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*J Immunol* 2001; 167:21-29; ;
doi: 10.4049/jimmunol.167.1.21
http://www.jimmunol.org/content/167/1/21

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Molecular Characterization of B Cell Clonal Expansions in the Liver of Chronically Hepatitis C Virus-Infected Patients

Vito Racanelli,* Domenico Sansonno,† Claudia Piccoli,* Francesca Paola D’Amore,* Felicia Anna Tucci,* and Franco Dammacco2*

PCR DNA amplification of IgH genes was performed on liver biopsy samples of 42 unselected hepatitis C virus (HCV)-positive patients. Genotypic analysis and signal amplification by branched DNA were used to characterize and quantitate HCV RNA genomic sequences. Intraportal lymphoid follicle-like structures were isolated from surrounding hepatocytes by microdissection technique. IgH VDJ PCR products were cloned and sequenced. IgH VDJ gene rearrangements were detected in the liver of 26 (62%) patients. Unequivocal monoclonal or oligoclonal patterns of B cell expansions were found in 14 (33.3%) and 12 (28.6%) patients, respectively. Patients with intrahepatic B cell monoclonal expansions showed liver HCV RNA levels higher than those with oligoclonal or polyclonal features (1106.4 ± 593.5 vs 677.3 ± 424.3 vs 406.2 ± 354.3 pg HCV RNA/g tissue; p = 0.048 and p = 0.001, respectively). Although a single dominant band was obtained with total DNA, characterization of DNA recovered from intraportal inflammatory aggregates resulted in the detection of multiple IgH VDJ gene rearrangements, pointing to an oligoclonal pattern of lymphoproliferation. Cloning and sequence analyses showed that B cell clonalities were differentially distributed in adjacent portal tracts of the same liver area. In addition, HCV RNA genomic sequences could be consistently amplified from each of the portal inflammatory aggregates examined. These data support the concept that in chronic HCV infection the intrahepatic B cell repertoire is frequently clonally restricted and that HCV may have a direct role in sustaining in situ B cell proliferation. The Journal of Immunology, 2001, 167: 21-29.

The most striking feature of hepatitis C virus (HCV) infection is that it tends to become chronic (1). Viral replication occurs despite evidence of both cellular and humoral responses (2, 3). The mechanisms responsible for tissue injury are poorly understood. It is now accepted that HCV is not cytopathic for the cells it infects and that the immune response plays a central role in the pathogenesis of both acute and chronic damage (4). Reinfection may even occur despite an apparently protective immune response, as demonstrated in animal models (5) and in polytransfused patients (6). Failure to generate an effective neutralizing immune response and the persistent production of nonneutralizing Abs result in both an uncontrolled virus spread with cell reinfection and formation of the circulating immune complexes (ICs) involved in many immunopathological aspects of chronic infection (7, 8).

Basically, ICs consist of HCV, IgG anti-HCV Abs, and IgM molecules directed against IgG (9). Approximately one-third of HCV-infected patients have circulating ICs with cryoprecipitating properties. HCV has indeed been implicated as the possible etiologic factor of mixed cryoglobulinemia (MC), a chronic IC-mediated disease with underlying B cell clonal proliferation (10). Morphologically, type II MC is characterized by bone marrow multifocal lymphoid infiltrates of monoclonal B cells (11). Despite the occurrence of a monoclonal component (IgMk) in the serum of patients with type II MC, their bone marrow B cell proliferation investigated at the DNA level showed to be oligoclonal in nature. This supports the concept that MC is a nonneoplastic disorder, in agreement with its indolent clinical course (12, 13).

Lymphoid aggregates morphologically similar to those found in bone marrow of type II MC patients are detected in the liver of chronically HCV-infected patients (14). Inflammatory infiltrates mainly recruited in the portal areas frequently occur as round aggregates of small lymphoid cells. Immunohistochemical characterization have demonstrated that they mainly consist of B cells surrounded by a T cell zone (15). These follicle-like structures often display a well-formed germinal center and may act as true functional follicular structures (16).

