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Accumulation of B Lymphocytes with a Naive, Resting Phenotype in a Subset of Hepatitis C Patients

Jianhua Ni,* Edgardo Hembrador,† Adrian M. Di Bisceglie,‡ Ira M. Jacobson,§ Andrew H. Talal,§ David Butera,* Charles M. Rice,* Thomas J. Chambers,† and Lynn B. Dustin²

Chronic infection with hepatitis C virus (HCV) is associated with disturbances of B lymphocyte activation and function: autoantibody production, mixed cryoglobulinemia, and B cell lymphomas. It has been proposed that these abnormalities reflect chronic antigenic stimulation or aberrant signaling through the B cell coreceptor, the latter mediated by binding of the HCV E2 glycoprotein to CD81. To test this hypothesis, we measured expression of activation and differentiation markers on peripheral blood B cells from patients with chronic HCV infection. Thirty-six HCV patients with and without mixed cryoglobulinemia were compared with 18 healthy control volunteers and 17 sustained virologic responders who had cleared HCV infection. Ten of the 36 HCV patient samples showed increased B cell frequencies; B cell frequency was higher in patients with more severe hepatic fibrosis. However, these samples lacked evidence of Ag-driven activation or proliferation. The expanded cells were low in the activation markers CD25, CD69, CD71, CD80, and CD86. Proliferation of circulating B cells was unchanged in HCV patients. These cells did not express the differentiation marker CD27, suggesting that they were not enriched in memory B cells. Furthermore, the expanded B cells expressed both IgD and IgM, suggesting that they were antigenically naive. Together, these results indicate that B cell expansion in the peripheral blood of HCV patients is not associated with Ag-mediated activation and differentiation. Instead, factors other than antigenic stimulation may promote the accumulation of peripheral blood B cells with a naive phenotype in a subset of HCV patients. The Journal of Immunology, 2003, 170: 3429–3439.

A

pproximately 170 million people worldwide are chronically infected with the hepatitis C virus (HCV) (reviewed in Ref. 1). Chronic infection can progress to cirrhosis, hepatocellular carcinoma, and liver failure, and HCV infection is now the leading indication for liver transplantation in the United States. In many patients, the liver graft is quickly reinfected with HCV and it, too, may fail. While combination therapy with pegylated IFN and ribavirin offers improved results, sustained viral clearance remains an unachieved goal in many patients (2). Both virus and host factors can contribute to poor therapy responses. Thus, IFN therapy is more successful for patients infected with HCV genotypes 2 or 3 than for those infected with genotype 1, the most common genotype in the United States (3). African-American (AA) patients are less likely to clear the virus during IFN treatment than are Caucasian patients (4, 5).

Studies of patients and chimpanzees who spontaneously clear acute HCV infection indicate that successful control of the infection requires a broad response of helper and cytolytic T cells (6–8). While Abs are produced in HCV infection, and likely mediate selective pressure on viral sequences (9), few studies have demonstrated an important role for the humoral immune response in the control or clearance of infection. Although control of HCV infection is likely to be largely T cell dependent, this infection is often associated with puzzling alterations in B cell function. Seroconversion, the development of Abs to HCV, lags behind viremia by weeks (10–13). The reason for delayed seroconversion is unknown. Mixed cryoglobulinemia (MC), with or without overt symptoms, occurs in a substantial fraction of patients (14, 15). It has been reported that almost all patients with symptomatic type II or type III MC are also infected with HCV (16). These observations suggest a causal link between HCV infection and MC. Sjögren’s syndrome may occur in a substantial fraction of HCV patients as well (17). More controversial is the association between HCV infection and B cell non-Hodgkins lymphomas. Patients with B cell lymphoma are reported to have higher than expected rates of HCV infection in some studies, but not others, suggesting that other factors may contribute to lymphomagenesis (18–24). MC may accompany low-grade or occult non-Hodgkins lymphoma (25) or may progress to overt lymphoma (26). In support of a direct role for HCV in the pathogenesis of B cell lymphomas, it was recently reported that eradication of HCV following IFN treatment was correlated with regression of splenic lymphoma with villous lymphocytes in seven out of seven patients with complete IFN responses (27). Transient or partial remissions of the lymphoma occurred in the two other patients in this study, whose viral infections did not respond completely to antiviral therapy. Some B lymphomas in HCV patients may express HCV-specific Ag receptors,
suggesting a role for chronic antigenic stimulation in the development of these malignancies (28).

The mechanisms by which HCV infection leads to MC and lymphoma are not yet known, nor is it known whether these conditions are related to delayed seroconversion. These abnormalities may result from direct modulation of B cell functions by viral proteins, from infection of B cells, from chronic antigenic stimulation in the context of a chronic infection, or from other, indirect effects of viral infection.

