Abstract. Aim: To evaluate the immune response in peripheral blood and liver tissue, through the measurement of T-cell subsets, in patients with chronic hepatitis B (CHB) and C (CHC). Patients and Methods: Thirty-four patients with CHB (21 with active HBV infection and 13 inactive HBV carriers) and 20 patients with CHC were included in the study. We also evaluated 21 biopsies from patients with active CHB infection and 20 patients with CHC. We measured CD3, CD4, CD8 and CD4/CD8 ratio in peripheral blood and liver tissue. Results: We found no differences in the numbers of all T-lymphocyte subpopulations between patients with active HBV infection and inactive carriers. We found a significant increase in the absolute numbers of CD3+, CD4+ and CD8+ T-lymphocytes in CHC compared to CHB patients (p=0.005, p=0.034 and p<0.0001 respectively). There was a significant increase in the number of CD3+ and CD8+ T-lymphocytes in the area of portal tracts (p=0.012 and p=0.009 respectively) and lobules (p=0.011 and p=0.01 respectively) in patients with CHC compared to those with CHB. In both groups there was a direct correlation between CD3+ cells in portal tracts and HAI score (r=0.783, p=0.008), while we noted a correlation between CD8+ cells in portal tracts and HAI score only in patients with CHC. Interphase hepatitis correlated to CD3+ cells in lobules of patients with CHC and CHB but a direct relationship between CD8+ cells and HAI score was found only in those with CHC. Conclusion: Insufficient cellular immune response is critical for the ineffective virus clearance and liver damage in chronic hepatitis B, while in chronic hepatitis C, immune response, as represented by CD8+ T-cells, is present in the peripheral blood and the liver. However, there is an immunological escape of HCV, which seems to survive in the presence of an adequate immune response. The significant correlation between portal and periportal CD8+ T-lymphocyte expression and interface hepatitis may be considered evidence of the occurrence of cytotoxic immune-mediated toxicity.

Hepatitis B (HBV) and C (HCV) virus infection is a global public health problem. It is estimated that approximately 2 billion people worldwide have serological evidence of past or present HBV infection and more than 350 million are chronically infected with HBV (1). Spontaneous resolution of acute HBV infection occurs in 90-95% of infected adolescents, whereas 5-10% develops chronicity. The majority (~70-80%) of patients with chronic HBV infection have a lifetime mild liver disease with persistent normal liver function tests and undetectable HBV DNA (1). Further persistent viral infection, however, may lead to chronic active hepatitis, liver cirrhosis and the development of hepatocellular carcinoma (1). Chronic HCV infection affects about 170 million people worldwide and it is the leading cause for liver transplantation in developed countries. It results in chronic disease in about 70-80% of infected individuals (1). Approximately 20% of those with chronic HCV will develop liver cirrhosis over a 20-year period post infection and up to 3% will develop hepatocellular carcinoma per year (2).

It seems that multiple arms of the immune response, mainly cellular immunity, play an important role in the immunopathogenesis of both HBV and HCV infection (3, 4). The type of early immune response that the infected host is able to mount following infection is believed to dictate strongly the outcome toward resolution or persistence of HCV or HBV infection. In patients with an acute self-limiting HBV or HCV infection, CD4+ and CD8+ T-cell responses are important for the control of the infection. Patients with a chronic HBV infection lack such vigorous, polyclonal and multi-specific T-cell responses, but instead exhibit a weak, narrowly focused T-cell response (5, 7). Similarly, in HCV infection, persistent infection appears to be due to weak CD4+ and CD8+ T-cell responses during acute infection, which fail to control viral
replication (8). On the other hand, recent studies suggest that hepatitis C virus persists, despite the presence of virus-specific CD8+ T-cell responses, in both the liver and the peripheral blood (9, 12). The reasons for this lack of responsiveness and the mechanisms that contribute to the failure of adaptive immune responses to eliminate viral infection in chronically infected patients are not well understood (13). Liver lesions in HCV and HBV infection appear to result from locally driven immune responses, which are mainly non-specific.