In infectious diseases, B cell activation, differentiation, and proliferation occur in the lymphoid follicles of secondary lymphoid organs, such as regional lymph nodes and spleen, or in the so-called “ectopic” germinal centers found in nonlymphoid organs on abnormal sites, namely rheumatoid synovial membrane (17), thyroid gland (18), choroid (19), or lung (20). The liver may indeed be considered an “ectopic” lymphoid organ, in that B cells bearing Ag-specific receptors are stimulated to proliferate and differentiate into Ab-secreting plasma cells within germinal centers of intraportal lymphoid follicles of HCV-positive patients. This concept is consistent with our previous observations conducted in the liver of these patients with and without MC (21). Molecular analysis of the IgH VDJ region on DNA extracted from the biopsy core showed that B cell clonal expansions occurred in almost 60% of HCV-positive patients without MC and in 90% of those with MC.
Analyzes of expanded clones also revealed that D genes were frequently mutated, as compared with the known germline segments (22), pointing to the presence of an Ag-driven process in the B cell growth and clonal evolution.

In this paper, we have defined the frequency of intrahepatic B cell clonal expansions of unselected patients with chronic hepatitis C and compared their presence to the intrahepatic viral load. Cloning and sequencing analyses were also performed on intrahepatic portal lymphocyte clusters isolated by microdissection. Results demonstrate that these clusters are often clonally restricted.

Materials and Methods

Patients

Forty-two consecutive patients (23 male, 19 female) aged 34–72 years (mean 53 years) attending the Liver Diseases Unit of the Department of Internal Medicine and Clinical Oncology of the University of Bari (Bari, Italy) were studied. All had clinical and serological evidence of chronic liver disease (see Table I). Sera obtained at diagnosis were all positive for anti-HCV Abs by ELISA (HCV 3.0; Ortho Diagnostic Systems, Raritan, NJ) and by recombinant-based immunoblot assay (second generation; Ortho Diagnostic Systems) as well as for HCV RNA. All patients were HIV seronegative and heterosexual, with no history of i.v. drug abuse. Twenty of them had been transfused 8–15 years before enrollment into this study, whereas in the remaining 22 patients the source of HCV infection remained undefined. None was positive for serological markers of active hepatitis B virus (HBeAg, IgM anti-HBc Abs), nor for antinuclear or antismooth muscle autoantibodies. None had received corticosteroids or IFN therapy before enrollment. Liver biopsy was performed for diagnostic purposes, and informed consent in writing was obtained from each patient.

Mononuclear cells from bone marrow aspirates and peripheral blood were also obtained from patients with concomitant lymphoproliferative diseases. Seven had type II MC and the purpura-weakness-arthritis syn-drome, and two had an IgG monoclonal gammapathy of undetermined significance (MGUS).

Serum cryoglobulins were determined as described elsewhere (23), and the monoclonal component in the serum or in the cryoprecipitate was characterized by immunofixation (Paragon; Beckman, Fullerton, CA) and classified according to the criteria described by Brouet et al. (24).

DNA purification from frozen liver tissue and circulating or bone marrow-derived lymphocytes by phenol-chloroform extraction according to standard protocols. Samples from heparinized blood and bone-marrow aspirates were immediately used for mononuclear cell isolation by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation. Recovered mononuclear cells (2 × 10^6 cells), liver tissue biopsy specimens (~5 mg), and lymphoid nodules microdissected from portal tracts of fresh-frozen liver sections were processed for DNA preparation according to the method previously described (25). Briefly, samples were digested overnight in a lysis buffer containing 5 μg/ml proteinase K, 1% SDS, 20 mM Tris-HCl (pH 8.0), and 5 mM EDTA (pH 8.0). DNA was extracted by phenol/chloroform in the presence of 300 mM sodium dithiothreitol, which resulted in a single band of 300 bp. In all cases, a radioactive PCR approach, which enhanced the identification of the number of multiple dominant bands in oligoclonal B cell expansions. Radioactive PCR was performed using a labeled nucleotide, i.e., [α³²P]dATP (Amersham, Little Chalfont, U.K.) in the second round of amplification in both Fr2 and Fr3 protocol. The radioactive PCR products were then subjected to a long run (20–40 cm) electrophoresis on a polyacrylamide gel. Dried gels were autoradiographed using a β-max film (Amersham).