The E2 envelope glycoprotein of HCV has been reported to interact with CD81 (29). CD81 is a widely expressed member of the tetraspanin family of proteins and may function as a “molecular facilitator,” promoting a network of interactions of other molecules in the plasma membrane (30). Thus, CD81 is found in association with a variety of molecules functioning in cell adhesion, signal transduction, and/or Ag presentation on different cell types (30, 31). In B cells, this molecule is a component of the B cell coreceptor complex, which links Ag recognition to recognition of the complement breakdown product C3d. Coligation of the B cell Ag receptor and the B cell coreceptor by Ag-complement complexes is believed to dramatically increase B cell Ag responsiveness (reviewed in Ref. 32). It can be proposed, then, that HCV binding to CD81 may mimic B cell coreceptor engagement and reduce the threshold for B cell activation, thereby contributing to polyclonal B cell activation.

Others have reported that anti-CD81 or recombinant HCV E2 glycoprotein can enhance T cell activation (33), inhibit NK cell activation (34, 35), or inhibit proliferation of a transformed B cell line in vitro (36). These observations support the notion that HCV may act on lymphocyte function by virtue of its ability to bind to CD81. However, the affinity of CD81 binding by E2 may vary considerably among different virus genotypes (37, 38). Furthermore, these studies have been restricted to in vitro manipulations. Whether the changes observed in vitro following treatment with anti-CD81 or recombinant HCV E2 correspond to actual in vivo changes has not been demonstrated. We hypothesized that chronic antigenic or polyclonal stimulation would lead to an increase in the frequency of B cells with an activated phenotype in HCV patients. To test this hypothesis, we compared B lymphocytes directly ex vivo from HCV patients to B lymphocytes from normal controls. The changes observed in HCV patient B cells are not easily explained either by a model involving CD81 ligation by HCV E2 glycoprotein, or by chronic antigenic stimulation.

Materials and Methods

Samples

The details of these studies were approved by the Institutional Review Boards at Saint Louis University School of Medicine, Weill Medical College of Cornell University, and The Rockefeller University. All donors gave written informed consent before blood was drawn. Thirty-six HCV patients, and 17 patients who had cleared HCV infection following antiviral therapy (sustained virologic responders; SR), were recruited by their hepatologists. SR were identified by their hepatologists as those patients whose serum HCV levels remained below the limit of detection by PCR for 6 mo at the time of viral therapy (39) and classified as detailed by Simmonds et al. (40).

Clinical tests

HCV RNA was quantified in clinical laboratories by the Roche Amplicor assay (Roche Diagnostics, Branchburg, NJ); results are standardized to international units (IU). HCV genotypes were determined by sequencing of the NS5B region (39) and classified as detailed by Simmonds et al. (40). Rheumatoid factor (RF) was measured by standard assays (42). To identify patients with MC, blood was allowed to clot at 37°C; serum was then incubated at 4°C and examined for the presence of a cryoprecipitate (CP) (43). Qualitative results for RF and CP are given here because of the use of different clinical laboratories in different cities. Liver biopsies were evaluated by pathologists according to well-accepted standards for the documentation of fibrosis (44) and inflammatory activity (45).

Antibodies

The following Abs were used for flow cytometry. FITC anti-CD3 (clone UCHT1), PE anti-CD4 (clone RPA-T4), allophycocyanin anti-CD5 (clone UCHT2), biotin anti-CD19 (clone HIB19), allophycocyanin anti-CD21 (clone B-ly4), allophycocyanin anti-CD25 (clone M-A251), PE anti-CD27 (clone M-T271), PE anti-CD38 (clone HIT2), allophycocyanin anti-CD69 (clone FN50), PE anti-CD71 (clone M-A712), FITC anti-CD80 (clone L307.4), PE anti-CD86 (clone 2331), FITC anti-human IgD (clone IA6-2), biotin anti-human IgM (G20-127), FITC anti-human IgG (clone G20-127), and streptavidin PerCP were obtained from BD PharMingen (San Diego, CA).

Flow cytometric analysis

Cells (2.5 × 10^6/sample) were stained with the indicated Abs in PBS supplemented with 2% (v/v) BSA (Fraction V; Fisher Biotech, Fair Lawn, NJ) and 0.02% NaN3. Stained cells were fixed for a minimum of 2 h in PBS/2% parafomaldehyde before analysis on a FACSCalibur flow cytometer equipped with argon and dye-head lasers (BD Immunocytometry Systems, San Jose, CA). Lymphocytes were identified by forward and side-angle light scatter characteristics. At least 20,000 lymphocyte-gated cells were analyzed for each sample.

