Early Prediction of Hepatitis C Virus (HCV) Infection Relapse in Nonresponders to Primary Interferon Therapy by means of HCV RNA Whole-Blood Analysis

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(See the editorial commentary by Shire and Koziel on pages 1761–3)

Background. Routine analysis of serum and/or plasma specimens for hepatitis C virus (HCV) RNA does not always correctly reflect the response to antiviral therapy. Analysis of whole-blood specimens for detection of viral RNA should provide more-accurate prognostic information.

Methods. Whole-blood, serum, and plasma specimens (268 sample sets) were obtained from 56 patients who did not respond to initial interferon (IFN)—α2b monotherapy (5 MU every 2 days for 3 months). Specimens were analyzed for HCV RNA by 4 different types of reverse-transcriptase polymerase chain reaction (RT-PCR) (Cobas Amplicor HCV-2.0 [Roche], LightCycler real-time PCR [Roche], and 2 in-house RT-PCRs) to determine whether specimen type can predict the rate of virologic response to high-dose treatment with IFN (10 MU every 2 days) and ribavirin (1–1.2 g/day).

Results. Of the 56 patients who provided specimens, serum and plasma obtained from 18 tested negative for HCV RNA at the end of treatment, indicating a complete virologic response. In contrast, analysis of whole-blood specimens obtained at the same time revealed the presence of viral RNA in 12 of these 18 patients. All 12 subjects had relapse of HCV in serum and plasma: 11 relapsed a median of 4 weeks after the end of treatment, and 1 relapsed 20 weeks after the end of treatment. None of these 12 patients—all of whom consistently had whole-blood specimens that tested positive and plasma and serum specimens that tested negative for HCV RNA up to 20 weeks before the end of treatment—showed a sustained virologic response (P = .0002).

Conclusions. Results of whole-blood tests for detection of HCV RNA were highly predictive of viral relapse (positive predictive value, 100%) and thus may be useful tools for monitoring and tailoring IFN/ribavirin therapy. Testing of only serum or plasma specimens underestimates the true circulating HCV load and leads to an overestimation of antiviral response rates.

Antiviral treatment response in patients with chronic hepatitis C virus (HCV) infection usually is defined by negative results of tests for detection of HCV RNA in serum or plasma specimens [1–4]. Thus, the virus population—which may be inside PBMCs [5–8], in precipitates of immune complexes and cryoglobulin aggregates [9–11], or attached to circulating peripheral blood cells—is excluded from detection [12–14]. Testing of only serum and/or plasma specimens instead of whole-blood samples may lead to false-negative results (i.e., a detection bias) for patients who are still carrying significant amounts of virus in blood components that...
are not accessible for analysis [15, 16]. Thus, viruses in compartments less affected by therapy probably constitute a significant source for the emergence of viral replication after the end of treatment [17]. Incorrect data on the “true” viral load in peripheral blood increase the risk for inadequate treatment decisions. However, the superior analytical sensitivity of whole-blood PCR for detection of HCV, which has been used elsewhere [12–15, 17], is still considered to be controversial [18].

At present, monitoring of antiviral hepatitis C therapy is usually performed with commercially available tests (e.g., the Cobas Amplicor HCV test [Roche] and the Versant HCV RNA assay [Bayer]) or with in-house RT-PCR techniques [7, 19–24] for qualitative detection of HCV RNA. Most of these procedures use serum or plasma specimens for viral RNA isolation. Thus, such methods may fail to detect virus present in the circulation of treated patients.

The aim of our study was to investigate whether the source tested for the presence of viral RNA (i.e., whole blood, serum, or plasma) has an impact on test results and whether additional information obtained from whole-blood testing can more accurately predict virologic response rates to combined IFN/ribavirin treatment than does serum or plasma analysis alone, to avoid overestimation of treatment response rates.