For each experiment, a control was included using primers for the β-ac-tin gene, which resulted in a single band of 300 bp. In all cases, a β-actin PCR product was identified by gel electrophoresis, which controlled for quality, quantity, and presence of potential contaminants.

A monoclonal B cell expansion was defined as one or two (if both alleles were rearranged) discrete narrow band(s) within the predicted size. Distinction of biclonal monoallelic rearrangement from monoclonal biallelic rearrangements was based on the results of subsequent sequence analyses, in that a nonfunctional rearrangement of one of the two alleles (i.e., one of the two dominant bands) was detected in the case of a monoclonal disorder, whereas both dominant bands were representative of a functional IgH rearrangement in biclonal disorders.

Cloning and sequencing analyses

PCR products amplified from liver DNA were run on agarose gel in TBE buffer, stained with ethidium bromide and visualized in standard procedures. Individual bands were excised from the gel, cloned, and sequenced. Cloning and sequencing were also performed on bone marrow and circulating lymphocytes obtained from patients with MC. In such cases, individual bands or the entire smear within the VDJ PCR range were cut from the gel. DNA was purified using the QIAEX II gel extraction kit (Qiagen, Hilden, Germany), ligated into a pGem-T cloning vector (Promega, Madison, WI), and transformed into Escherichia coli (Top10F). Transfected cells were plated onto Luria-Bertani-ampicillin agar plates containing 5-bromo-4-chloro-3-indolyl-β-d-galactoside and isopropylthio-galactose. Uncolored colonies were selected at random and cultured. Plasmid DNA was purified with the Wizard Plus Miniprep DNA Purification System (Promega).

All PCR reactions were conducted on an ABI Prism 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA). All sequences were confirmed by sequencing in both directions with primers T7 and SP6. At least 10 different clones were sequenced for each dominant band. Sequences were considered to be related if they shared the same complementarity-deter-mining region (CDR) b, but had differences in the number of point mutations. Where a sequence showed similarity to more than one D region, possible D regions in the CDR b were identified, and those that could be assigned without overlap, and with the smallest number of nucleotides in between, were used.

A clonospecific oligonucleotide probe based on the third CDR DNA sequence labeled at the 5'-end with T4 polynucleotide kinase (Boehringer Mannheim, Mannheim, Germany) and [γ-³²P]ATP were used to detect predominant B cell clones in different areas of the same liver and in different livers. IgH VDJ PCR products of the liver biopsy specimens were blotted on nylon membrane (Pall, Portsmouth, U.K.) and hybridized with the clonospecific oligonucleotide at a melting temperature of 5°C in 5× SSC/7% SDS and washed at the same temperature in 2× SSC/0.2% SDS. Appropriate controls were included. The reliability of the method was ensured by repeating the experiments and comparing recurrent cases with the initial presentation to show identical bands.

To minimize the risk of contamination, each step of tissue digestion, PCR mixture/preparation/reaction, and electrophoresis was conducted in a separate room using a category II laminar air flow cabinet restricted to PCR use together with separate pipettes dedicated to PCR. Samples referred to different specimens, and each specimen to different areas of the liver were processed in different experiments and in different days. Clone sequencing was processed and confirmed in two independent laboratories.

**RT-PCR for HCV RNA**

For qualitative detection of HCV RNA sequences, RNA was extracted from serum, mononuclear cells, and liver tissue. cdNA was synthesized using random reverse transcriptase primers. HCV RNA was detected by
two-stage PCR using primers from the 5′ noncoding region of the HCV genome, as previously described (27).

Genotype of HCV RNA

HCV genotypes were determined by two-stage PCR using universal and type-specific primers from the putative C gene of the HCV genome. The first stage was performed with primers consisting of 5′-TGCCTCAGCGG-3′ (sense) and 5′-ATGATCCCCTATGAGCTGGC-3′ (antisense). The second stage was performed with a sense primer consisting of 5′-AGGAAGATTCGAGGCCTCT-3′ and a mixture of four HCV type-specific antisense primers: 5′-GCTTGGGATAGGC-3′ (type 1a), 5′-GACCATCTTGCCACCCCA-3′ (type 1b), 5′-CAGGAGGACGGGAACCTCC-3′ (type 2a), and 5′-ACCCCTGTTTCGTAACAG-3′ (type 2b). The genotype nomenclature proposed by Simmonds (28) was used.

