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Clonal Analysis of Intrahepatic B Cells from HCV-Infected Patients With and Without Mixed Cryoglobulinemia

Domenico Sansonno,* Salvatore De Vita,† Anna Rina Iacobelli,* Vito Cornacchiulo,* Mauro Boiocchi,† and Franco Dammacco*‡

Clonal rearrangements of Ig heavy chain (IgH) genes and hepatitis C virus (HCV) genomic sequences were assayed on intrahepatic B lymphocytes isolated from HCV chronically infected patients with and without type II mixed cryoglobulinemia (MC). Liver tissue samples from eight patients with and nine without MC were subjected to routine histologic studies, immunophenotyping, and genotypic analysis including IgH V-D-J region gene rearrangements by PCR. RT-PCR, signal amplification by branched DNA assay, and in situ hybridization technique were used to detect and quantitate HCV RNA genomic sequences in selected B cells purified from each tissue sample. Although HCV infection of intrahepatic B cells was shown in all patients both with and without MC, frank B cell monoclonal and oligoclonal patterns were found in only three and four patients with MC, respectively. No monoclonal profile was seen in the noncryoglobulinemic patients, whereas an oligoclonal profile was demonstrated in four of them. No clonalities were shown in HCV-unrelated patients matched for age and severity of liver disease. No obvious difference in HCV genotype distribution was found in relation to the clonal expansion profile. Noncryoglobulinemic patients showing clonal expansion in liver tissue had higher titers of serum rheumatoid factor (RF). Spontaneous production of RF was shown in cell cultures of intrahepatic B cells, suggesting their persistent stimulation in vivo. These data indicate that HCV infection of B cells and B cell clonal expansions occur in the liver microenvironment and preferentially involve RF-producing cells. The Journal of Immunology, 1998, 160: 3594–3601.

Hepatitis C virus (HCV) is a major human pathogen that causes acute and chronic infections (1). Although acute infection rarely results in severe illness, >80% of subjects become chronic HCV carriers and may progress to a broad spectrum of liver diseases, ranging from chronic hepatitis to cirrhosis and hepatocellular carcinoma (2), as a consequence of complex interactions between viral and host factors (3). The occurrence of HCV infection and replication in sites outside the liver is a prominent biologic feature of this variable clinical outcome. The peculiar tropism of HCV for immunologically privileged tissues helps to explain its persistence and the development of immunologic abnormalities. The presence of HCV in lymphocytes (4) and lymphoid organs (5), indeed, may result in a high mutation rate of HCV genome and the production of variant strains that escape the immune response (6). In addition, the involvement of lymphocytes may help to elucidate the mechanisms that seem to link HCV infection with autoimmunity, B cell dyscrasia, and lymphomagenesis.

In the course of B cell clonal proliferation, somatic mutations arising in the IgV region genes generate different types of mutants (7). PCR has been successfully employed to detect B cell clonality in malignant lymphoproliferative disorders (8, 9), as well as in mixed cryoglobulinemia (MC) (10). PCR directed against the V-D-J region of the Ig gene has been advocated as a reliable alternative to Southern blot analysis and conventional immunotyping. The unique combination of N regions along with variations in the D_H and J_H regions can be used as a clonal marker of the cell progeny (11).

It has been strongly suggested that HCV plays a primary role in the induction of type II MC, a disorder characterized by bone marrow multifocal lymphoid infiltrates of monoclonal B cells (12). It is most likely a lymphoproliferative process with an indolent clinical course (13). The liver histology of HCV-infected patients with MC, in fact, shows a combination of portal and/or lobular inflammatory cell infiltration frequently associated with lymphoid nodules resembling secondary lymphoid organs (14, 15), in which follicular B cells display single-Ag specificity.

