Profibrogenic chemokines and viral evolution predict rapid progression of hepatitis C to cirrhosis

Patrizia Farci1,2, Kurt Wollenberg3, Giacomo Díaz4, Ronald E. Engle5, Maria Eliana Lai6, Paul Klenerman1, Robert H. Purcell7, Oliver G. Pybus8, and Harvey J. Alter9,10

1Hepatic Pathogenesis Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; 2Bioinformatics and Computational Biosciences Branch, Office of Cyber Infrastructure and Computational Biology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; 3Department of Biomedical Sciences, University of Cagliari, 09123 Cagliari, Italy; 4Hepatitis Viruses Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; 5Department of Medical Sciences, University of Cagliari, 09042 Monserrato (Cagliari), Italy; 6Peter Medawar Building for Pathogen Research, University of Oxford, Oxford OX1 3VY, United Kingdom; 7Department of Zoology, University of Oxford, Oxford OX1 3PS, United Kingdom; and 8Department of Transfusion Medicine, Warren G. Magnuson Clinical Center, National Institutes of Health, Bethesda, MD 20892

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Chronic hepatitis C may follow a mild and stable disease course or progress rapidly to cirrhosis and liver-related death. The mechanisms underlying the different rates of disease progression are unknown. Using serial, prospectively collected samples from cases of transfusion-associated hepatitis C, we identified outcome-specific features that predict long-term disease severity. Slowly progressing disease correlated with an early alanine aminotransferase peak and antibody seroconversion, transient control of viremia, and significant induction of IFN-γ and MIP-1α, all indicative of an effective, albeit insufficient, adaptive immune response. By contrast, rapidly progressive disease correlated with persistent and significant elevations of alanine aminotransferase and the profibrogenic chemokine MCP-1 (CCL-2), greater viral diversity and divergence, and a higher rate of synonymous substitution. This study suggests that the long-term course of chronic hepatitis C is determined early in infection and that disease severity is predicted by the evolutionary dynamics of hepatitis C virus and the level of MCP-1, a chemokine that appears critical to the induction of progressive fibrogenesis and, ultimately, the ominous complications of cirrhosis.

Hepatitis C virus (HCV) is an important human pathogen that causes persistent infection in up to 80% of infected individuals (1). It is the most common cause of chronic liver disease in the United States, and the leading indication for liver transplantation (LT) (2). The clinical presentation and outcomes of chronic hepatitis C are highly variable. Although the disease is mild and either stable or slowly progressive in about 70% of chronically infected patients, the remaining 30% develop progressive liver disease culminating in cirrhosis and possibly hepatocellular carcinoma (HCC), leading to death or orthotopic LT (1, 2). Progression to cirrhosis usually takes 20–30 y from the onset of infection (3). Presently, it is not possible to predict which individuals will rapidly evolve to severe fibrosis and liver failure.

The mechanisms responsible for the different rates of disease progression in chronic hepatitis C are unknown, but likely reflect differences in the complex interplay between the virus and host immunity. Although HCV infection elicits strong cellular and humoral immune responses (4–7), evidence suggests that the virus has evolved numerous strategies to avoid immune recognition (8). One of the most effective mechanisms relates to the remarkable genetic diversity of HCV, which circulates in vivo as a dynamic distribution of divergent but related genomes termed a “quasispecies” (9). This genomic heterogeneity enables rapid adaptation to selective constraints imposed by the host (10). We previously demonstrated that the evolutionary dynamics of HCV during early infection predicts the outcome of acute hepatitis C, such that a decrease in viral diversity correlates with viral clearance whereas an increase correlates with progression to chronicity (11). These data, subsequently corroborated elsewhere (12–14), are consistent with the concept that aspects of viral evolution reflect the strength of virus-specific adaptive immune responses and therefore the ability of the host to control the virus (8, 15). However, little is known about the correlations among adaptive immunity, HCV evolution, and rates of disease progression once chronic hepatitis C is established.

We have been able to study the complex interplay between virus and host throughout the course of infection in a cohort of patients with acute hepatitis C that progressed to chronicity while under observation during a prospective study of posttransfusion non-A, non-B hepatitis (16); these patients have been followed from the day of transfusion for up to 30 y. Here, we investigate the relationship among biochemical markers of liver damage, viral kinetics, humoral immunity, serum cytokine and chemokine profiles, and HCV evolution in patients with slowly or rapidly progressive disease to obtain insights into the mechanisms underlying variable rates of disease progression.