Cell cycle analysis

CD19+ B lymphocytes were magnetically purified from PBMCs using the CD19 MicroBeads purification kit (Miltenyi Biotech, Auburn, CA) according to the manufacturer’s protocol, except that cryopreserved PBMCs were thawed for use as starting material. B cells obtained from this kit were >95% pure (data not shown). Cell cycle analysis was performed on MACS-purified B cells by a modification of published methods (46). Purified B cells were fixed overnight in ice-cold 70% ethanol, spun down, and resuspended in 0.1% (w/v) sodium citrate, 0.3% (v/v) Igepal, pH 7.8, containing 20 μg/ml RNase A and 50 μg/ml propidium iodide. Cells were stained at 37°C and kept on ice until analysis. A minimum of 40,000 cells was analyzed on a FACSCalibur. Coefficient of variation (CV) values were routinely well below 8%, as required for accurate S-phase assessment by the cell cycle analysis software, ModFit LT (Verity Software House, Topsham, ME).

Data analysis

Flow cytometric data were collected using CellQuest software (BD Immunocytometry Systems) and analyzed using FlowJo (Tree Star, San Carlos, CA). Cell cycle data were analyzed using ModFit LT (Verity Software House). Statistical analyses were performed using Prism (GraphPad Software, San Diego, CA). Nonparametric statistics were used because many of the data were not normally distributed. Tests used included the Mann-Whitney test for comparisons of groups, the Wilcoxon signed-rank test for paired comparisons, and the Spearman rank sum test for correlations. Contingency tables were analyzed by Fisher’s exact test. Two-tailed p values were calculated for all comparisons.

Results

HCV patient population

Patients with chronic HCV infection were recruited from the liver clinics at Saint Louis University Hospital (St. Louis, MO) and New York Presbyterian Hospital (New York, NY). Blood samples were drawn after informed consent was obtained. Eighteen healthy volunteer donors provided control samples. Patients were therapy-naïve or had been off antiviral therapy for >6 mo at the time of...
sampling. Those patients who were off therapy remained infected with HCV. Patient characteristics are summarized in Table I. Twenty male and 16 female patients participated; their ages ranged from 18.6 to 71.3 years. Most (27 patients) were infected with HCV genotype 1a, but genotypes 1b, 2a, and 2b were also represented. Patients were negative for anti-HIV and for hepatitis B surface Ag, except for patient 33, who was seropositive for hepatitis B surface Ag. HCV infection is more difficult to treat in AA patients (4, 5), six of whom participated in this study.

Because HCV patients may differ from healthy controls in lifestyle or health history, a control group of SR was also studied. These individuals, who had been treated successfully for HCV infection, were expected to resemble current HCV patients in terms of risk factors that may have led to the acquisition of HCV infection. SR in this study were all Caucasian; 10 were male, and 7 were female. Their average age was 49 years. They had previously been infected with HCV genotypes 1a (4 patients), 1b (6 patients), 2a (2 patients), 2b (3 patients), and 3a (1 patient). In one former patient, the genotype with which he had been infected was unknown.

Table I.  Patient demographic and clinical data

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* Grade, Histological assessment of inflammation.
* a Stage, Histological assessment of fibrosis.
* b % CD19, Frequency of CD19 B cells among PBMCs.
* c % CD21, Frequency of CD21 B cells among PBMCs.
* d C, Caucasian.
* e Neg, Negative.
* f ND, Not done.
* g Values in italics are above the 75th percentile for the patient group and are higher than all values obtained from normal controls (p < 0.0001 by Mann-Whitney test).
* h Pos, Positive.
In controls, the frequency of CD19\(^+\) B cells ranged from 4.68 to 19.9% of gated lymphocytes (mean 13.02%, SD 4.18). The range in HCV patients was much greater, with CD19\(^+\) B cell frequencies ranging between 1 and 34.9% of gated lymphocytes (mean 15.82%, SD 7.31). Among SR, CD19\(^+\) B cells made up between 6.1 and 22.0% of gated lymphocytes (mean 14.09%, SD 4.31). Ten of the 36 patients analyzed, but only one of the SR and none of the healthy controls, had CD19\(^+\) B cell frequencies of >20%. Three of the six AA patients had CD19\(^+\) B cell frequencies of >20%. B cell frequencies were higher overall in the AA patients (mean 18.39%) than Caucasian patients (mean 15.4%), but this difference was not statistically significant (p = 0.329). Population variances were compared as a measure of the range of values obtained. The variance of the HCV patients' B cell frequency data was greater than that for the normal controls (p = 0.008) or the SR population (p = 0.014). The variance of the SR patients' B cell frequency data was not significantly different from that for the normal controls (p = 0.442). Thus, HCV patients showed greater heterogeneity than the normal controls or SR in the frequency of B cells within a lymphocyte-gated PBMC population.