**PATIENTS AND METHODS**

**Study population.** Of 304 patients treated with IFN-α2b monotherapy (5 MU every 2 days for 3 months) (Intron-A; Schering-Plough), 157 had positive results of serum HCV RNA tests, constituting the group of primary nonresponders. All 157 primary nonresponders were randomized in a multicenter, prospective, controlled clinical trial to receive high-dose IFN-α2b (10 MU every 2 days) alone or in combination with ribavirin (dosage range, 1 g/day [body weight, <70 kg] to 1.2 g/day [body weight, ≥70 kg]) (Aesca/Schering-Plough). The major characteristics of the IFN-α2b study population and treatment schemes are summarized in table 1; the trial protocol and course of the study have been described in detail elsewhere [22, 23].

To elucidate the impact of the source (i.e., serum and plasma vs. whole blood) on HCV RNA test results, we investigated 56 consecutive primary nonresponders participating in the high-dose IFN-α2b/ribavirin study. Twenty-seven subjects received IFN-α2b monotherapy (group A), and 29 received combination IFN-α2b/ribavirin therapy (group B). Informed consent was obtained from all study participants. The study was performed according to the guidelines of the ethics commission of the University of Vienna (Austria).

**Samples used for HCV RNA RT-PCR analysis.** Serum, plasma, and whole-blood specimens obtained simultaneously were assayed by means of an in-house single-step RT-PCR (in-house PCR I) every 4 weeks, from the time of randomization (24 weeks before the end of treatment) through the end of the follow-up period (24 weeks after the end of treatment). If the result of the serum test was negative, all 3 specimens in the sample set were then tested with Cobas Amplicor HCV-2.0 and with real-time RT-PCR (LightCycler; Roche). Serum samples were also tested by means of an in-house nested RT-PCR (in-house PCR II).

Overall, 755 sample sets (each containing 1 whole-blood specimen, 1 serum specimen, and 1 plasma specimen) were obtained during the study. A total of 268 of these 755 sample sets were obtained from 18 patients for whom HCV RNA was not detected in serum and plasma samples at the end of treatment (which indicated a complete virologic response to therapy), including additional sample sets collected 96 weeks after the end of therapy (i.e., during extended follow-up). Four of these 18 end-of-treatment responders showed a sustained virologic response, and additional sample sets were collected 48, 72, 120, and 144 weeks after the end of treatment. All sample sets collected from these 18 patients underwent analysis by means of the 4 RT-PCR methods.

The remaining 487 sample sets, which were obtained from 38 patients during the course of the study, were tested only by in-house PCR I, because results of serum tests for these specimens were consistently positive. Seven sample sets (<1%, all of which were obtained from secondary nonresponders) were not evaluable during the study period.

**Sample collection.** Serum, plasma, and whole-blood sam-

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**Table 1. Baseline characteristics of patients who did not respond to primary IFN monotherapy and who received secondary treatment with either IFN monotherapy (group A) or IFN/ribavirin combination therapy (group B).**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group A (n = 76)</th>
<th>Group B (n = 81)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographic/clinical</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male sex/female sex, n/p</td>
<td>54/22</td>
<td>57/24</td>
</tr>
<tr>
<td>Age, median years (range), n/p</td>
<td>48.2 (20–70)</td>
<td>45.7 (19–70)</td>
</tr>
<tr>
<td>ALT level, mean U/L ± SD, n/p</td>
<td>65.4 ± 34.5</td>
<td>69.8 ± 39.9</td>
</tr>
<tr>
<td>Cirrhosis, n/p</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Chronic hepatitis, n/p</td>
<td>42</td>
<td>51</td>
</tr>
<tr>
<td><strong>Genotype of isolated HCV</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a, n/p</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>1a and 1b, n/p</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>1b, n/p</td>
<td>52</td>
<td>51</td>
</tr>
<tr>
<td>3, n/p</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>4, n/p</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><strong>Therapy received</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-α2b, MU every 2 days, n/p</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary, n/p</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Secondary, n/p</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Ribavirin, g/day, n/p</td>
<td>…</td>
<td>1–1.2</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. of patients, unless otherwise indicated (from [23]). ALT, alanine aminotransferase.
amples (200 μL each) were stored at −80°C within 2 h after blood was collected. Whole-blood samples were collected in EDTA-coated tubes and split into 2 aliquots after gentle shaking. One aliquot, representing the whole-blood specimen, was frozen immediately. For RNA extraction, the first aliquot was thawed and shaken gently but did not undergo centrifugation. Plasma was recovered from the second aliquot by means of centrifugation at 850 g for 10 min immediately before freezing. Serum specimens were prepared from blood collected in uncoated tubes on the same occasion.

