Pretreatment Intrahepatic CD8+ Cell Count Correlates with Virological Response to Antiviral Therapy in Chronic Hepatitis C Virus Infection


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We analyzed the relationship between virological response and baseline immune factors in 17 patients chronically infected with hepatitis C virus (HCV) who received interferon-α–ribavirin therapy for 26 weeks. The number of intrahepatic CD8+ cells present in the portal tract before the start of treatment was found to be significantly higher in patients who responded to treatment than in nonresponders. The relationship between portal CD8+ cell counts and the response to therapy could be described by a logistic curve. Neither peripheral cytokine levels nor HCV-specific T cell reactivity in peripheral blood mononuclear cells showed a relationship to response to therapy.

The ability of interferon (IFN) to inhibit hepatitis C virus (HCV) replication and to stimulate the clearance of infected cells is the basis of current standard antiviral therapy that uses IFN-α and ribavirin. Most patients exhibit an initial response, with the loss of circulating HCV RNA. Unfortunately, complete virus clearance is not observed in all patients [1, 2]. Although the mechanism responsible for the eradication of HCV-infected hepatocytes is not well understood, there is consensus that this process is immune mediated. Revealing mechanistic studies have been hampered by the lack of representative laboratory models and by the difficulty of studying the intrahepatic compartment.

In peripheral blood, HCV-specific T lymphocytes are detected easily during acute HCV infection, exhibiting a broad reactivity toward different HCV antigens [3, 4]. In chronic hepatitis, HCV-specific T cell frequencies are low [5], and response to subsequent IFN-α therapy is difficult to predict on the basis of this parameter. Higher frequencies of HCV-specific T cells may be found in the liver [6, 7]. Accordingly, liver biopsies may prove to be instrumental for the study of immune factors associated with response to subsequent IFN-α therapy. The frequency of virus-specific intrahepatic T cells [8, 9] and absolute numbers of these cells present in the liver can possibly mirror the ability of the immune system to generate a sufficient antiviral response after the start of antiviral therapy. We examined baseline localization of diverse subsets of intrahepatic immune cells, by quantitative immunohistochemistry (CD4+, CD8+, and CD68+ cells), in pre- and end-of-treatment liver biopsy specimens and assessed causality to response to therapy for chronic HCV infection. For comparison, peripheral immune parameters, such as plasma interleukin (IL)–10, IL-12, and IFN-γ levels and circulating HCV-specific T cells, were also determined.

**Patients and methods.** Seventeen patients with chronic HCV infection who were assigned to receive IFN-α2b (3 MU 3 times/week) and ribavirin (1000–1200 mg/day) (Schering-Plough) for 26 weeks in our center and from whom a pretreatment biopsy specimen was available were studied. Informed consent was obtained from all patients, and the institutional review board of the Erasmus MC approved the study.

All patients were treatment naive and HCV RNA positive, had biopsy-proven chronic HCV infection, and were seronegative for hepatitis B virus (HBV) and human immunodeficiency virus. None had clinical or biochemical evidence of other liver diseases. Blood samples for HCV RNA quantification were obtained at 0, 4, 12, and 26 weeks of therapy. Response to treatment was defined as HCV RNA levels <100 copies/mL at the end of therapy, determined by means of the Supercuant assay (National Genetics Institute; limit of detection, 100 copies/mL).

Before treatment and during the last week of treatment, biopsies were performed percutaneously with a 14-gauge Tru-Cut needle. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded liver biopsy sections. After an overnight incubation at 37°C, 4-μm-thick sections were de-waxed in xylene and rehydrated in graded alcohols. Epitope retrieval was achieved by temperature-controlled incubation for 20 min at 98°C in citrate buffer (pH 6.0). The primary antibodies against CD8 (clone cd8/144B; Dako), CD68 (clone PG-
considered to be statistically significant.

The number of cells staining positive within the portal tract and lobular region was determined by counting the number of positive cells per equivalent microscopic field, at a magnification of 400×, with a Zeiss light microscope. In every slide, 10 fields for the lobular region and 3 equivalent fields for the portal tracts were examined, and the mean count of these fields was expressed as cell count per microscopic field. The cell counting was performed by 2 investigators, in an independent fashion, with slides blinded by codes (interobserver correlation, 0.996).

HCV-hepatitis activity, interface hepatitis, intralobular degeneration, fibrosis, and the number of apoptotic bodies were histologically assessed and routinely graded in all specimens, according to the Knodell classification, by an experienced liver pathologist blinded to the clinical status of the patient.