CD21 staining gave similar results in most patients (Table I and Fig. 1C). In normal controls, between 4.8 and 19.3% of gated lymphocytes stained with anti-CD21 (mean 12.78%, SD 4.33); in HCV patients, between 0.84 and 36% of gated lymphocytes stained with anti-CD21 (mean 14.12%, SD 7.32). Three of the 17 SR had CD21\(^+\) B cell frequencies of >20%. While the means for the three groups were not significantly different, their variances were (p = 0.018). Of note, in most HCV patients, the frequency of CD21\(^+\) B cells was lower than that of CD19\(^+\) B cells (Wilcoxon signed rank test, p < 0.0001). There was no significant difference between the frequencies of CD19\(^+\) and CD21\(^+\) B cells in either normal controls or SR.

These results were confirmed in patients with and without positive CP or RF tests (Fig. 1D). Nine out of 27 patients infected with HCV genotype 1a had elevated B cell frequencies, but elevated B cell frequencies were also seen in 1 out of 6 patients infected with HCV genotype 1b and 1 out of 2 patients infected with genotype 2b (Fig. 1E). The presence of genotype 1a infection was not a significant predictor of increased B cell frequency (Table II). There was also greater heterogeneity in the frequency of circulating CD3\(^+\) T cells among patients (Fig. 1F), but the difference in variances was not statistically significant (p = 0.24). T cell frequencies did not vary in any way that was connected to B cell frequencies in either normal or HCV-infected samples (Fig. 1G).

Because most of the patients had been tested for the presence of CP and/or RF, we compared the frequency of B cells among those with and without these features of altered B cell function (Table I and Fig. 1D). Among the 18 patients who had a positive test result for CP and/or RF, CD19\(^+\) B cell frequencies ranged between 6.75 and 34.4% of total PBL. Five patients were confirmed to have both CP and RF; surprisingly, their peripheral blood B cell frequencies were all in the normal range. Of the five patients in whom both tests were performed and in whom both CP and RF were negative, all had B cell frequencies outside of the normal range. However, when all of the patients with only negative test results were factored in, there was not a significant difference between the groups with positive and negative test results. A total of 15 patients had negative results in whichever of these tests had been performed in the clinic, and the frequency of CD19\(^+\) B cells in this group ranged from 1 to 31.9% of total lymphocytes. Thus, the presence of a positive laboratory test for RF or CP did not correlate with peripheral B cell expansion in these patients (Fig. 1D and Table I).

**Correlation between disease severity and B cell frequency**

Disease severity was monitored by liver biopsy as a part of patient care. Most patients had moderately severe hepatic damage, with an average histological activity grade of 2.19 and an average fibrosis stage of 2.23, both on a scale of 0–4 (44, 45) (Table I). A correlation was noted between the degrees of inflammation and fibrosis and the frequencies of circulating B lymphocytes (Fig. 2). Increased B cell frequencies were seen only in patients with both grade 2 or greater inflammation and stage 2 or greater fibrosis (Fig. 2C). Patients with milder inflammation (grade 1) had B cell frequencies in the normal range, even if significant fibrosis was present. Similarly, patients with milder fibrosis (stage 1) had B cell frequencies in the normal range, even if significant inflammation was present. Five of the 9 patients whose biopsies revealed grade 3 inflammation had B cell frequencies of >20%. Four of the 8 patients whose biopsies revealed stage 3 fibrosis also had elevated B cell frequencies. Three out of three patients with stage 4 fibrosis...
Table II. **Comparison of patients with and without increased B cell frequency**

<table>
<thead>
<tr>
<th>Patient Characteristic</th>
<th>B Cells &lt; 20%</th>
<th>B Cells &gt; 20%</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)(^b)</td>
<td>47.6 ± 11.6</td>
<td>46.1 ± 10.2</td>
<td>0.75(^c)</td>
</tr>
<tr>
<td>Sex</td>
<td>13 M/13 F</td>
<td>7 M/3 F</td>
<td>0.46(^d)</td>
</tr>
<tr>
<td>Race</td>
<td>23 C/3 AA</td>
<td>7 C/3 AA</td>
<td>0.32(^e)</td>
</tr>
<tr>
<td>Genotype 1a vs others</td>
<td>18 (genotype 1a)</td>
<td>9 (genotype 1a)</td>
<td>0.39(^f)</td>
</tr>
<tr>
<td></td>
<td>8 (other genotypes)</td>
<td>1 (other genotypes)</td>
<td></td>
</tr>
<tr>
<td>Genotype 1 vs 2</td>
<td>24 (genotype 1)</td>
<td>9 (genotype 1)</td>
<td>1.00(^g)</td>
</tr>
<tr>
<td></td>
<td>2 (genotype 2)</td>
<td>1 (genotype 2)</td>
<td></td>
</tr>
<tr>
<td>Viral load (IU)</td>
<td>898,000</td>
<td>548,700</td>
<td>0.93(^h)</td>
</tr>
<tr>
<td>Grade (1–4)</td>
<td>2.08</td>
<td>2.5</td>
<td>0.078(^i)</td>
</tr>
<tr>
<td>Stage (1–4)</td>
<td>1.92</td>
<td>3</td>
<td>0.0007(^j)</td>
</tr>
<tr>
<td>CP</td>
<td>10 pos/7 neg</td>
<td>1 pos/6 neg</td>
<td>0.078(^k)</td>
</tr>
<tr>
<td>RF</td>
<td>10 pos/10 neg</td>
<td>2 pos/7 neg</td>
<td>0.23(^l)</td>
</tr>
</tbody>
</table>

\(^a\) Demographic and clinical data about patients with low to normal B cell frequencies (<20%) compared to data from patients with increased B cell frequencies (>20%).