**Determination of detection limits for in-house RT-PCR.** The standardized Nucleic Acid Panel NAP HCV-RNA (AcroMetrix, Berkley, CA), calibrated to the first World Health Organization International Standard for HCV RNA (96/790), was used to establish the detection limits of the in-house RT-PCR methods. Standards containing the same amounts of virus and a 10⁻² dilution were tested in triplicate by means of a minimum of 8 different RT-PCR runs [25].

**Qualitative, in-house, recombinant Thermus thermophilus (rTth) single-step RT-PCR (in-house PCR I).** For the qualitative, in-house, single-step RT-PCR, a recently published protocol [22–24] was used, with some modifications. In brief, RNA was isolated from 200-μL specimens of serum, plasma, or whole blood (with no prior centrifugation step) by the HighPure Viral Nucleic Acid Kit (Roche Diagnostic Systems), according to the manufacturer’s instructions. Five microliters of RNA were added to a 45-μL PCR reaction mixture containing 2.5 U of MasterAmp rTth DNA Polymerase (Epigenic Technologies) and 10 pmol of biotinylated primers KY78 and KY80 [19] (distributed by ViennaLab). After synthesis of cDNA (20 min at 60°C, followed by 1 min at 95°C), amplification was performed for 45 cycles (30 s at 92°C, followed by 30 s at 60°C and 45 s at 68°C). Amplification products were analyzed by means of a microplate-based hybridization assay (HepaTest-C Detection Assay; ViennaLab). Specimens that tested negative for HCV RNA during the first run were retested in duplicate. The limit of detection was 75–200 IU/mL.

**In-house nested RT-PCR (in-house PCR II).** We used the oligonucleotides 5'-GGCGAAGCTCCACCAGGTTAACAATG-3' (F1) and 5'-GGTGGACAGTCTAGAGACT-3' (R1) as outer primers and 5'-GTTGAGGAACTGTGTCCTC-3' (F3) and 5'-CTCTGATCGGAGTACACCACAA-3' (R3) as inner primers. In-house PCR II analysis was performed for serum samples only, as described elsewhere [7]; all such specimens underwent duplicate testing. The detection limit was 100–250 IU/mL.

**Cobas Amplicor HCV-2.0 (reference assay).** This commercially available, semiautomatic, qualitative RT-PCR assay with a detection limit of 50 IU/mL was used to analyze 200-μL samples of serum and plasma, according to the manufacturer’s instructions [19, 20]. RNA from 200-μL samples of whole blood were tested in the same way as described for serum and plasma analysis, without centrifugation or additional preparation. Samples that tested negative for HCV RNA during the first run were retested in duplicate.

**Real-time RT-PCR analysis with the LightCycler assay.** RNA was extracted from 200-μL specimens of serum, plasma, and whole blood (with no previous centrifugation step) by means of the HighPure Viral Nucleic Acid Kit (Roche), according to the manufacturer's instructions. A 2-μL aliquot of the isolated RNA was added to 18 μL of the LightCycler PCR master mix containing 10X PCR buffer (200 mmol/L Tris HCl; 500 mmol/L KCl; pH 8.4), 4 mmol/L MgCl₂, 3 mmol/L MnSO₄, 0.8 mmol/L deoxyribonucleoside triphosphate, 0.125 μmol/L of each primer (KY78 and K80 [19]; distributed by ViennaLab), SYBR Green I fluorescence dye (dilution, 1:10,000), and 0.5 U/μL rTth DNA Polymerase mix. PCR conditions were as follows: reverse transcription, 10 min at 55°C; initial denaturation of cDNA, 30 s at 95°C; and amplification of target DNA, 1 s at 95°C, 10 s at 60°C, and 12 s at 72°C for 45 cycles. Melting curve analysis was performed to identify the specific HCV PCR amplicon. All RNA extracts were tested in duplicate. The detection limit was 100–300 IU/mL [26, 27].