Intrahepatic measurement of HCV RNA

HCV RNA levels were measured by signal amplification with a branched DNA probe assay (Quantiplex; Chiron, Emeryville, CA) whose detection limit was 200,000 HCV genome equivalents (Eq)/mL. RNA was recovered from −5 mg liver tissue specimens for all patients. Samples were stored at −80°C without any preservative solution. Then 0.5 ml of cold guanidine-HCl homogenizing solution (8 M guanidinium thiocyanate, 50 mM Tris-HCl, pH 7.5, 25 mM EDTA, 8% (v/v) 2-ME containing 3 M sodium acetate, pH 5.2) was added to the frozen tissue and homogenized with a pellet pestle mixer. Next, 0.025 ml 10% Sarkosyl was added to each tube and gently mixed. After 5 min, tubes were centrifuged to sediment particulates. Supernatants (0.5 ml) were removed and added to tubes containing 0.01 ml of poly(A) (10 mg/ml). Then 0.25 ml of 100% ethanol was added to each tube and mixed very thoroughly. Tubes were placed at −20°C overnight and then centrifuged for 20 min at 4°C. Supernatants were aspirated, and the pellets were dried down with a Speedvac rotatory vacuum device (Eppendorf-Netheler-Hinz, Hamburg, Germany). After solubilization in nuclease-free H2O, HCV RNA was measured. Duplicate samples were added to the wells in which lysis, hybridization, capture, and signal amplification occurred. A mixture of synthetic oligonucleotides, which included probes that mediated capture and probes that bound to the basic DNA amplifier molecule, hybridized equally well to the highly conserved 5′ noncoding and core regions of the HCV RNA of all known genotypes, thereby capturing the RNA molecules onto the surface of a microwell plate and linking the target to synthetic basic DNA molecules added to the well. Multiple copies of an alkaline phosphatase-linked synthetic probe hybridized to the immobilized complex, resulting in amplification of the target signal. Detection was achieved by incubating the complex with a chemiluminescent reagent, resulting in the formation of a chemiluminescent signal. Detection was achieved by incubating the complex with a chemiluminescent reagent, resulting in the formation of a chemiluminescent signal. The standard curve was constructed from a diluted sample from a patient whose serum HCV RNA had been quantitated by a highly purified RNA transcript covering the first 3200 nucleotides from the 5′ end of the HCV genome. Results were expressed in picograms per milliliter by multiplying the conversion factor 0.52 obtained by dividing HCV RNA molecular mass by the number of viral copies/mL (3.13 × 10^10 copies/mL) × 10^7). Based on the weight of liver processed, results were normalized to picograms HCV RNA per gram of biopptic specimen. When considering bone marrow-derived mononuclear cells and PBMC, picograms were divided by the number of cells and results were normalized to picograms HCV RNA per gram of biopptic specimen.

Micromanipulation of liver tissue sections

Crystalline sections from patients 1 and 10 showing a single band feature in PCR IgH VDJ gene rearrangement were micromanipulated and harvested under an inverted microscope (model IX-50; Olympus, Tokyo, Japan) equipped with ×4, ×10, and ×40 long-distance lenses. Two three-dimensional hydraulic micromanipulators equipped with a joystick (Narishige, Tokyo, Japan), a reflex camera, and a TV monitor were used. Manipulation tools consisted of a reception pipette prepared from glass capillaries (model GD-1; Narishige) through a vertical pipette puller (model P-87; Sutter, Novato, CA) and a manipulator rod (model RG-1; Narishige). The agarose gel was produced by excising a 3 mm × 3 mm × 0.5 mm block from a 1% agarose gel. HCV RNA sequences were successively amplified in all specimens, either in bone marrow or circulating lymphocytes.