\(^b\) Mean ± SD.

\(^c\) Values of \(p\) calculated by \(^c\) Mann-Whitney test (two-tailed) and \(^d\) Fisher’s exact test (two-tailed).

(cirrhosis) had elevated B cell frequencies. These results suggest that more severe liver damage is associated with an accumulation of circulating B cells.

Patient variables associated with increased B cell frequencies are summarized in Table II. The strongest association with increased B cell frequency was fibrosis stage as determined by liver biopsy. Marginally significant associations were found with inflammation grade and with the absence of CPs. No significant associations were found with patient age, gender, race, HCV genotype, or the results of laboratory testing for RF. The mean viral load was somewhat lower in patients with an increased frequency of circulating B lymphocytes, as previously noted in HCV/HIV coinfected patients (47), but the difference was not statistically significant.

![Figure 2](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org)

**FIGURE 2.** Relationship between disease severity and B cell frequency. A, Patients stratified according to histological evidence of inflammation (Grade). The frequency of CD19\(^+\) B cells in the peripheral blood is plotted for patients in each group. The median for each group is shown as a line across the column. B, Patients stratified according to histological evidence of fibrosis (Stage). The frequency of CD19\(^+\) B cells in the peripheral blood is plotted for patients in each group. Lines across each column represent the median CD19\(^+\) B cell frequency for each set of patients. Different from controls by Dunnett’s multiple comparison test, \(p < 0.05\); ***, \(p < 0.001\).

**Expanded B cells have a resting phenotype**

It has been suggested that HCV infection may cause polyclonal B cell activation due to binding of the E2 glycoprotein to CD81, which is a component of the B cell coreceptor (e.g., Ref. 51). Others suggest chronic antigenic stimulation of B cells by persistent virus as a cause of B cell activation and transformation in HCV patients (e.g., Ref. 28). To determine whether the increased frequency of peripheral blood B cells in HCV patients is the result of antigenic or polyclonal stimulation of B cells, we examined circulating B cells for expression of activation markers (Fig. 4). The activation markers studied are typically found at very low levels on circulating B cells from healthy adult donors (52). Most
HCV patient B cells resembled normal, resting B cells. The IL-2 receptor α-chain, CD25, was detected on a fraction of circulating B cells in a few individuals from both the normal and HCV patient groups. CD25 expression was not increased overall in patient B cells, nor was it higher in B cells from those patients with increased B cell frequencies (Fig. 4A). CD69, a marker of early lymphocyte activation, was nearly completely absent from circulating B cells in normal controls and in HCV patients, even those with increased B cell frequencies (Fig. 4B). CD71 (transferrin receptor) was expressed by a population of circulating B cells in normal volunteers and in HCV patients. An inverse correlation was observed between B cell frequency and the percentage of CD71⁺ B cells in both groups, but this correlation was weaker in the HCV patient samples, so that expression of CD71 was evident on B cells from controls and patients; the frequency of CD71⁺ B cells was somewhat lower in patients (Fig. 4D). CD86 expression was observed on <2% of circulating B cells in most patient and normal control samples (Fig. 4E). This was true whether the patients had increased B cell frequencies or not. However, CD86 expression was easily detected on monocytes in the same sample (data not shown). All of the activation markers above were detected following polyclonal B cell activation in vitro (J. Ni and L. B. Dustin, unpublished data). Overall, these results indicate that the expanded or accumulated B lymphocytes in HCV patients do not resemble activated cells, suggesting that the accumulation of these cells in the blood does not result from chronic antigenic or polyclonal stimulation.

To determine whether ongoing B cell proliferation could explain the accumulation of B cells with a resting phenotype in HCV patients, we performed cell cycle analysis on CD19⁺ B cells purified from the available HCV patient and control samples (Fig. 5). As in normal donors, most circulating B cells from HCV patients were in the G₀ - G₁ phase of the cell cycle. No evidence was found for increased proliferation among circulating B cells even among patients with an increase in B cell frequency.