In the present study, we investigated the effect of chronic hepatitis B and C infection on T-cell subpopulations in both the peripheral blood and liver tissue of chronically HBV and HCV infected patients.

**Patients and Methods**

*Patients.* The study included 34 patients with chronic HBV and 20 patients with chronic HCV. In all 54 cases, the diagnosis of chronic hepatitis was made according to standard criteria (14). Twenty one of our HBV patients had a positive serum hepatitis B surface antigen (HBsAg) for at least 6 months, positive serum HBV DNA with high viral load (>10^5 copies/ml) measured at least two times in a period of 12 months, aminotransferase levels higher than 2 times the upper normal limits, and a positive liver biopsy. The rest of the HBV patients (n=13) had a positive serum HBsAg for at least 6 months, undetectable HBV-DNA and normal serum aminotransferase levels (inactive carriers). All HBV patients were HBeAg negative and anti-HBe positive.

All HCV patients had positive HCV serology (second- or third-generation ELISA) confirmed by positive polymerase chain reaction (PCR) for HCV, elevated aminotransferase levels (at least 1.5 times the upper normal limit) and liver biopsy indicative of chronic hepatitis C according to the Ishak classification (14). The study also included 31 healthy individuals who were free of HBsAg and negative for HCV serology and served as controls.

Exclusion criteria were the following: Decompensated cirrhosis, HBV/HCV co-infection, alcohol abuse, human immunodeficiency virus (HIV) or human T-lymphotrophic virus (HTLV) infection, immunosuppressive treatment, presence of other liver disease such as drug hepatotoxicity, alpha-1 antitrypsin deficiency, Wilson’s disease, hemochromatosis, autoimmune hepatitis and liver cancer. Blood tests were obtained before the initiation of any kind of treatment. Liver biopsies were obtained only from the patients with chronic HCV infection and from those with chronic HBV infection who had elevated aminotransferase levels and positive HBV DNA.

All patients and controls gave written informed consent before entering the study which was approved by the Ethics Committee of the Institute.

**Serological liver functions tests.** Serum alanine aminotransferase (ALT), aspartate transaminase (AST) were tested with routine automated techniques (upper limit of normal: 40 U/L and 40 U/L respectively).

**Peripheral blood T lymphocyte subset measurement.** Blood samples were collected in heparinized Vacutainer® (BD, NJ, USA) tubes. Whole blood samples were analyzed with a Multi-Q-Prep processor (Beckman Coulter, FL, USA) and then the Epics-XL (Beckman Coulter) flow cytometer. Lymphocytes were analyzed using a gate set on forward scatter versus side scatter, and a three-color flow cytometry combination reagent of CD3, CD4 and CD8. Anti-human monoclonal antibodies CD3,PE-CY5/CD4-FITC/CD8-PE were from Immunotech, Ltd, MO, USA. For each sample, the detection was analysed with the CELLQuest software (Beckman Coulter). The results were expressed as the percentages of CD3*, CD4* and CD8* T-cells and the ratio of CD4*/CD8* cells found to be positive for the marker antigen in the total T-cell population.

**Liver biopsies and T-lymphocytes subsets measurement.** All patients underwent a liver biopsy for diagnostic and therapeutic reasons. All liver tissues obtained by needle biopsy (14 gauge) and fixed in 98% solution of ethanol and acetic acid for 16 hours. Thereafter, the tissues were rinsed with water (3 times × 5 min each) and fixed in 10% neutral formalin for 10 min. Subsequently, tissues were embedded in paraffin. For each liver biopsy, one hematoxylin–eosin (H&E), one periodic acid-Schiff, one periodic acid-Schiff-D, one Pearl’s, one reticulin, one rodhanine and one Masson’s trichrome stain were available. All slides were reviewed in a blind fashion and without knowledge of the clinical laboratory profile of the patient. In each biopsy, several histologic features were assessed, and finally the hepatitis activity index (HAI) was applied and the architectural stage was recorded (14). Table I displays the clinicopathologic profile of the patients included in the study.