Since the liver is the primary site of productive HCV infection (16), we investigated its possible hosting of infected B cells undergoing clonal expansion. A PCR technique was applied to detect HCV genomic sequences and Ig heavy chain (IgH) V-D-J region gene rearrangements in B cell populations from HCV-infected liver tissues of patients with and without MC. Our data indicate that B cell clonal expansions and local rheumatoid factor (RF) production are strictly related events in the liver microenvironment.

Materials and Methods

Liver tissue specimens from 17 patients with chronic hepatitis were studied (Table I). Eight had MC and the purpura-weakness-arthralgia syndrome.
Table I. Clinical and laboratory parameters of 17 HCV-infected cryoglobulinemic and noncryoglobulinemic patients

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Age (yr)/sex</th>
<th>Cryocrit (%)</th>
<th>Monoclonal Component/ Fr3 protocol</th>
<th>Liver Histology</th>
<th>Liver Disease (yr)</th>
<th>Autoantibodies</th>
<th>Ig Concentration (mg/dl)</th>
<th>HBV Status (HBSAg/a-HBs/a-HBe/ HBsAg/a-HBe)</th>
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Noncryoglobulinemic

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<th>Liver Disease (yr)</th>
<th>Liver Histology</th>
<th>Monoclonal Component/ Fr2 protocol</th>
<th>Fr3 protocol</th>
<th>Autoantibodies</th>
<th>Ig Concentration (mg/dl)</th>
<th>HBV Status (HBSAg/a-HBs/a-HBe/ HBsAg/a-HBe)</th>
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Without intrahepatic

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<th>Monoclonal Component/ Fr2 protocol</th>
<th>Fr3 protocol</th>
<th>Autoantibodies</th>
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With intrahepatic

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<th>Fr3 protocol</th>
<th>Autoantibodies</th>
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<th>HBV Status (HBSAg/a-HBs/a-HBe/ HBsAg/a-HBe)</th>
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<td>1388 404 447 883</td>
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Isolation and characterization of cryoglobulins were conducted as described elsewhere (14). All patients were positive for Abs to HCV and HCV RNA. Epidemiologic data included age at time of liver biopsy, duration of liver disease, and exposure to risk factors. After giving their informed consent, all patients provided a liver biopsy specimen taken with the Menghini needle. No patient had received corticosteroids, IFN, or other systemic treatment. The study was approved by the ethics committee of the University of Bari Medical School.

The main controls were 10 patients (6 women and 4 men; mean age 65.3 ± 9.7 yr) without signs of progressive liver disease (mild steatosis in 6 and mild portal fibrosis without inflammatory infiltrates in 4) who underwent liver biopsy in the course of laparoscopic cholecystectomy. These patients were anti-HCV and HCV RNA negative. All were HBsAg negative and five had anti-HBs, anti-HBc, and anti-HBe Abs. Six additional patients with chronic HBV-related liver disease formed a second set of controls. All were HBsAg-positive males with a mean age of 58 ± 12 yr. Four were anti-HBe positive and 2 were HBsAg and anti-HBe negative. Liver histology showed chronic hepatitis in 3 and cirrhosis in the others. None had serologic evidence of anti-HCV Abs or HCV RNA genomic sequences.

Histology and immunohistochemical analyses of paraffin-embedded tissues and cryostat sections were performed using routine procedures, including detection of B and T cell-associated differentiation Ags, as well as restriction of B cell surface κA light chains.