Both cloning and sequence analyses of the single dominant band showed that for each patient of group 1, each tissue compartment yielded a different VDJ sequence, and was therefore dominated by a different B cell clone (Fig. 2).

Results

The main clinical and laboratory features of 42 unselected chronically HCV-infected patients with histologically proven chronic active liver disease in relation to the presence of intrahepatic B cell clonal expansions are summarized in Table I. All patients were shown to harbor productive infection by repeated confirmation of anti-HCV positivity and HCV viremia. Analysis of IgH VDJ gene rearrangement was performed on DNA extracted from liver biopsy specimens and compared with intrahepatic HCV RNA levels on the same specimens. According to PCR-based criteria, the occurrence of one or two distinct bands, considered diagnostic of B cell monoclonality, was found in 14 (33.3%) patients (group 1), whereas oligoclonality, defined as three or more bands, was displayed by 12 (28.6%) patients (group 2). The presence of a smear of products suggestive of polyclonality was detected in the remaining 16 (38.1%) patients (group 3).

Group 1 patients showed a lower mean age (50.8 ± 8.5 vs 53.8 ± 3.7 and 59.8 ± 10.2 years, mean ± SD for group 2 and group 3) and a shorter duration of liver disease (5.8 ± 2.5 vs 5.8 ± 4.1 and 8.9 ± 5.5 years; mean ± SD). No significant differences in terms of serum alanine aminotransferase were found (group 1, 94.3 ± 38.0 IU/ml; group 2, 77.8 ± 69.9 IU/ml; group 3, 99.8 ± 78.7 IU/ml, mean ± SD). A similar distribution of superimposed cirrhosis occurred: it was diagnosed in three (21.4%) patients of group 1, two (16.7%) of group 2, and four (25%) of group 3. In addition, the distribution of HCV genotypes showed no significant difference, type 1b being prevalent in each group.

The overall incidence of associated MC and MGUS was significantly higher in patients with intrahepatic B cell expansion than in those without (42.3% vs 6.2%, p = 0.01). Type II MC occurred in five (35.7%) patients of group 1, five (41.7%) of group 2, and none of group 3. IgGk and IgGλ MGUS were found in one (8.3%) patient of group 2 and one (6.3%) of group 3.

Intrahepatic HCV RNA levels were analyzed in relation to intrahepatic B cell clonal expansion (Fig. 1). Group 1 patients showed significantly higher mean values than group 3 (1106.4 ± 593.5 vs 406.2 ± 354.3 pg/g, mean ± SD, p = 0.001) and group 2 (677.3 ± 424.3 pg/g; mean ± SD, p = 0.048). No direct relation was established between intrahepatic levels of HCV RNA and the occurrence of type II MC either in group 1 (p = 0.38) or in group 2 (p = 0.07).

The relationship between the biological compartments was studied in three patients with type II MC (1, 9, and 10) of group 1 and four patients (15, 16, 17, and 18) of group 2. PCR assay for IgH VDJ gene rearrangements and HCV RNA genomic sequences were performed on nucleic acids of lymphocytes recovered both from bone marrow aspirates and peripheral blood and compared with features displayed by liver tissue ampiclons. The agarose gel electrophoretic patterns of PCR products revealed similar profiles of B cell clonal expansion in the three compartments in each patient. A single dominant band in the appropriate base pair range was found in the liver, bone marrow, and circulating lymphocytes from each patient of group 1. All randomly examined cloning products of each band displayed an identical nucleotide sequence. On the contrary, multiple bands were detected in the three compartments in the four patients of group 2. Furthermore, HCV RNA genomic sequences were successfully amplified in all specimens, either in bone marrow or circulating lymphocytes.

Both cloning and sequence analyses of the single dominant band showed that for each patient of group 1, each tissue compartment yielded a different VDJ sequence, and was therefore dominated by a different B cell clone (Fig. 2).
PCR has been increasingly used for the rapid detection of gene rearrangements during B lymphocyte development. Even so, considerable variations in many aspects of its experimental design lead to marked differences in its results. One of the major problems of this technique is the large number of false negatives. It can be assumed that consensus primers currently used amplify the CDR3 of most B cells, but the products of a small clonal population may be obscured by the smear of polyclonal B cells in the sample. This drawback becomes particularly critical when considering total DNA extracted from the entire bioptic samples, which contain a mixture of normal and abnormal nucleic acids that likely interfere with the molecular analysis of cells of interest.