**Immunohistochemical detection of CD3+, CD4+ and CD8+ T-lymphocytes.** The expression of CD3+, CD4+ and CD8+ T-lymphocytes was evaluated in 21 biopsies from patients with chronic HBV (elevated serum aminotransferases and detectable HBV-DNA) and 20 hepatic biopsies from patients with PCR-positive chronic HCV. Identification of the aforementioned markers relied on immunohistochemistry, performed on 4-μm thick paraffin sections. For the

| Table I. Clinical and histological characteristics of patients included in the study (n=54) |
|-----------------------------------------------|-----------------------------------------------|
| **HAI score** | **Chronic hepatitis** | **Chronic hepatitis** |
|               | **type C (n=20)** | **type B (n=34)** |
| **No. of cases** | **Mean±SD (median) [range]** | **Mean±SD (median) [range]** |
| Category A | 2.05±0.84 [2 (1-4)] | 2.57±1.08 [2 (1-4)] |
| Category B | 0.52±0.21 [0 (0-1)] | 0.87±0.32 [0 (0-1)] |
| Category C | 2.71±0.25 [2 (0-3)] | 2.88±1.31 [2 (0-3)] |
| Category D | 2.54±0.88 [3 (1-4)] | 3.65±1.12 [3 (1-4)] |
| Total score | 5.32±2.31 [5 (2-12)] | 7.21±3.12 [7 (4-13)] |
| Stage | No. of cases | No. of cases |
| 1 | 6 | 12 |
| 2 | 4 | 11 |
| 3 | 5 | 7 |
| 4 | 2 | 4 |
| 5 | 1 | 0 |
| 6 | 2 | 0 |
| Stage mean±SD (median) | 2.65±1.1 (3) | 2.01±0.9 (2) |
purposes of CD3+ T-lymphocyte expression, the immunohistochemical method used was as follows (15): after standard deparaffinization, hydration and blocking of endogenous peroxidase, sections were processed in a microwave oven twice (5 min each) at high power. Subsequently, a standard immunohistochemical method, using the ChemMate™ EnVision™ Detection Kit, Peroxidase/DAB, Rabbit/Mouse, DAKO, CA, USA, was applied to detect the antigen. Sections were incubated with the polyclonal anti-CK19 antibody (ready to use, DAKO, USA) for 30 min at 37˚C. Diaminobenzidine (Sigma Fast DAB tablets-D-4293, St. Louis, MO, USA) was used as the chromogen.

Furthermore, for the detection of CD4+ and CD8+ T-lymphocytes, we employed an immunohistochemical method described earlier (16). We used the BenchMark® XT automated slide stainer (Ventana, USA) and a standard streptavidin biotin method [i view DAB-DAB MapTM Detection kit (streptavidin horseradish peroxidase detection kit; Ventana USA)]. Antibodies used included: anti-CD4 (Neomarkers, USA), anti-CD8 (Novocastra, UK).

For control purposes, 10 wedge biopsies from normal liver parenchyma were used. These were obtained from 10 patients, during operation for cholelithiasis (without coexisting cholecodolithiasis). All these 10 biopsies were histologically normal by routine light microscopy, without apparent hepatic, cholangiolytic, or vascular injury, i.e., there was no acute or chronic cholestasis, perportal ductular reaction, edema, or scarring of the portal tracts, sinusoidal dilatation or congestion or hepatitis. These 10 biopsies were obtained after patients gave informed consent.

**Morphometric analysis.** All biopsies were evaluated simultaneously in a blinded (to treatment and date) fashion. As has been previously reported (15, 16, 17) cell counts were performed manually at ×400 magnification using a 10×10 microscope grid. Both the numbers of immunoreactive cells (CD3+, CD4+ and CD8+) T-lymphocytes and the total number of lymphocytes were determined (separately in portal tracts and lobules) by visual inspection of five different fields per section. For each field, the percentage immunoreactivity for the aforementioned antigens was obtained by dividing the number of positive T-lymphocytes (CD3+, CD4+ and CD8+) by the total number of lymphocytes counted. Finally, the CD4+/CD8+ ratio was recorded by dividing the %CD4+ T-lymphocytes by the %CD8+ T-lymphocytes. The values in the same field did not differ by more than 10%. The average scores were then calculated. All the counts were correlated with the clinical laboratory and liver pathologic features at each patient.