Results

Acute-Phase Viral Kinetics Differ in Slow and Rapidly Progressive Hepatitis C. Six patients infected with HCV genotype 1 were selected for intensive study because they represented two different clinical outcomes and had serial serum samples available throughout the acute and chronic phases of infection, in addition to clinical and liver pathology data. Disease was mild and stable or slowly progressive for up to 30 y in three patients (slow progressors) and rapidly progressive in the remaining three (rapid progressors), leading to liver-related death within 7 y of the onset of infection (Fig. S1 and Table S1). Strikingly, in two of the three rapid progressors, active cirrhosis was documented within the first 2 y of infection (patients 5 and 6 in Table S1), suggesting that extremely rapid progression to cirrhosis can occur outside the setting of immunosuppression as has been observed following liver transplantation (17) or in HIV-1 coinfected individuals (18). Comparisons were censored at 7 y because all of the rapid progressors had died from end-stage liver disease by that time. In all patients, viral RNA became detectable in serum within 1–2 wk of transfusion, after which viral titers rose very rapidly. The subsequent course of disease could not be predicted by the onset or magnitude of early viremia. However, a difference in viral load between slow and rapid progressors emerged later in acute infection. In slow progressors, HCV viremia became transiently...


The authors declare no conflict of interest.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. JQ747752–JQ749627).

To whom correspondence may be addressed. E-mail: pfarci@niaid.nih.gov or halter@dtn.cc.nih.gov.

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undetectable in two of three patients and decreased by 3 logs in the third patient during the first 4 mo of infection; in contrast, none of the rapid progressors lost detectable HCV RNA, although fluctuations in viral level were observed. In slow progressors, serum HCV RNA reappeared at a high level between 6 and 12 mo and subsequently persisted (Fig. 1).

Rapid Disease Progression Correlates with Delayed Alanine Aminotransferase Peak and Antibody Seroconversion. In slow progressors, the decline in viremia coincided with the alanine aminotransferase (ALT) peak (Fig. 1) as previously reported (7, 19, 20). Furthermore, ALT peaked earlier in slow than in rapid progressors, with means of 9 and 15 wk (median 8 and 17) from the time of infection, respectively. ALT levels tended to be higher in rapid than in slow progressors. The temporary loss of detectable viremia seen after the ALT peak in slow progressors was associated with transient ALT normalization, whereas none of the rapid progressors normalized ALT. Because liver damage in acute hepatitis C is believed to be immunologically mediated (7, 8), these findings suggest that slow progressors have an earlier and more effective viral-specific immune response than rapid progressors. Analysis of specific anti-HCV antibodies by enzyme immunoassay (EIA3) documented a delay in anti-HCV antibody seroconversion in rapid progressors (mean, 11.7 wk; median, 12 wk) compared to slow progressors (mean, 8.7 wk; median, 8 wk) from the time of infection (Table S1).

Cytokine and Chemokine Levels in the Acute Phase Predict the Pace of Disease Progression. Because viable cellular samples were not available from this unique historical collection, to evaluate virus-specific cellular immune responses we studied the long-term profile of 39 cytokines and chemokines in serial serum samples during the acute and chronic phase. In both slow and rapid progressors, cytokine and chemokine profiles initially increased, before the rise in ALT levels, and then declined following the ALT peak, with minor fluctuations during chronic infection. The most striking observation of our study was the correlation between cytokine and chemokine expression and the pace of disease progression (Fig. 2 A–E). Overall, slow progressors tended to have higher cytokine and chemokine levels than rapid progressors, but only 5 of the 39 cytokines tested were significantly different between the two groups. We found that during the entire acute phase (both preceding the ALT peak and in the period post-ALT peak until 6 mo), the levels of IFN-γ and MIP-1β—two markers associated with effective, multispecific CD4+ and CD8+ virus-specific T-cell responses (19–22)—were significantly higher in slow than in rapid progressors (P < 0.05) (Fig. 2 C and D). By contrast, the levels of MCP-1 (CCL2) (Fig. 2E), IL-8 (CXCL8) (Fig. S2A), and IP10 (CXCL10) (Fig. 2B), three chemokines associated with inflammatory reactions (23–26), became significantly higher (P < 0.05) in rapid progressors in the period between the ALT peak and 6 mo post the onset of infection, reflecting a prolonged inflammatory state following the onset of liver injury.