To overcome this critical point, IgH VDJ gene rearrangement and HCV RNA were assayed on DNA and RNA, respectively, extracted from portal tract-containing lymphoid aggregates directly isolated from liver biopsy sections. Fine microdissection of fresh-frozen sections of two of three patients (1 and 9) showing B cell monoclonality was used to precisely separate inflammatory cells from the surrounding contaminating hepatocytes. Lymphoid follicle-like structures were isolated from the remaining infiltrating mononuclear cells in the portal tract, as depicted in Fig. 3.

Patterns of B cell clonalities from three portal tracts were compared with those from the entire liver tissue. Results showed that despite one distinct band obtained on total DNA, oligoclonal features of B cell expansions were demonstrated in intraportal inflammatory cells. Multiple amplicons of different size were present in two of the three portal tracts of patient 1. A smear with no distinct band compatible with a fully polyclonal profile was found in the third. An oligoclonal feature was also demonstrated in the portal tract isolated from patient 9.

A further significant aspect of PCR analysis of microdissected samples was that HCV RNA genomic sequences were successfully amplified in each inflammatory aggregate (Fig. 4 A). The differences from the putative germline genes in the D\textsubscript{H} region sequences were identified and analyzed in relation to the portal tracts. Results showed that the prevalent clone was homologous

### Table I. Clinical, laboratory, virological, and histological parameters in 42 chronically HCV-infected patients

<table>
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<tr>
<th>Patients</th>
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<th>Age (yr)</th>
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<th>Liver Disease Duration (yr)</th>
<th>Liver Histology</th>
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\textsuperscript{a} CAH, Chronic active hepatitis; C, cirrhosis.
to the D3-22 germline, and in both patients it shared unique mutations and identical CDR3 rearrangements (Fig. 5). To assess specificity of the results, PCR-derived products hybridized with a clonospecific oligoprobe using oligonucleotide specific for the CDR3 sequences to detect the same B cell clone originated in patients 1 and 9. A positive hybridization signal was detected with DNA extracted either from the core liver biopsies or from microdissected portal tracts in both patients. No hybridization was detected in the liver samples that lacked D3-22-related genes. (Fig. 4B). To further exclude nonspecific hybridization signals, both the hybridization protocol and washing temperatures were optimized in repeated experiments.

Discussion
Molecular analyses of B cell clonalities in liver tissue of patients with chronic HCV infection have corroborated previous indications that B lymphocytes recruited at the disease sites are activated and clonally expanded (21, 29). The present study shows that monoclonal or oligoclonal B cell expansions were established in 62% (26/42) of unselected patients with HCV-related chronic active liver disease. From a clinical point of view, the presence of intrahepatic frank B cell monoclonality defines a subgroup of HCV-infected patients with a lower mean age and a shorter duration of liver disease than those with a polyclonal profile of intrahepatic B cell infiltrates.

Measurement of intrahepatic HCV RNA levels confirms conclusions of other studies, namely that hepatic viral load does not distinguish patients with more severe liver damage from those with higher serum alanine aminotransferase levels, and probably reflects the lack of a direct contribution of ongoing viral replication

![FIGURE 1](Relation between tissue HCV RNA levels and profiles of B cell clonalities in the liver of HCV-infected patients.)

![FIGURE 2](Molecular analysis of B cell clone expansions in different biologic compartments in three HCV-infected patients with concomitant type II MC. Lower case shows changes from the germline sequence identified in the EMBL/GenBank database.)
to the liver cell injury (30, 31). The direct relation between intrahepatic B cell monoclonal feature and viral load suggests that they are closely related events and is consistent with the notion that HCV plays a direct role in stimulating and maintaining in situ expansion of these B cell clones.