**Results**

**Peripheral blood T-lymphocytes.** Table II displays the results of T-cell marker expression in the blood samples of the patients included in the study. There was no difference in the absolute and percentage numbers of CD4+, CD8+, CD3+ T-lymphocyte subpopulations, or in CD4/CD8 ratios, in the peripheral blood, between patients with CHB and elevated serum aminotransferases and detectable HBV DNA (group A) and those with normal levels of serum aminotransferases and undetectable HBV DNA (inactive carriers-, group B).

On the contrary, we found a significant increase in the absolute numbers of CD3+, CD4+ and CD8+ T-lymphocyte subpopulations (p=0.005, p=0.034 and p<0.0001 respectively), but not in the CD4/CD8 ratio in patients with chronic HCV (group C) compared to those with chronic HBV (groups A and B).

No differences were detected in the absolute or the percentage numbers of T-cell subsets between both group A and group B and the healthy controls (group D), while there was a significant increase in the absolute number of CD8+ cells in HCV+ patients compared to controls. In addition, no correlation between the levels of viral load or serum aminotransferases and the number of T-lymphocyte subpopulations in patients with chronic HBV (group A) and those with chronic HCV (group C) was recorded [data not shown].

**Liver pathology and T-lymphocyte subpopulations in liver tissues.** Control biopsies showed rare lymphocytes. This was statistically significant when compared to the study group (p<0.001). In all liver biopsies from patients with chronic hepatitis, T-cells (and their subsets) were located in portal tracts (Figure 1a) and lobules (Figure 1b). Tables II and III and Figures 2 and 3 (respectively) present the results regarding the expression of T cells (and their subsets) in liver biopsies.

There was a significant increase in the number of CD3+ and especially in the CD8+ T-lymphocytes in portal tracts in patients with chronic HCV compared to those with HBV. However, this increase did not affect the CD4+/CD8+ ratio.

<table>
<thead>
<tr>
<th>Group</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
<th>p-Value</th>
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</thead>
<tbody>
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<td>n=21</td>
<td>n=31</td>
<td></td>
</tr>
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<td>891+269</td>
<td>1118+391</td>
<td>1029+306</td>
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<tr>
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<td>2.1+0.8</td>
<td>1.9+0.9</td>
</tr>
<tr>
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<td>2.2+0.8</td>
<td>1.7+0.8</td>
<td>1.9+0.9</td>
</tr>
</tbody>
</table>

Table II. T-Cell subset values in all groups.
both groups, there was a direct correlation between CD3⁺ cells in portal tracts and HAI category D (r=0.657, \( p=0.011 \)). Furthermore, a significant correlation between CD8⁺ cells in portal tracts and HAI category D was noted only in the liver biopsies of patients with chronic HCV (r=0.783, \( p=0.008 \)).

Similarly, regarding lobules, there was a significant increase in CD3⁺ and CD8⁺ TL in patients with HCV in comparison to those with HBV. Again, this increase did not affect CD4⁺/CD8⁺ ratio. In both groups, there was a direct correlation between CD3⁺ cells in portal tracts and HAI category C (r=0.612, \( p=0.021 \)). Furthermore, a significant correlation between CD8⁺ cells in portal tracts and HAI category C was noted only in the liver biopsies of patients with HCV (r=0.763, \( p=0.012 \)).

There was no significant difference in the number of CD4⁺ cells between the two groups and there was no correlation between CD4⁺ T-lymphocytes and the degree of perportal hepatitis. Furthermore, no correlation was recorded between the numbers of CD3⁺, CD4⁺ and CD8⁺-lymphocytes and the degree of fibrosis.