The expanded B cells in HCV patients are antigenically naive
CD27, a member of the TNF receptor family, is expressed on memory B cells (53, 54). If HCV infection caused B cell proliferation as a consequence of chronic antigenic stimulation, we predicted that the expanded B cell populations would be enriched in memory B cells. As shown in Fig. 6, this was not the case. Among the normal donors, the frequency of CD27⁺ B cells ranged from 16.7 to 60.1% of total peripheral blood B cells (mean 33.3%, SD 12.7) (Fig. 6A). Among HCV patients, the frequency of CD27⁺ B cells ranged from 4.5 to 77.3% of peripheral blood B cells (mean 34.1%, SD 16.9). Neither the means nor the variances of the two groups differed in a statistically significant manner. In both groups, further analyses revealed that the CD27⁺ B cells were mostly

### FIGURE 3. No expansion of CD5⁺ B cells in HCV patient peripheral blood. PBMCs were stained with Abs to CD3, CD19, and CD5 and were analyzed by flow cytometry. A, Analyses of representative samples from (left to right) a normal control donor, an HCV patient (patient 9, Table I) with positive CP and RF tests, and an HCV patient (patient 7, Table I) with negative CP and RF tests. CD5⁺ B cells were gated as described (50, 52). CD5⁻ B cells in the upper left quadrant of each plot were almost exclusively CD3⁺ (data not shown). B, Frequency of CD5⁺ B cells among gated B cells (CD19⁺, CD3⁺) in 18 normal donors (open symbols) and 36 HCV patients (filled symbols), regardless of CP or RF. Lines across each column represent median values for each group. C, Frequency of CD5⁺ B cells plotted against the frequency of CD19⁺, CD3⁻ lymphocytes in the same sample from 18 normal donors (left, open symbols) and 36 HCV patients (right, filled symbols). D, Frequency of CD5⁺ B cells plotted according to histological evidence of inflammation (Grade). Lines across each column represent the median CD5⁺ B cell frequency for each set of patients. E, Frequency of CD5⁺ B cells plotted according to histological evidence of hepatic fibrosis (Stage). Lines across each column represent the median CD5⁺ B cell frequency for each set of patients.
In contrast, CD27 expression was also high in the expanded B cell population in HCV patients, consistent with antigenically naive B cells. IgD expression is lost following B cell activation and differentiation (reviewed in Ref. 55), and cells may retain or lose surface IgM expression. HCV patients with increased B cell frequencies showed an inverse correlation between B cells and memory B cells (Fig. 6E–H). This suggests that in that subset of patients, B lymphocyte activation is present or that activated B cells are selectively retained or recruited in the circulation.

As another measurement of Ag-driven B cell activation and differentiation, we measured the frequency of IgD<sup>+</sup> B cells (Fig. 7). In normal donors, there is a large population of antigenically naive, recirculating B cells expressing both IgM and IgD (Fig. 7, A and C). IgD expression is lost following B cell activation and differentiation (reviewed in Ref. 55), and cells may retain or lose surface IgM expression. HCV patients with increased B cell frequencies also had high frequencies of IgD<sup>+</sup>/IgM<sup>+</sup> B cells (Fig. 7, B and D), consistent with antigenically naive B cells. IgD<sup>-</sup>, IgM<sup>+</sup> mature/activated B cells and IgM<sup>−</sup>, IgD<sup>-</sup> activated/memory B cells were proportionally reduced in blood samples from patients with increased B cell frequencies (Fig. 7, E–H). These results indicate that the increased population of B cells represents the expansion or accumulation of a naive B cell pool in the circulation. IgD<sup>+</sup> B cells in patients with increased B cell frequencies expressed low levels of CD38, consistent with a resting phenotype (56) (J.N. and L.B.D., unpublished data). In contrast, there was an apparent increase in the relative frequency of IgM<sup>+</sup>, IgD<sup>+</sup> activated/memory B cells in the circulation of patients with normal to low overall B cell frequencies (Fig. 7, E–H). This suggests that in that subset of patients, B lymphocyte activation is present or that activated B cells are selectively retained or recruited in the circulation.

**Discussion**

To better understand the source of B cell abnormalities associated with HCV infection, and to test the hypothesis that these abnormalities are a result of direct B cell activation by the virus, we have characterized circulating B cells in patients chronically infected with HCV. PBMCs were obtained from 36 patients chronically infected with HCV and not currently on antiviral therapy. Total lymphocyte frequencies were similar in HCV patients and in normal volunteers (data not shown), consistent with data reported by Streiff and colleagues, who found that while lymphocyte frequencies were not altered in HCV patients, platelet and neutrophil frequencies were reduced (57). We found that the range of B cell frequencies was much greater in samples obtained from HCV patients than from normal volunteers. Elevated B cell frequencies were correlated with the degree of hepatic fibrosis, but did not
correlate with laboratory evidence of MC, or with viral load, patient age, sex, or race. HCV genotype did not appear to determine whether or not elevated B cell frequencies were present, although in this patient cohort, as in most American HCV patients, genotypes 1a and 1b predominated. The accumulation of circulating B cells in HCV patients was not accompanied by evidence of widespread B cell activation; there was, if anything, an inverse correlation between the frequency of circulating B cells and the frequency of B cells expressing activation markers. In patients whose B cell frequencies were in the normal range, activated B cells were evident, but not present at higher levels than among normal controls. Cell cycle analysis did not reveal any changes in the proliferative status of circulating B cells. In those patients whose circulating B cell population was increased, the B cells were enriched in a subset that resembled naive resting B cells. The accumulated B cells did not express the memory B cell marker CD27 (53, 54). They expressed both IgD and IgM, consistent with naive B cells (55). In contrast, activated/memory B cells made up a larger fraction of circulating B cells among patients with lower B cell frequencies. Together, these results indicate that the increased frequency of circulating B cells in a subset of HCV patients is not a result of Ag-driven or polyclonal activation.

