection, liver and salivary gland involvement were significantly more frequent in HCV patients. Primary location in these sites is extremely uncommon in HCV-negative patients with B-cell lymphoma [95].

Although many studies have provided evidence that HCV infection is associated with development of both indolent and aggressive B-cell NHL [96], lymphoplasmacytoid lymphoma (immunocytoma) is closely associated with HCV infection [97]. However, a recent case control study of patients with various B-cell NHL subtypes indicated that HCV infection was detected most frequently among those with diffuse large B-cell lymphoma [98] and splenic lymphoma with villous lymphocytes [99, 100].

The similarities shared by rearranged Ig genes present in B cells from patients with type II MC and malignant B-cells from HCV-positive patients with B-cell NHL support the possibility that the antigens that promote type II MC and B-cell NHL in HCV-positive patients are the same [101, 102]. These similarities also suggest that type II MC may be a precursor of B-cell NHL [103].

Type II MC probably plays a central role in the development of B-cell lymphoma in HCV-positive patients with Sjögren's syndrome (SS) [104]. Lymphoma is conventionally regarded as the main complication in the natural history of SS. In the SS population, B-cell lymphomas are predominantly extranodal, mainly located in the salivary glands. Interestingly, lymphomas in SS and HCV patients share several characteristics, such as predominance of

low-grade, marginal zone histological type, frequent mucosal involvement, possible transformation into a large B-cell lymphoma, a close association with MC and the appearance of lymphomas in organs where HCV or SS are active [105].

Treatment

The HCV aetiology of MC has shifted its treatment away from the usual combination of steroids and cyclophosphamide [106]. Interferon-α (IFN-α) therapy as a means of achieving a rapid decrease in HCV RNA levels and a sustained response has met with only limited success [107]. About 80% of responders relapse within 6 months after its suspension [108]. Clearance of the virus is possible and a sustained viral response is achieved in almost 20%. Reduction of the cryocrit in responders follows clearance of HCV RNA. By analogy with the treatment of chronic HCV infection without MC, low HCV RNA levels are predictive of a favourable response [109], whereas other parameters including cryocrit, serum RF titre, or frequency of vasculitic spurts are of little value [108].

Combination of IFN-α and ribavirin (RBV) improved the clinical response [110]. A more favourable response rate was reported with the use of pegylated IFN-α (PEG-IFN-α) and RBV [111]. Results of these studies, however, are not conclusive and controlled trials are needed.

Rituximab, a humanized murine monoclonal antibody directed to CD20 antigen, a transmembrane protein expressed on pre-B lymphocytes and mature lymphocytes, has been used as a new way of reducing or depleting B-cell clonal expansions [112], since it is highly effective for in vivo B-cell depletion [113]. Peripheral blood lymphocytes become undetectable after a single infusion and recover after 3–5 months from discontinuation of treatment [114].

The two largest studies showed rituximab used as a single therapeutic agent in MC elicited a favourable response in most patients [114, 115]. Molecular monitoring of B-cell response revealed disappearance/deletion of peripheral clones [114]. However, since rituximab had a deep impact on hepatitis C viraemia in that HCV RNA increased [114], use of rituximab combined with PEG-IFN-α and RBV has been proposed. A randomized study with PEG-IFN/RBV vs PEG-IFN/RBV plus rituximab in MC is warmly urged.

In view of MC’s complexity, some patients with rapidly progressing MCV may benefit from steroids combined with cytotoxic agents and cryofiltration [116].

Conclusions

Advances in knowledge of the mechanism of HCV persistence and the pathogenesis of sustained lymphoproliferation, and the introduction of more effective therapies are the major aims for future research.

Particular objectives will be clarification of the genetic differences of host factors, especially the differential expression levels of HCV-binding seen as critical in the virus–lymphocytes interaction; determination of the mechanisms whereby HCV or/and its gene products modulate B-cell functions to generate
host components of cold-insoluble ICs; definition of cytokine signalling sustaining B-cell activation and proliferation and the promoting DNA accessibility required for IgH VDJ recombination as well as the regulated expression of VDJ recombination machinery; elucidation of the intrinsic role of HCV core protein in the mechanism leading to deposition of ICs in the vascular bed and in the transcriptional signals promoting proliferation of infected cells; identification of genetic accidents providing growth advantages of expanded B cells and conversion to malignant phenotype; acquisition of new treatment options capable of neutralizing the biological activities of deregulated cytokines (i.e. Belimumab, an anti-BAFF antibody) [117] in MC patients.

Acknowledgements
The authors are indebted to G. Lauletta, MD, F.A. Tucci, BSc and M. Montrone, MD for their invaluable scientific and intellectual contribute, and Mr V. Padolecchia for his skillful technical assistance during many years of collaborative work. This study was supported in part by grants from: Associazione Italiana per la Ricerca sul Cancro (AIRC, Milan, Italy)—Regional Grants 2003; University of Bari, Italy; Italian Ministry of University and Research, PRIN 2006 and Cassa di Risparmio di Puglia Foundation, Bari, Italy.

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Acknowledgements
The authors are indebted to G. Lauletta, MD, F.A. Tucci, BSc and M. Montrone, MD for their invaluable scientific and intellectual contribute, and Mr V. Padolecchia for his skillful technical assistance during many years of collaborative work. This study was supported in part by grants from: Associazione Italiana per la Ricerca sul Cancro (AIRC, Milan, Italy)—Regional Grants 2003; University of Bari, Italy; Italian Ministry of University and Research, PRIN 2006 and Cassa di Risparmio di Puglia Foundation, Bari, Italy.

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