Discussion

This study demonstrates that in patients with chronic hepatitis type C or B, a T-cell-dependent immune response (in the peripheral blood and liver), exhibits a different immune profile, depending on the disease (HCV/HBV). Additionally, insufficient cellular immune response seems to be critical for the ineffective virus clearance and liver damage in chronic HBV, while in chronic HCV, immune response, as represented by CD8⁺ T-cells, is present in the peripheral blood and the liver. It is also probable that there is an immunological escape of HCV, which seems to survive in the presence of an adequate immune response. Finally, the significant correlation between portal and periportal CD8⁺ T-lymphocyte expression and interface hepatitis may be considered as evidence of the occurrence of a cytotoxic immune mediated toxicity.

To the best of our best knowledge, this is the first study of its kind to compare the expression of T-cell subsets markers in patients with chronic HBV and HCV, and which measures T-cell markers both in blood and liver tissue samples. Additionally, this study included HBeAg-negative chronic HBV patients, the common type of HBV patients living in Greece. No investigations regarding the presence of T-cell subpopulations has been conducted in the past for this category of patient.
Figure 2. Photomicrograph showing CD4+ cell expression in a portal tract of liver biopsy from a patient with HCV.

Figure 3. Photomicrograph showing CD8+ cell expression in the lobules of liver biopsy from a patient with HBV.
However, a limitation of our study is that we focused on the absolute and percentage numbers of T-lymphocyte subpopulations, while the HBV-specific T-cell response and T-cell function was not examined. Nevertheless, it has been suggested that most liver-infiltrating cells are not antigen-specific and that these cells might be able to influence the local immune response by producing pro-inflammatory cytokines and could reflect the functional potential of antigen-specific CD8+ T-cells.

The immunopathogenesis of persistent infection and liver damage induced by chronic HBV and HBC infection remain poorly understood, and little is known regarding the role of adaptive immune responses driven by CD4+ and CD8+ T-lymphocytes within the peripheral blood and the liver in chronically infected patients. Therefore, comparative analysis of the immune response during the course of the above viral infections may provide important insights into the pathogenetic mechanisms which are involved in both viral persistence and liver damage. To address this issue, we investigated the immune profile of T-lymphocyte subpopulations in both the peripheral blood and liver tissue of patients with chronic HBV, asymptomatic HBV carriers and chronic HCV, in order to focus on the pathogenesis of chronicity and liver injury. We also investigated the correlation of the expression of CD4+ and CD8+ T-cells in the portal and periportal areas of hepatic biopsies from patients with chronic HCV and HBV and the intensity of interface hepatitis.

In the current study, we did not detect any difference in T-lymphocyte subpopulations between chronic HBV patients and healthy controls. When we compared peripheral blood CD3+, CD4+, CD8+ T-lymphocytes and T4/T8 ratio between patients with HBV-DNA(+) chronic HBV infection and HBV-DNA(−) asymptomatic HBV carriers, we did not find any significant difference to explain the different profile of adaptive immune response between the two groups of patients. Previous studies (18, 24) investigated either the HBV-specific or non-specific T-cell response and suggested a T-cell failure in patients with HBV disease which is mainly expressed as a decrease in CD4+(+) T-cells and secondarily as an increase in CD8(+) T-cells. Tian and Li (25) investigated the role of HBV specific T-cells and demonstrated that virus specific CD4+ T-cells decreased in all 33 CHB patients and 21 asymptomatic HBV carriers, although the decrease was significantly higher in patients with symptomatic disease. You et al. (26) showed an impaired balance of T-cell subsets (increased proportion of CD8+ T-lymphocytes and decreased proportion of CD4+) in patients with asymptomatic chronic HBV. It was also shown that this T-cell impairment was significantly associated with viral replication levels. Other studies (27, 30) indicate that efficient antiviral therapy of persistently infected patients appeared to increase the frequency of HBV-specific CD4+ T-cell response, or to overcome CD8+ T-cell hypo-responsiveness in these patients.