**PCR analysis of B cell clonal expansion**

DNA was recovered from fresh-frozen liver samples by standard methods (17, 18). While it was insufficient for Southern blot analysis of B cell clonality, PCR analysis for B cell clonal expansion was performed in all of the cases using two different seminested protocols of amplification according to well-established procedures (18). In the first protocol, the upstream primer was complementary to the third framework V region (Fr3) of the IgH gene, whereas in the second, the upstream primer was complementary to the second framework V region (Fr2). In both protocols, the downstream primer was the same and was directed to an outer conserved region of the IgH J region in the first round of amplification and to an inner conserved sequence of the same J region in the second round (18, 19) (Fig. 1). Each sample was tested in duplicate, and any positive or negative result was confirmed by at least two separate PCR experiments. Positive and negative controls were always included. The sensitivity of the technique was checked by the amplification of serial dilutions of DNA from clonal B cells admixed with DNA from polyclonal B cells. The detection threshold of a discrete band was 0.5 to 1% (18). First, a “cold” nonradioactive PCR was performed (18). At the end of the second-round amplification, 20 μl of reaction mixtures were analyzed in parallel by electrophoresis (150 V) in 5% (Fr3 protocol) or 3% (Fr2 protocol) agarose gel (Seakem LE, FMC Bioproduct, Rockland, ME) in TBE (100 mM Tris, 100 mM boric acid, 2 mM EDTA) buffer, stained with ethidium bromide, and optically evaluated by UV transillumination. Clonal expansion was indicated by one or more dominant bands within the predicted size range and a fully polyclonal pattern by a smear with no specific dominant bands. The clonal patterns detected by nonradioactive PCR were subsequently confirmed by a radioactive PCR approach, which enhanced the identification of the number of multiple dominant bands in oligoclonal B cell expansions, as described (18). Radioactive PCR was performed using a labeled nucleotide, i.e., α-[32P]-labeled dATP (Amersham, Bucks, U.K.) in the second round of amplification in both the Fr2 and Fr3 protocol. The radioactive PCR products were then subjected to a long run (20–40 min).
Liver biopsy specimens (~10 mg) washed in ice-cold PBS were cut into small pieces, washed twice with RPMI 1640, passed through a mesh, and resuspended in calcium- and magnesium-free HBSS medium containing EDTA (2 mM). After 15 min, they were resuspended in RPMI 1640 containing collagenase (0.2%) and a trypsin inhibitor (0.01%). The suspension was gently shaken in an incubator at 37°C for 2 h.

Inflammatory mononuclear cells were purified from the suspension by two-step gradient density centrifugation, using a Percoll followed by a Ficoll gradient as previously described (20). After washing, cells were counted and tested for viability by trypan blue exclusion. The B cell/monocyte-macrophage fraction was monitored by FACS analysis with an anti-CD19 mAb (Ortho Diagnostic Systems, Raritan, NJ). CD19+ cells were purified by immunomagnetic separation using Dynabeads (DynaI, Oslo, Norway), according to the manufacturer’s instructions. Briefly, mononuclear cells were incubated with anti-CD19-conjugated magnetic beads at a bead-to-cell ratio of 3:1 for 30 min at 4°C on a rotating platform. Positively selected cells were recovered by further incubation for 45 min at room temperature with an affinity-purified polyclonal antisera against the Fab portion of mAb bound to the beads (Detect-a-Bead; Dynal). Isolated cells were suspended at 0.5 × 10^6 cells/ml in RPMI 1640 supplemented with 1% foetal calf serum (FCS), 2 mM l-glutamine, and 10% FCS and assayed for HCV RNA. When enough intrahepatic B cells were recovered, cell cultures were set up with 0.5% FCS and assayed for HCV RNA. When enough intrahepatic B cells were recovered, cell cultures were set up with 0.5% FCS and assayed for HCV RNA. When enough intrahepatic B cells were recovered, cell cultures were set up with 0.5% FCS and assayed for HCV RNA.

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but without RF activity. There were differences in the prevalence of females (M/F ratio 0.6 in the cryoglobulinemic and 2.0 in the noncryoglobulinemic group, \(p < 0.05\)) and mean age (61.6 ± 12.6 yr vs 47.9 ± 14.2 yr; \(p = 0.05\)). The mean length of liver disease was shorter in noncryoglobulinemic than in cryoglobulinemic patients (4.8 ± 2.2 yr vs 7.6 ± 4.3 yr; \(p = 0.10\)), although the histologic features of active liver disease were equally distributed. There were no histologic differences (Knodell score), even when inflammation and fibrosis were analyzed separately. Cirrhosis was detected with almost the same frequency: 12.5 vs 11.1%. All patients were HBsAg negative and the frequency of HBV markers (anti-HBs, anti-HBc, anti-HBe) was similar in the two groups.