With respect to patients associating type II MC, intrahepatic levels of HCV RNA were found to lack a direct correlation with cryoglobulin production, further suggesting that the occurrence of cryoglobulinemia is an independent variable of HCV infection (13).

In the first part of this study, we investigated the B cell repertoire in different biologic compartments in three patients showing a gel electrophoretic monoclonal profile of intrahepatic B cell expansion and concomitant type II MC. Conclusions that emerged when B cell clonal profiles either from peripheral blood or bone marrow were compared in the same patient provided evidence that monoclonal profiles of B cell expansion do occur in each compartment.

Features of monoclonal B cell expansions appeared in the liver just when examined on total DNA extracted from the core biopsy specimen. When analysis was performed on DNA obtained from microdissected portal lymphoid infiltrates directly isolated from the relative cryostat sections of the same liver biopsy, definite oligoclonal pictures were achieved. Multiple IgH bands, whose number likely corresponded to the number of proliferating B cell

FIGURE 3. Example of microdissection. A, Section of liver biopsy. B, High magnification showing enlarged portal tract with heavy inflammatory infiltrate and lymphoid aggregates with appearance of follicle structure. C and D, Dissection of lymphoid follicle-like structure is halfway complete with the tip of dissecting needle clearly visible. E, Dissection is complete and a cleared area remains in the context of the inflammatory cluster. F, The target tissue is mobilized and processed for molecular analyses.
clones, were consistently demonstrated. This, indeed, likely reflects methodological problems mainly related to the poor efficiency of the PCR assay used for detecting IgH VDJ gene rearrangements. IgH VDJ PCR sensitivity mainly depends on the proportion of polyclonal B cells present in the sample. This issue was clearly demonstrated by the present experiments, in that cloning and sequence analyses showed that the B cell clone obtained from total DNA was included in those present in the portal tract inflammatory aggregates.

It was of interest that the same B cell clonotypes that expanded commonly in different areas of the same liver had identical size and identical nucleotide sequence in the liver samples from the two patients we studied (1 and 9). These results strongly suggest that common B cell clones in HCV-related lesions are likely induced by a relatively restricted number of Ags. Furthermore, it can be emphasized that common clonotypes can be the result of mutational and division of the same type of B cells, revealing the relatively conserved sequence motifs in CDRH3 region.

The presence of identical clones in these samples might have resulted from contamination during DNA processing. However, the isolation of rearranged D genes from a single clone is not a fake due to selection bias during the PCR amplification, as shown by identification of multiple independently rearranged D genes. All samples were processed in duplicate, and the utmost care was taken to prevent contamination (30). Previous limiting dilution experiments suggested that when the starting DNA concentration is

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**FIGURE 4.** A. Agarose gel electrophoresis of IgH VDJ PCR products (Fr3 protocol) and HCV RNA genomic sequences in the liver samples of patients 1 and 9. Monoclonal B cell expansion is shown in lanes 3 and 7, which refer to PCR products of DNAs extracted from entire liver biopsy tissues of patients 1 and 9, respectively. B cell clonal profiles shown in lanes 4 and 5 (portal tracts A and B, respectively; oligoclonal B cell expansion showing >2 dominant bands within the predicted size range, 80–120 bp) and lane 6 (portal tract C, showing a fully polyclonal pattern; smear with no dominant band). A B cell oligoclonal feature is shown in lane 8 corresponding to the lymphoid aggregate isolated from the portal tract of patient 9. HCV RNA genomic sequences amplified in entire liver biopsy are reported in lanes 11 and 15 for patients 1 and 9, respectively. HCV RNA amplificates are reported in lanes 12–14 for portal tracts of patient 1 and in lane 16 for that of patient 9. Lane 2, Positive control (DNAs from B cell non-Hodgkin’s lymphoma). Lane 9, Negative control with no DNA in the PCR mixture. Lane 1, Molecular mass markers (HaeIII-digested DNA of phiX 174). Lanes 17 and 18, Negative and positive controls of RT-PCR for HCV RNA assay performed on well-characterized HCV-negative and -positive liver tissues, respectively. B, Clonospecific oligoprobe hybridization of the PCR products with a radiolabeled oligoprobe based on the CDRH3 sequences of the D3-22-related gene segment (5’-GGATTACTCTGATAGTAGTGATTCTACTTCAAT-3’). Under stringent experimental conditions, positive hybridization signals were detected in entire liver tissue specimens and in portal tracts of both patients. No signal was seen in lane 10 with a DNA segment lacking D3-22 gene (IgH VDJ PCR product obtained from DNA of entire liver tissue sample of patient 10).