Sustained Elevations of Profibrogenic MCP1 Correlates with Rapid Progression to Cirrhosis. A different pattern was observed during chronic infection. Whereas the levels of IFN-γ and MIP-1β remained significantly higher (P < 0.05) in slow progressors (Fig. 2 C and D), MCP-1, a major chemotactic factor for hepatic stellate cells (HSCs) (23), which play a central role in fibrogenesis (26–30), was the only chemokine that remained significantly higher (P < 0.05) in rapid progressors until the patient’s death from end-stage liver disease. Notably, persistence of MCP-1 elevation was associated with significantly higher ALT levels (Fig. 2B). The relationships among the levels of ALT, HCV RNA, and five cytokines/chemokines (IFN-γ, MIP-1β, IL-8, MCP-1, and IP-10) during the acute and chronic phase of infection in the six patients studied were also investigated by multivariate methods. Q-mode Principal Component Analysis showed a complete separation of slow and rapid progressors and patterns of ALT, HCV RNA, and cytokine/chemokines consistent with the above observations (Fig. 3). A marked separation of slow and rapid progressors was also obtained by hierarchical
Collectively, the results of our study indicate that slow and rapidly progressive liver disease showed distinctive biochemical, virological, and immunological profiles.

**Viral Evolution and the Pace of Disease Progression.** We subsequently investigated the relationship between HCV molecular evolution and disease progression using powerful analytical methods. Because serum was longitudinally sampled after a known date of infection and none of the patients had received antiviral therapy, this data set allowed for a comprehensive analysis of HCV evolution in relation to disease outcome. Comparisons were censored at 7 y because all of the rapid progressors had died from end-stage liver disease by that time. Viral sequences within the HCV envelope 1 and 2 genes, including hypervariable region 1 (HVR1), were analyzed in all PCR-positive samples during the entire course of infection. DNA amplified by PCR from the E1 and E2 genes was cloned, and a mean of 29 molecular clones for each time point were sequenced, resulting in a total of 1,876 sequences, each 558 nucleotides long. The six individuals analyzed here constituted a subset of those previously selected for a study in which we investigated the evolution of HCV during the first 4 mo of infection and its relationship with the outcome of acute hepatitis C (resolution vs. chronic evolution) (11). In this study, we independently analyzed the genetic diversity and divergence of the HCV quasispecies within E1, HVR1, and E2 outside the HVR1. The greatest diversity and divergence were detected in HVR1 (Fig. 4A and B), consistent with the fact that it is a major target for neutralizing antibodies (31), followed by E2 outside the HVR1, and then by E1 (Fig. S3A–D). Both common and outcome-specific changes were observed. During acute infection, HVR1 diversity initially decreased, but then increased dramatically after antibody seroconversion, especially in slow progressors, followed again by a sharp decrease between 6 and 12 mo of infection. Then, the levels of HVR1 diversity remained lower in slow progressors until 2–4 y post infection, but not later. From 5 to 7 y post infection, viral diversity dramatically increased in slow progressors, reaching values higher than those seen in rapid progressors before their death (Fig. 4A and Fig. S2A and B). In both slow and rapid progressors, divergence from the initial viral population increased over time, but a difference in the degree of these changes between the two groups became evident. HVR1 divergence before 1 y was constantly higher in rapid progressors and remained higher throughout follow-up (Fig. 4B and Fig. S3C and D).

To study the relationship between disease progression and viral evolutionary dynamics, we used the phylogenetic approach implemented in Beast v1.6.2 to reconstruct for each patient the evolution of all E1/E2 sequences, inclusive of the HVR1, which were obtained sequentially over the entire course of the disease. Our data set is unique in that it permitted us to investigate the relationship between long-term disease progression (over several decades) and viral evolutionary dynamics in both acute and chronic hepatitis C. Bayesian molecular clock phylogenies revealed a distinct change in viral evolutionary dynamics during the transition from acute to chronic hepatitis in all patients. During the acute phase, lineages sampled at different times intermingled, with multiple lineages in one sample leading back to multiple lineages in the previous one (Fig. 4C and D). However, around 1 y post infection this pattern changed, and a strong genetic bottleneck occurred in all patients with only one, or a few, of the lineages present during acute infection giving rise to the viral population in chronic infection (Fig. 4C and D). This dynamic signature indicates that the vast majority
γ analysis. The map of the variables points to an increase of IFN-γ the acute and the chronic phase. Higher ALT and HCV-RNA levels are also in slow progressors and of MCP-1, IL-8, and IP-10 in rapid progressors in both progressors (3 of 10) (trated in the HVR1 in slow progressors (9 of 12) than in rapid codons under positive selection was signifi
ction of positive selection across the E1/HVR1/E2 region between (mos substitutions (Fig. 4
mean number of substitutions per site was greater in rapid pro-
tion with respect to the amount of synonymous substitution was considered to be indicative of positive selection. Overall, the mean number of substitutions per site was greater in rapid pro-
sitions (Fig. 4E). Next, we analyzed individual codons for positive selection. The most striking observation from our analysis was the finding of a significantly different distribution of positive selection across the E1/HVR1/E2 region between slow and rapid progressors (P < 0.01) (Fig. 4F). The number of codons under positive selection was significantly more concentrated in the HVR1 in slow progressors (9 of 12) than in rapid progressors (3 of 10) (P < 0.01), suggesting that host humoral immunity imposes the strongest selection pressure in slow pro-
gers, whereas selection pressures in rapid progressors are less focused (Fig. 4F). Interestingly, none of the slow progressors showed positively selected codons in the E1 region, whereas three positively selected sites in E1 were identified in rapid progressors.