The relative frequency of circulating CD5+ B cells was somewhat reduced in HCV patients compared with normal volunteers, although those patients with elevated B cell frequencies had higher numbers of CD5+ B cells as well. Some groups of European and Israeli HCV patients are reported to have expanded CD5+ B cells in the peripheral blood (50, 51) or the liver (58). Curry and colleagues noted a negative correlation between CD5+ B cell frequency and disease activity, as assessed by inflammatory scores of liver biopsies in HCV patients (50). In contrast, Zuckerman and colleagues (51) found a positive correlation between CD5+ B cell frequency and disease activity. We found no correlation between CD5+ B cell frequency and hepatic inflammation or fibrosis. It seems from these widely different results that the factors affecting the frequency of circulating CD5+ B cells remain to be identified in HCV patients. The role of these cells is not clear in the setting of HCV infection.

Peripheral blood B lymphocyte accumulation is apparently unrelated to MC, as it is neither limited to patients with MC nor is it seen in all patients with MC. Of the seven patients with increased B cells who were also tested for serum CP as a laboratory marker of MC, six had negative results. HCV infection with MC or B cell lymphoma is associated with the presence of B cells with limited Ag receptor variability, suggesting oligoclonal expansion (59–61). Antiviral therapy may reduce the prominence of oligoclonal populations in the bone marrow and the circulation (62, 63). Such clonal dominance among B cells supports the hypothesis that Ag-driven B cell activation and proliferation play a role in the development of MC and lymphoma. Of interest, a related set of clones also predominates among the B cells that accumulate in the salivary glands in Sjögren’s syndrome (64), a condition sometimes associated with HCV infection (17), and HCV may localize to the salivary gland in some patients (65). Work is ongoing to determine whether particular B cell clones predominate in the patient samples...
analyzed in this report. In the patients studied here, there was no clear association between B cell frequency and CP or RF. This suggests that the origins of B cell expansion and of MC may not be closely related in this group of patients. Furthermore, the expanded B cell population observed here does not resemble activated B cells; thus, it is possible that B cell accumulation is not mediated by the same factors as those that drive the production of oligoclonal RF and CP.

One potential mechanism for B cell accumulation is suggested by the possibility that HCV infects a subset of hemopoietic cells. HCV RNA has been found associated with B lymphocytes (66–69) or monocytoid cells (66, 68) in a number of studies; however, it has been difficult to demonstrate HCV replication in hemopoietic cells (67, 68). If productive infection of B lymphocytes does occur, such infection could potentially lead to either increased cell division or altered regulation of cell lifespan. These possibilities are under investigation.

It is not known how chronic HCV infection and hepatic fibrosis may be related to increased B cell frequency in the circulation. Serum globulins are frequently elevated in cirrhosis; however, to our knowledge, it has not previously been reported that advanced liver disease leads to a specific accumulation of resting B lymphocytes in the peripheral circulation. Plasma cells were not observed in the blood in these patients (data not shown).

HCV may use multiple strategies to evade immune clearance and establish chronic infection. The high rate of virus production may contribute to immune exhaustion. HCV-specific T cells often display a phenotype suggestive of incomplete activation (6, 70, 71), and HCV-specific T cells in the blood do not approach the frequencies seen for virus-specific T cells in other viral infections (reviewed in Ref. 72). The high rate of sequence variation permits the selection of CTL escape mutants (73, 74). Some reports also suggest direct immune modulation by structural protein components of HCV, including the core protein (75, 76) and the E2 glycoprotein. There are reports that recombinant E2 glycoprotein can bind to CD81 (29), and that such binding by recombinant E2 may alter T lymphocyte (33) and NK cell (34, 35) function in vitro. In addition, recombinant truncated E2 was shown to bind to a B cell line, inducing aggregation and decreasing spontaneous cell division (36). However, these reports do not address the question of whether these effects occur in vivo in the context of circulating virus particles and normal lymphocytes.