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Intrahepatic B cell clonalities were revealed by PCR in liver tissue otherwise negative by conventional immunophenotyping. \(\kappa\lambda\) Light chain restriction of B cell infiltrates was never observed.

HCV RNA was shown in the plasma and purified intrahepatic lymphocytes and in the liver from all patients regardless of the presence or absence of circulating cryoglobulins.

Morphologically, localization and distribution of HCV RNA sequences in the liver sections were studied by ISH. No consistent differences were seen between cryoglobulinemic and noncryoglobulinemic patients. The autoradiographic signal was shown in

![Diagram](http://www.jimmunol.org/)

**FIGURE 2.** Assessment of B cell clonal expansion in liver samples from patients with chronic HCV infection by V-D-J PCR. Agarose gel stained with ethidium bromide, showing nonradioactive V-D-J PCR products (Fr3 protocol). A fully polyclonal pattern is shown in lane 3 and 4. An oligoclonal B cell expansion (>2 dominant bands within the predicted size range of 80–120) is shown in lane 2. A monoclonal pattern (1 or 2 dominant bands) is shown in lanes 1, P1, and P2. Lanes 1 to 4, PCR products of DNAs extracted from liver biopsy samples. Lane N, negative control (PCR products without DNA). Lane M, m.w. markers (HoeII-digested DNA of &X-174). Lanes P1 and P2, Positive controls (DNAs from two B cell non-Hodgkin’s lymphomas).
individual hepatocytes or clusters of hepatocytes, with no obvious topographical relationship between the stained hepatocytes and the sites of acinar inflammation or hepatocyte degeneration (Fig. 3A). The hybridization signal was predominantly located in the cytoplasmic compartment of hepatocytes. No signal was found in the Kupffer cell component nor in the portal tracts and terminal hepatic venules. In addition, the labeling signal involved small round cells, probably inflammatory cells, scattered within the intralobular areas and frequently far from necroinflammatory foci (Fig. 3B). Hybridization staining was considered specific, in that: 1) no signal was found on any liver section when the 3′-end dATP-labeled reverse antisense probe was used; 2) hybridization signal was abolished by prehybridization of sections with a 200-fold excess of cold unlabeled antisense probe; 3) incubation of the sections with ribonucleases before hybridization abolished detection of HCV RNA signal; 4) no signal was found in liver samples from HCV-unrelated patients nor with the use of irrelevant probe.

Intrahepatic B lymphocytes from two patients (No. 5 and 6) with and one (No. 14) without MC were cultured for 5 days to determine whether they were capable of supporting in vitro a productive HCV infection and of spontaneous production of RF. Cells and supernatants were harvested at 24 h-intervals and tested for HCV RNA levels by bDNA assay. The relationship between cells and supernatants in each fraction was almost the same in all three patients. HCV RNA titers gradually increased in the supernatants, but were not detectable in the first sample. In contrast, HCV RNA levels decreased in the cells after a transient peak. A spontaneous production of RF was demonstrated in vitro in the two patients with MC, whereas IgM molecules bearing 17.109 XId were found in the supernatants of all three patients (Fig. 4).

The presence of HCV RNA genomic sequences within B lymphocytes was demonstrated morphologically using an ISH technique on the starting sample (Fig. 3C). The autoradiographic signal was preserved in situ while the cell membrane was permeabilized with proteinase K. The range of HCV RNA-positive cells in samples taken just before the culture varied from 0.1 to 3.0%.

The same HCV genotype was regularly demonstrated in the bloodstream and in intrahepatic B cells. Except for a higher prevalence of HCV genotype 2a in the cryoglobulinemic compared with the noncryoglobulinemic patients (37.5% vs 11.1%; p = 0.261), no obvious difference in HCV genotype distribution was found in relation to the clonal expansion profiles.