Discussion

Our study provides insights into the clinical, virological, and immunological determinants of slow versus rapidly progressive hepatitis C. Our patients were devoid of confounding factors such as viral coinfections and i.v. drug use or alcohol abuse, and all were Caucasian and infected with HCV genotype 1. Despite these limitations inherent within an historical cohort, particularly the lack of viable cellular samples for analysis of virus-specific T-cell responses and the limited number of patients who could be included in this intensive study, our data indicate that slow and rapid progressors exhibited distinct outcome-specific features that became evident during the acute phase and persisted into chronicity. We found that stable or slowly progressive disease is associated with the induction of earlier and stronger adaptive immune responses against HCV, as documented by an earlier ALT peak and antibody seroconversion, transient clearance or marked reduction of viremia, significant induction of IFN-γ and MIP-1β, and evidence of increased selective pressure on HVR1, along with a significant decrease of proinflammatory chemokines. By contrast, patients with rapidly progressive disease exhibited reduced or delayed adaptive immune responses associated with significantly higher levels of HCV viremia and persistent elevation of proinflammatory and profibrotic chemokines, such as MCP-1, IL-8, and IP-10, beginning in the late acute phase of hepatitis. The higher expression of these chemokines in rapid progressors during the early phase of hepatitis C suggests that these markers might be predictive of rapidly progressive liver disease. Strikingly, MCP-1 persisted at significantly elevated levels throughout the course of the disease. MCP-1 is a critical mediator of liver fibrogenesis via an autocrine loop enacted by HSCs, the primary effector cells in hepatic fibrogenesis (26–30), which both produce and are attracted by this chemokine (26–28). Interestingly, an MCP-1 gene polymorphism was found to be associated with significantly higher hepatic expression of MCP-1 in patients with chronic hepatitis C and more advanced liver fibrosis (32). In addition to its potent pro fi-

Fig. 3. Q-mode Principal Component Analysis showing the relations among the six patients resulting from the median levels of ALT, HCV RNA, and five cytokines/chemokines (IFN-γ, MIP-1β, IL-8, MCP-1, and IP-10) observed during the acute phase (0–6 mo from infection) and the chronic phase (6 mo–7 y from infection) of the disease. A complete separation of low (blue) and rapid (red) progressors is given by the first principal component, which accounts for nearly 50% of the total variance of the 14 variables included in the analysis. The map of the variables points to an increase of IFN-γ and MIP-1β in slow progressors and of MCP-1, IL-8, and IP-10 in rapid progressors in both the acute and the chronic phase. Higher ALT and HCV-RNA levels are also associated with rapid progressors, although in HCV RNA only during the acute phase. Hierarchical clustering (inset) using the same set of variables confirmed the separation between slow and rapid progressors.

of HCV strains were cleared. The viral nucleotide-substitution rate was estimated independently for the E1, HVR1, and E2 regions of the HCV genome using Beast v1.6.2 (Table S2). Rapid progressors showed higher substitution rates on average compared with slow progressors across all regions. In both rapid and slow progressors, the highest rate was observed within the HVR1. To investigate whether the difference in the evolutionary rates between slow and rapid progressors was due to positive selection, we estimated the mean number of nonsynonymous and synonymous substitutions per nonsynonymous and synonymous site, respectively, across the first 7 y of the Bayesian molecular clock phylogenies. An excess of nonsynonymous substitution with respect to the amount of synonymous substitution was considered to be indicative of positive selection. Overall, the mean number of substitutions per site was greater in rapid pro-
gressors, and this effect was especially pronounced for syn-

brosis progression following liver trans-

plantation (38, 39). Altogether, these results suggest that accelerated fibrogenesis in rapid progressors is secondary to sustained intrahepatic inflammation, leading to iterative hepatocyte injury, consistent with the hypothesis that the liver damage in rapidly progressive hepatitis C is not induced by virus-specific and viral effector T cells, as occurs during the acute phase, but rather by massive infiltration and activation of nonspecific inflammatory cells and continuous release of proinflammatory and profibrotic soluble mediators.