Based on the reported functional and chemical associations between CD81 and other cell surface molecules (31), CD81 engagement by viral proteins might be predicted to affect B cell signal transduction by reducing the threshold for antigenic stimulation (32). Anti-CD81 has been reported to have antiproliferative effects in some cell types, including B cells. In addition, CD81 engagement by virus could possibly induce changes in lymphocyte migration and adhesion to stromal cells and the extracellular matrix. The effects of anti-CD81 Abs are likely mediated by aggregation or rearrangement of molecules associated with CD81 in a membrane web (30), rather than by direct signal transduction by CD81 itself. In B cells, one role for CD81 is as a component of the B cell coreceptor complex. This complex synergizes with the B cell receptor to promote B cell activation and differentiation (32). However, ligation of the B cell coreceptor complex is not expected to induce B cell activation or differentiation in the absence of Ag receptor engagement.

Our results indicate that circulating B lymphocytes from HCV patients, while their numbers can be increased, do not show increased evidence of Ag receptor or coreceptor mediated activation. This may be especially pertinent in light of observations that not all HCV genotypes encode an E2 glycoprotein with the capacity for high-affinity CD81 binding in vitro (37, 38). Most of the patients studied here are infected with genotype 1a virus, whose E2 glycoprotein is expected to interact with CD81 in vitro (29). Despite this, the phenotype of the circulating B lymphocytes from these patients is not consistent with the model that HCV E2 binds to and directly stimulates B cells in vivo. The B cells in circulation instead resemble normal resting, mature B cells. Indeed, in those patients in whom a lymphoproliferative process arguably is present, the accumulated cells are enriched for naive, resting B cells. PBMCs from these patients were largely devoid of activated B cells or memory B cells.

If HCV binds to the B cell coreceptor in vivo, it is possible that the outcome of such binding is not B cell activation but, instead, inhibition of activation. Such a model might explain the clinical observations of delayed seroconversion (10–13) and the lack of evidence for neutralizing Abs to HCV. This hypothesis may be supported by the observation that circulating B cells, although more common in many chronically infected HCV patients, are not in an activated state. It is not supported by the observation that activated and memory B cells are more abundant in the circulation of other patients, those with low to normal B cell frequencies. This hypothesis remains to be tested directly in vitro.

The lack of evidence for widespread B cell activation in the peripheral blood suggests that the accumulation of circulating B cells in a substantial minority of HCV patients is not a direct result of activation and proliferation. Indeed, we found no evidence to suggest that proliferation is increased above normal levels among circulating B cells in HCV patients. Proliferation may occur in the bone marrow, lymphoid organs, or in lymphoid follicles in the liver in these patients. However, it is not clear how activation-induced proliferation in these sites would contribute to the accumulation of apparently naive, resting B cells in the circulation.

B cell accumulation in the circulation may reflect altered homeostatic regulation of circulating B cell numbers. This could result from increased virgin B cell release from the bone marrow. We have noticed that the frequency of B cells expressing the C3d receptor CD21low is significantly ($p < 0.0001$) lower than that of B cells expressing CD19 in peripheral blood samples from most HCV patients (Table I and data not shown); this was not observed in normal donors or SR. Preliminary results suggest that the CD21low B cells resemble a transitional subset recently emerged from the bone marrow (49) (J. Ni, D. Butera, and L. B. Dustin, unpublished data). If the increase in circulating B cells reflects accelerated release of newly formed B cells from the bone marrow, this might interfere with the normal processes of negative selection and receptor editing that reduce the threat posed by autoreactive B lymphocytes.

Increased numbers of circulating B cells could reflect altered trafficking or motility of mature B cells such that they spend more time in the circulation, rather than in the tissues and secondary lymphoid organs, in HCV patients with significant fibrosis. Alternatively, it could reflect alterations in the lifespan of mature resting B cells. If B cell survival is prolonged, this may allow B cells that normally undergo apoptosis following activation with self Ags to survive and produce Ab. Furthermore, the loss of normal B cell senescence in the absence of antigenic stimulation might constitute a step toward transformation.

Recent reports have shown that HCV infection is associated with subtle to dramatic alterations in the circulating levels of some cytokines (77–82). These results may support a model in which changes in the cytokine milieu resulting directly or indirectly from HCV infection contribute to 1) increased bone marrow production, 2) increased or accelerated release from the bone marrow, 3) an increase in the relative amount of time that a given B lymphocyte

\[ \text{HCV} \times \text{B cell} \rightarrow \text{CD81} \rightarrow \text{activation} \]

\[ \text{HCV} \times \text{B cell} \rightarrow \text{senescence} \rightarrow \text{CD81} \rightarrow \text{inhibition of activation} \]

\[ \text{HCV} \times \text{B cell} \rightarrow \text{proliferation} \rightarrow \text{CD81} \rightarrow \text{clonal expansion} \]
spends in the circulation, or 4) prolonged survival of naive recirculating B cells in infected patients. Experiments are ongoing to measure the circulating levels of cytokines and chemokines that may affect B cell survival and compartmentalization.

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References