All of the aforementioned studies enrolled exclusively HBeAg(+) patients with high viral load, while to our knowledge there is no study investigating the role of T-cells in HBeAg(−)/anti-HBeAb(+) CHB patients. In a very recent study, You and colleagues demonstrated significant differences in peripheral T-cell subpopulation profiles in different clinical stages of chronic HBV infection, which were significantly associated with HBV load (31). However, detailed analysis of the study results indicate that the above study characterized mainly HBeAg(+) patients, while in HBeAg(−) patients higher levels of CD3(+) and CD4(+) cells and lower levels of CD8(+) cells were found. We believe that HBeAg may play an important role in immunomodulating chronic HBV patients by suppressing the T-cell response. As in HBeAg(−) patients with chronic HBV, the viral load has not been associated with peripheral blood CD4 and CD8-cell response. This has been shown by other investigators (32, 35) who proposed that HBeAg may have an immunoregulatory function and establish T-cell tolerance and may have an immunomodulatory role in antigen presentation and recognition by CD4+ cells.

In contrast, the present study demonstrates a significantly different immune response between chronic HBV and chronic HCV infection, as expressed by T-cell subsets in the peripheral blood and liver tissue. We found a strong CD8+ T-cell response in the peripheral blood and liver in the chronic HCV group but not the chronic HBV group. Our findings support the notion that the CD8+ T-cell response is not necessarily absent during chronic HCV and that the CD8+ T-cells are recruited to the liver of persistently infected patients, where they probably contribute significantly to ongoing immunopathology and liver damage. Our findings are supported by previous studies (36, 39) which demonstrated the existence of virus-specific CD8+ T-cells in the peripheral blood and liver of patients with chronic HCV. It seems therefore, that the HCV may induce a strong CD8 response in the peripheral blood and in the liver, a response which contributes to liver damage but without the ability to eliminate the virus, as HCV has the ability to persist despite a vigorous CD8+ T-cell response. The underlying mechanisms responsible for the failure of CD8+ T-cell response to clear HCV are not defined. Data exists (40, 43) which supports the idea that HCV-specific CD8+ T-cells in peripheral blood are functionally impaired with respect to IFN-γ production or are anergic. Consistent with this loss of function, these cells exhibit phenotypic changes which are characteristic of the early stages of differentiation. In experimental models, HCV-specific CD8+ cells do not have the ability to produce INF-γ, having a so-called ‘stunned’ phenotype (44). Other investigators (45) describe the accumulation of hypo-responsive CD8+ T-cells in the liver of patients of CHC. These CD8+ cells are poorly responsive.
to T-cell receptor-mediated stimulation, compared with those obtained from uninfected subjects. Mutational escape from adaptive immune response has been thought to be one of major viral evasion strategies used by HCV. Finally, Erickson et al. (46) first demonstrated an immune escape mechanism carried out through mutations which affect virus-specific CD8+ T-cell responses. These mutations decrease the binding affinity between the epitope and the MHC molecule. Thus, the persistence of viral load in patients with chronic HCV could lead to mutational escape, ultimately affecting the function of CD8+ T-cells. The dysfunctional effector CD8+ T-cells are recruited to the liver of chronically infected patients where they are not able to control the infection and end up contributing to liver damage.

Finally, in the current study we recorded a strong correlation between the strength of CD8+ T-cell response and necroinflammatory activity within the liver of these patients. These findings are in agreement with previous studies (47, 48). This supports the hypothesis that HCV, but not HBV evolution depends on the action of intrahepatic CD8+ lymphocytes, emphasizing the role of immune-mediated mechanisms in the pathogenesis of chronic HCV.

In conclusion, this study shows that in patients with chronic HCV or HBV, T-cell-dependent immune response in the peripheral blood and the liver exhibits a different immune profile for these two diseases. It seems that insufficient cellular immune response is critical for the ineffective virus clearance and liver damage in chronic HBV, while in chronic HCV, immune response, as represented by CD8+ T-cells, is present in the peripheral blood and the liver. However, there is an immunological escape of HCV, which seems to survive in the presence of an adequate immune response. The significant correlation between portal and periportal CD8+ T-lymphocyte expression and interface hepatitis may be considered as an evidence of the occurrence of cytotoxic immune-mediated toxicity. More research is warranted in order to clarify the exact mechanisms that involved in local immune responses in those cases and specify the functional roles of antigen-specific CD8+ T-cells.

References

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