Interestingly, all noncryoglobulinemic patients with intrahepatic clonal expansions (subgroup 1) had higher levels of serum RF activity compared with the levels in patients with no evidence of IgH gene rearrangements (subgroup 2) (50.75 ± 33.12 IU/ml vs 11.00 ± 3.81 IU/ml, p = 0.03). The mean age of patients was higher in subgroup 1 than in subgroup 2 (52.75 ± 15.59 yr vs 44 ± 13.38 yr; p = 0.394). The two subgroups differed neither in terms of length of liver disease (4.00 ± 1.63 yr vs 5.40 ± 2.51 yr; p =
0.370) nor of IgM serum levels (175.25 ± 44.59 mg/dl vs 288.6 ± 297.88 mg/dl; p = 0.445).

Discussion

The present results consistently show that: 1) intrahepatic B lymphocytes harbor HCV regardless of the occurrence of cryoglobulinemia; 2) B cell clonal expansion is demonstrable in the liver in seven of eight cryoglobulinemic and in four of nine noncryoglobulinemic patients; 3) generally, patients showing intrahepatic B cell clonal expansion associate higher serum levels of RF activity; 4) in vitro, intrahepatic HCV-infected B cells are capable of spontaneous production of RF and IgM molecules displaying 17.109 XIr. The preceding data support the notion that, in chronic HCV infection, lymphocytes recruited at the disease site are infected, and in a fraction of cases they are also expanded and activated to secrete molecules with RF activity. Thus, it seems reasonable to speculate that HCV infection of B lymphocytes, clonal B cell expansion, and RF production are closely related events in the natural history of hepatitis C. However, the definite phenotypic identification of RF-secreting, HCV-infected B cells will be crucial to the verification of this hypothesis. In addition, the possibility cannot be excluded that B cells not infected by HCV might also undergo clonal expansion in the liver microenvironment.

Our findings add further evidence of the striking lymphotropism of HCV. However, although viral RNA can be consistently detected in nucleic acid samples from either peripheral or bone marrow mononuclear cells of HCV-infected patients, it has recently been questioned whether its presence really reflects the result of intracellular replicating virus (28). Indeed, because no suitable experimental controls are available, viral particles may bind very tightly to the cells. It can be inferred that the virus cannot be washed away and remains detectable for many days (29).
to blood mononuclear cells is not necessarily followed by membrane penetration and active infection.

Results from spontaneous cultures of B cells obtained from hepatic inflammatory infiltrates of two MC patients were compared with those obtained from one noncryoglobulinemic patient. All showed roughly the same kinetics of HCV RNA levels in cells and supernatants. A productive infection was strongly suspected to be sustained by these cells, in that a time-dependent increase of HCV RNA was detected in the supernatants, whereas no signal was demonstrable at time 0. At the beginning, purified intrahepatic B cell samples showed that 0.1 to 3.0% of the cells were specifically stainable with HCV radiolabeled oligonucleotide probe. Unfortunately, subsequent culture samples were not available to establish whether the dynamic changes observed in cultured cells or in supernatants paralleled the number of ISH-positive cells and/or the intensity of hybridization signal. These results were further corroborated by ISH studies of the liver, which confirmed the presence of HCV RNA in the hepatocytes of HCV-infected patients and defined the presence of the hybridization signal in cells resembling infiltrating inflammatory cells, irrespective of the occurrence of cryoglobulinemia.

These data strongly support previous immunomorphology (20, 30) and in situ hybridization studies (16, 31, 32) indicating that HCV actively replicates in blood mononuclear cells, providing new clues as to its putative role in the development of certain B cell dyscrasias including MC (12) and malignant lymphoproliferative disorders (33).