**FIGURE 5.** Nucleotide sequence of VDJ gene region of portal tract lymphoid aggregates isolated by microdissection. The closest identified germline genes are specified in parentheses. Lower case shows changes from the germline sequence.
very low, there is preferential amplification of some Ig genes so that one or few monoclonal bands may be detected in polyclonal DNA. The microdissection technique by which we have been able to isolate the smallest lymphocytic structural units in the portal tract of liver tissue sections likely increased PCR sensitivity. Negative controls were conducted through the entire DNA extraction and PCR protocol for each sample. Tissue specimens were divided into two separate samples in the early stages of processing as an added precaution to provide back-up samples, and a clonospecific oligogrope based on common CDR\textsubscript{3} DNA sequence specifically hybridized with amplified PCR products.

The D\textsubscript{H} regions of our four panels share some structural features with those that display few random mutations compared with germline D sequences, suggesting that they are daughters of a parental cell that divided within the liver. This implies that intrahepatic clones develop by proliferation of a common precursor accompanied by IgV gene somatic diversification. Indeed, the origin of the parental B cell from which the clones arose is not known, but the small number of mutations in D3-22 germline-related clones found in the portal tract clusters suggests that it arose from a naive B cell, whereas those more heavily mutated may have arisen from memory B cells.

Our technique may not have been sensitive enough to detect a single germline gene in the microdissected clusters. Even so, the present findings suggest that there is a marked heterogeneity in H chain gene usage by individual patients. Hypermutation within a germinal center is closely related to Ag-induced B cell proliferation, so it is reasonable to deduce that the response within the liver is also driven by multiple Ags. In this context, of particular interest are the findings that HCV RNA genomic sequences could be amplified in each microdissected intraportal lymphoid cluster in support of the evidence that HCV is directly involved in the pathogenesis of B cell clonal expansions. The present occurrence of HCV in the inflammatory cells of portal tracts suggests that viral Ags are necessary for the continuous maintenance of B cell clones. Though no formal proof exists that this reflects direct infection of B lymphocytes, it is likely to explain the possible attachment of viral particles by means of cell surface molecules, perhaps CD81 determinants, a member of tetraspanin family, which is considered to represent the putative HCV receptor (31). Interactions with components of the B cell receptor complex would switch on a signal complex to sustain B cell activation (32).

If confirmed by future studies using direct Ab characterization, these findings may have substantial pathobiologic implications because it can be assumed that the intrahepatic B cell response is mainly not polyclonal and that selected Ags are preferentially recognized, thus conferring a strong survival advantage on a distinct B cell subset (i.e., RF-positive cells) already implicated in type II MC and HCV-related lymphoproliferation (33).

This study extends and reinforces previous observations in that the clonally restricted B cell response appears to be a hallmark of HCV infection. Although we have no data on "genuine" autoimmune chronic hepatitis, tissue B cell clonal restriction was rarely detected in HBV-related chronic liver disease (15, 21, 22).

The relationships between the natural pathobiology exhibited by the virus and the cellular origin of B cell clonal restriction must be considered in the context of a limited host response to a pathogen capable of undergoing long-term spontaneous mutations that heavily contribute to the viral burden (34). This Ag overload could initially expand a normal B cell response. Saturation of the APC system could also paralyze subsequent recognition of closely related evolving virus populations. If an array of cross-reactive viral epitopes exists, their presence continues to expand the initially derived and "higher affinity" B cell clones (35). In contrast, this mechanism might constitute a trigger for subsequent transforming events. Although the bearing of B cell clonal expansion on malignant transformation is not clear, it seems reasonable to envisage a predisposing role.

References