Peripheral blood mononuclear cells (PBMCs) obtained at the start of therapy were isolated from heparinized blood by density gradient centrifugation, after which cells were frozen in liquid nitrogen. After thawing, PBMCs were cultured in 96-well microtiter plates (NUNC); 6 replicates for each condition, with 2 × 10^5 cells/well were used. Cells were cultured in RPMI 1640 medium and supplemented with 10% heat-inactivated human serum, at 37°C with 5% CO₂ and 100% humidity for 6 days, in the presence or absence of HCV proteins at a concentration of 3 μg/mL. Recombinant HCV proteins used included HCV core (aa 2–120) and NS3-NS4 (aa 1192–1931), which were provided by Dr. M. Houghton (Chiron, Emeryville, CA). All antigens were expressed as COOH-terminal fusion proteins with human superoxide dismutase (SOD) in yeast. Controls included phytohemagglutinin-L, inactivated influenza virus (Duphar), and SOD. After 6 days, cultures were labeled by incubation for 16 h with 1 μCi of [3H]thymidine (Amer sham). Cells were harvested, and radioactive incorporation was estimated by a beta-counter (Wallac). Results were expressed as mean counts per minute. The stimulation index (SI) was calculated as the ratio between counts per minute obtained in the presence of antigen and that obtained without antigen; an SI ≥3 was considered to be significant.

Plasma obtained from EDTA blood, taken before the start of treatment, was stored at −70°C. Plasma cytokine concentrations were measured by means of commercially available ELISAs for IFN-γ, IL-10, and IL-12-p40 (purchased from CLB).

Data were analyzed by use of SPSS for Windows (version 10.1; SPSS). Mann-Whitney nonparametric rank-sum tests and Pearson’s correlation analyses were applied. Logistic regression models with backward selection procedures were used to explore baseline factors that predict response (virus load, genotype, sex, and lobular and portal CD8+ cell counts). P < .05 was considered to be statistically significant.

Results. Baseline patient characteristics, as well as outcome and immunohistochemistry results, are presented in table 1. The median duration of infection was 14.5 years (range, 5–30 years); in 3 patients, the duration of infection could not be estimated. Median baseline virus load was 9.0 × 10^5 copies/mL (range, 8.0 × 10^5–3.5 × 10^7 copies/mL; mean, 5.3 × 10^5 copies/mL); 11 patients were infected with HCV genotype 1a/b.

All patients completed 26 weeks of IFN-α2b–ribavirin therapy. Nine patients responded by the end of therapy (HCV RNA, <100 copies/mL), whereas, in 8 patients, HCV RNA remained detectable during the entire treatment period. No significant differences were observed with respect to pretreatment virus load or genotype between patients who responded to therapy and patients who did not. The average rate of virus load decline within the first 4 treatment weeks was 2.32 log (1.45 log in nonresponders vs. 3.32 log in responders; P = .015). The rate of decline in virus load was not different between genotype 1 and non-1 genotypes. In 4 patients, the quantitative polymerase chain reaction result for HCV RNA was negative after a 24-week treatment-free follow-up period.

No significant difference was observed between responders and nonresponders in baseline alanine aminotransferase levels (P = .321). HCV-specific lymphoproliferative responses were detected in 6 of 17 patients. However, baseline HCV-specific lymphocyte proliferation did not differ significantly between responders and nonresponders (table 1). In addition, other pretreatment peripheral markers of immune activity, such as amounts of circulating CD8+ T cells (P = .535) and plasma IL-12-p40 (P = .336), IL-10 (P = .321), and IFN-γ (P = .236) levels did not differ significantly.

Liver biopsy sections were evaluated histologically by determination of the Knodell score and the presence of CD4+, CD8+, and CD68+ cells. Knodell scores were similar in responders and nonresponders (table 1). CD8+ and CD68+ cells were present both in the portal tract and lobular region, but CD4+ cells were observed less frequently and were restricted mainly to the portal tracts. Amounts of CD68+ or CD4+ cells did not differ significantly between responders and nonresponders. Amounts of lobular CD8+ cells in pretreatment liver biopsy sections were similar in both groups (P = .815), but the amounts of CD8+ cells within the portal tracts were higher in responders, compared with nonresponders (P = .002) (figure 1, top). On the basis of univariate logistic regression modeling, the predicted probability of response to therapy as a function of the number of CD8+ cells in the portal tract in biopsy samples taken before treatment could be made (figure 1, bottom). The number of pretreatment CD8+ cells in the portal tract alone predicted response better than did either virus load or patient characteristics (multivariate logistic regression analysis including sex, age, genotype, stage of fibrosis, and virus load). Amounts of intrahepatic CD8+ cells were not significantly different in re-
Table 1. Demographic and virological characteristics of 17 patients chronically infected with hepatitis C virus (HCV) before and at the end of treatment.

<table>
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<tr>
<th>Patient</th>
<th>Sex</th>
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<th>Race</th>
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<th>HCV load, copies/mL</th>
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<th>Known duration of infection, years</th>
<th>ALT level, U/L</th>
<th>Knodell score</th>
<th>Pretreatment CD8+ cell counta</th>
<th>Portal tracts</th>
<th>Lobular region</th>
<th>LST</th>
<th>End-of-treatment CD8+ cell counta</th>
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**NOTE.** ALT, alanine aminotransferase; LST, lymphocyte stimulation test (shown is the antigen to which a specific response could be detected); NA, not available; ND, not detected (stimulation index [SI] <3.0); NR, no portal tracts; NP, no response; R, response.

**a** Ten fields for the lobular region and 3 equivalent fields for the portal tracts were examined, and the mean cell count of these fields was expressed as cells/microscopic field.

**b** Experienced a sustained virological response.

Sponders and nonresponders in end-of-treatment biopsy samples (figure 1, top).