Although the molecular mechanisms leading to increased liver inflammation in rapid progressors remain to be fully elucidated, the failure of a sufficiently effective virus-specific adaptive immune response during a critical window early in HCV infection appears to play a pivotal role in determining the extent of hepatic damage mediated by liver inflammation, necrosis, and early fibrogenesis. In contrast, subjects with stable or slowly progressive disease not only were able to raise a timely virus-
specific immune response, but also were able to maintain throughout the course of the infection higher levels of IFN-γ, a cytokine that has been shown to exert antifibrogenic effects (40). Consistent with our findings, patients with intrahepatic CD8+ T cells producing IFN-γ were reported to have less fibrosis and lower progression rates than those lacking such cells (41). Although the level of IFN-γ detected in slow progressors was presumably not sufficient to eradicate the virus, it may have effected a degree of viral containment that was clinically relevant and sufficient to alter the course of disease.

This longitudinal study also provided unique insights into the role of viral evolution in disease progression. In all patients, we documented a strong genetic bottleneck that reduced viral
diversity around 1 y post infection. Slow progressors also showed a significantly higher positive selection on individual codons within the HVR1, indicative of strong immunologic pressure on this region, which is a major target of neutralizing antibodies (31). It is likely that in slow progressors the recurrence of HCV viremia after a marked, but transient, reduction in viral load during early infection was due to the emergence of immune escape variants (42). In contrast, faster progression correlated with greater HCV sequence divergence across all regions examined, particularly within the HVR1, and with preponderance of synonymous substitutions as previously reported in HCV and HIV infections (43, 44). This greater accumulation of synonymous substitutions across all of the regions is consistent with a shorter viral generation time and a more rapid turnover of infected hepatocytes, as confirmed by the significantly higher ALT levels detected throughout the disease course. A shorter hepatocyte
life span with continuous replacement of infected cells can explain why the levels of viremia were not significantly higher in rapid progressors (43).

In summary, our data provide a model for the pathogenesis of chronic HCV-induced liver disease whereby rapid disease progression is the consequence of an early ineffective induction of the adaptive immune response followed by the sustained release of chemokine and cytokine mediators of hepatic inflammation and fibrosis. In essence, severe chronic injury may result from an excess of soluble inflammatory mediators rather than from cytotoxic T cells. This model may provide means for the early identification of patients at increased risk of rapid HCV disease progression and for the use of anti-inflammatory and antifibrotic therapy to limit or prevent accelerated liver fibrosis progression.

**Materials and Methods**

**Study Subjects and Design of the Study.** Six patients with acute progressing hepatitis C who were enrolled in a prospective study of posttransfusion non-A, non-B hepatitis conducted at the National Institutes of Health (16) were selected for this intensive study because they represented two clearly different outcomes of chronic hepatitis C, had serial serum samples available, and had long-term follow-up. Three patients whom we defined as slow progressors had a mild and stable liver disease for up to 30 y, and three whom we defined as rapid progressors had a rapidly progressive liver disease leading to liver-related death within 7 y of infection (SI Materials and Methods).

**Materials and Methods**

**Statistical Analysis.** Differences in serum ALT, HCV-RNA, and cytokine/chemokine levels between slow and rapid progressors were assessed by the Mann–Whitney U test and multivariate methods. The distribution of significant positive selection across domains was assessed using the Kolmogorov–Smirnov test. Details are given in SI Materials and Methods. Statistical analyses were performed using Statistica (Statsoft) and R software (R Development Core Team, http://www.R-project.org.).

**Molecular Evolution of HCV.** Details on the methods used are provided in SI Materials and Methods.

**Methods.** Details of serologic and virologic assays including anti-HCV, HCV-RNA extraction, reverse transcriptase reaction, and nested PCR and quantification of serum HCV RNA by TaqMan, HCV genotype, molecular cloning and sequencing, and multiplex cytokine analysis are provided in SI Materials and Methods.

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