The present results indicate that the liver is a major site of lymphocyte infection by HCV that likely stimulates B cells to produce IgM molecules bearing the 17.109 XId. These proteins, thought to be germline gene products of the WA group (34), have recently been shown to be a constant component of soluble, nonpapicitating immune complexes in patients with acute and chronic HCV infection (21). WA XId-positive Igs are molecules without RF activity and are thought to play the role of “natural” Abs to common pathogens (34). The close association between WA XId IgM with RF activity and V-D-J Ig gene rearrangements suggests that this activity is clonally related and derives from somatically mutated molecules. Indeed, differences in XId and fine specificities between RFs of different origin have been confirmed by the analysis of RF-encoding Ig V genes (35). In addition to a broader use of different nonmutated germline heavy and light chain variable region genes, somatically mutated V genes, suggestive of an Ag-driven response, were found (36).

The accumulation of somatic mutations in Ig V genes forms the molecular basis for the production of Abs with high affinity. Somatic mutations take place in the germinal centers of secondary lymphoid organs and characterize B cells (37). Since germinal center-like aggregates of lymphocytes are a consistent feature in the liver of patients with HCV-induced chronic disease, analysis of Ig V genes amplified directly from lymphoid aggregates of the liver becomes especially important. Like the synovial membrane in patients with rheumatoid arthritis (38), the liver may represent a microenvironment, apart from lymphoid tissue, in which a germinal center-like reaction is induced by HCV infection.

Clonal V-D-J products were amplified from foci of liver B cells. Different foci may derive from different B cells within the polyclonal repertoire of liver-infiltrating B cells, and different foci may therefore contain unrelated B cell clones. The frequent detection of oligoclonal B cell expansions is consistent with this hypothesis, and such oligoclonal expansion may indeed represent a key pathobiologic feature of HCV-associated, nonmalignant B cell lymphoproliferation. The preferential expansion of one clone would in turn lead to a monoclonal pattern. It should be emphasized that this pattern was observed only in patients with cryoglobulinemia. The study of multiple liver biopsies (synchronous and/or metachronous) in a larger series of HCV-infected patients should be relevant to better defining the pattern of B cell clonal expansion in the liver microenvironment. Furthermore, whether clonal B cells are primarily generated in the liver or whether they infiltrate this organ after preselecion in germinal centers of lymph nodes is another critical point to be clarified.

Interestingly, mean age and disease duration, in terms of clinical and biologic signs, were higher in patients with intrahepatic B cell expansion than in those without, suggesting that clonal expansion in the liver may be time related and closely associated with HCV biology. No clonal expansions were found in liver biopsy specimens from age-matched HCV-negative patients without progressive liver disease nor from HBsAg-positive patients with comparable chronic progressive liver damage.

A major question is why consistent RF production occurs in cryoglobulinemic patients only. Possibly, affinity maturation through hypermutation can take place specifically in lymphocytic infiltrates of a subgroup of HCV-infected patients in which there is a failure of the mechanisms for the silencing of higher affinity, potentially pathologic RF-expressing B cells, as recently indicated (39).

Furthermore, it can be inferred that intrahepatic infection of immunocytes induces environmental immunoregulatory defects, which predispose to progressive inflammatory liver damage. Liver B cell expansion associated with the production of RF molecules may be of great pathogenetic relevance, since these molecules react with human class I HLA molecules involved as part of Ag-binding pockets, thus influencing peptide recognition by cell-mediated immune response (40). The role of T cells at the site of lesions has been recently emphasized (41). Intrahepatic T cells have been shown to be clonally expanded. They express particular TCR Vβ gene products, suggesting an HCV-driven intrahepatic immune response.

Based on the present evidence, the possible pathogenetic role of HCV should be better addressed both in indolent stages of B cell lymphoproliferation (putative Ag-dependent), and in malignant lymphoproliferative disorders (putative Ag-independent) (33, 42, 43). A common origin from B cells selected by the triggering Ag may be supposed both in low grade and high grade non-Hodgkin’s lymphomas (44).

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References

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