**Discussion.** Intrahepatic localization of HCV-specific T cells may be crucial for controlling acute HCV infection [3, 4] but it may also augment inhibition of viral replication and clearance of infected hepatocytes when patients with chronic HCV infection are treated with IFN-ribavirin [11, 12]. In the present report, we demonstrate that, when analyzed by quantitative immunohistochemistry, the number of intrahepatic CD8+ cells present in the portal tract before the start of treatment with IFN-ribavirin is significantly higher in patients who respond to treatment. On the basis of univariate logistic regression modeling, an S-shaped logistic curve displays the predicted probability of response to therapy as a function of the number of portal CD8+ cells in biopsy samples taken before treatment. The number of CD8+ cells in the portal tract alone predicts response better than do viral and demographic characteristics. Our immunohistochemical analysis did not find any correlation of other subsets analyzed (CD4+ and CD68+ cells) with response to antiviral treatment.

The patients described in the present study were assigned to an experimental treatment schedule of 26 weeks of therapy combining IFN-α2b (3 MU 3 times/week) with ribavirin (1000–1200 mg/day). Only 4 of 17 patients had a sustained response, making the analysis with respect to the end of follow-up status difficult. The results suggest that the number of pretreatment intrahepatic CD8+ cells in the portal tract is a significant and independent predictor for the impairment of viral replication, leading to undetectable HCV RNA levels at the end of treatment. However, in the end-of-treatment biopsy samples, portal CD8+ cell counts were comparable in responders and nonresponders (figure 1, top). Reduced intrahepatic CD8+ cell counts in responders at the end of therapy might relate to the high relapse rate observed (5 of 9 patients experienced a virological relapse).

IFN-α most likely has a pronounced direct antiviral activity in patients with HCV infection. The first dose-dependent phase of the HCV decrease curve, which lasts ∼24 h, can be explained assuming that IFN-α mediates direct antiviral activity [13]. The slower subsequent second phase is thought to be partly immune mediated. Indeed, we observed a significant correlation between pretreatment CD8+ cell counts and the decrease of virus titers...
Figure 1. Intrahepatic CD8+ cell count in the portal tract of liver biopsy samples in relation to response to treatment with interferon (IFN). A, Box plots of intrahepatic CD8+ cells in the portal tract of pretreatment (white bars) and end-of-treatment (gray bars) biopsy samples in relation to response to treatment with IFN. The CD8+ cell count in pretreatment portal tracts is significantly higher in patients who responded to therapy than in patients who did not. The upper and lower limits of the boxes and the middle line across the boxes indicate the 75th and 25th percentiles and the median (50th percentile), respectively. The length of the box is thus the interquartile range; the box represents 50% of the data. The upper and lower horizontal bars indicate the 90th and 10th percentiles, respectively. Comparison of end-of-treatment nonresponse and response data was performed by Mann-Whitney nonparametric rank-sum tests. B, The predicted probability of response to following therapy displayed as a function of the no. of CD8+ cells in the portal tract in biopsy samples taken before treatment. The curve is based on univariate logistic regression modeling (P < .001, log likelihood; P = .002, score test).

during the first month of therapy (r = 0.514; P = .05). Both innate and virus-specific immune responses may further amplify HCV clearance during antiviral therapy, either because of their presence at the start of therapy or by IFN-ribavirin–mediated activation. Several reports have demonstrated the appearance of HCV-specific T cell activity after treatment with IFN-α [11, 12, 14], even when baseline HCV-specific T cell reactivity was undetectable [5]. We could not differentiate responders on the basis of lymphocyte stimulation test responses measured at the start of therapy. Rather, actual numbers of infiltrating CD8+ cells may have to be taken into account. Our observations are consistent with a study by Nelson et al. [8], who demonstrated that the presence of HCV-specific cytotoxic T cells in the liver was associated with biochemical response to IFN-α. CD8+ cells could be directly involved in the clearance of HCV from the liver during antiviral treatment through production of antiviral cytokines or the killing of infected hepatocytes. Alternatively, CD8+ cell count may correlate with other antiviral mechanisms implicated in the clearance of HCV during therapy.

The portal localization of CD8+ cells is of interest. As demonstrated in HBV infection, patients with high virus load were characterized by the presence of high amount of CD8+ cells located in the portal tracts, whereas relatively more CD8+ cells were found in the lobular region in cases of controlled HBV [9]. It is possible that high CD8+ cell counts in the portal tract may benefit patients at later stages during therapy, when virus load is diminished and the migration of CD8+ cells into the lobular region may have been facilitated. Future studies need to further analyze frequency and localization of HCV-specific CD8+ cells in the liver.

In conclusion, the pretreatment CD8+ cell count in the liver, but not in peripheral blood, is higher in patients who respond to IFN-ribavirin therapy than in patients who do not. We suggest that immunohistological evaluation of biopsy samples may be considered with respect to factors reflecting a patient’s immune system capacity to respond adequately to therapy. Moreover, these findings suggest that significant prognostic immune markers are to be found in the liver and should encourage further study of hepatic immune cells as important predictive factors.

Acknowledgments

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References