**Statistical analysis.** All analyses to determine the statistical significance of the differences in specimen-specific treatment outcome rates and relapse data were calculated by binomial distribution analysis on an intent-to-treat basis (table 2). SPSS, version 10.0 (SPSS), was used for all statistical calculations.

**RESULTS**

**End-of-treatment responders.** At the end of treatment, 18 of 56 patients (3 in group A and 15 in group B) showed no HCV RNA in serum or plasma samples, indicating a complete virologic response to therapy. However, 12 (67%) of these 18 patients (3 in group A and 9 in group B) had HCV RNA detected in whole-blood specimens at the end of treatment (figure 1). Eleven patients (92%)—all of whom had whole-blood specimens that tested positive for HCV RNA up to 20 weeks before the end of treatment—had serum and plasma specimens that demonstrated early relapse 4–8 weeks (median, 4 weeks) after the end of treatment. One (8.3%) had a late relapse 20 weeks after end of treatment (figure 1). Four of 6 patients who had whole-blood, serum, and plasma specimens that were concordantly free of HCV RNA demonstrated a sustained virologic response until 3 years after the end of treatment (all 4 patients were in group B); the other 2 relapsed (table 2 and figure 2). There were no differences in HCV genotype distribution among discrepant samples and concordant samples between end-of-treatment responders and patients with a sustained virologic response and between group A and group B (table 1) (data not shown).

**Secondary nonresponders.** A total of 38 patients had HCV RNA detected in serum, plasma, and whole-blood specimens.
Predicting HCV Relapse by Whole-Blood Analysis

We present evidence that testing of whole-blood specimens for HCV RNA has an analytical sensitivity that is superior to that of traditional serum and plasma testing and that a combination of whole-blood analysis with serum and plasma analyses allows prediction of early viral relapse. Viral clearance is a crucial parameter used to tailor the duration of IFN/ribavirin therapy [1–4, 28]. Thus, HCV detection systems offering the lowest possible detection limits should provide optimal prognostic in-

Table 2. HCV RNA detection in whole-blood and serum and plasma specimens at the end of treatment (ETx) and outcome of secondary treatment for 56 patients who did not respond to primary IFN therapy.

<table>
<thead>
<tr>
<th>Result of serum and plasma tests at ETx, treatment outcome</th>
<th>Result of whole-blood test at ETx, no. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive for HCV RNA, no response</td>
<td>38</td>
</tr>
<tr>
<td>Negative for HCV RNA</td>
<td>12</td>
</tr>
<tr>
<td>Relapse</td>
<td>0</td>
</tr>
<tr>
<td>Sustained virologic response</td>
<td>0</td>
</tr>
</tbody>
</table>

a Outcome was determined on the basis of HCV RNA status 6 months after ETx.
b Results of ETx analysis had a positive predictive value of 100% for the outcome of viral relapse (12 of 12 patients; \( P = .0002 \)), with a specificity of 100% (4 of 4 patients; \( P = .06 \)) and a sensitivity of 86% (12 of 14 patients; \( P = .0056 \)).

DISCUSSION

Concordance of results of different PCR procedures. Of a total of 804 samples (268 sample sets of whole-blood, serum, and plasma) analyzed by all 4 PCR techniques, only 18 samples produced PCR results that differed from results of Cobas Amplicor HCV-2.0 analysis (figures 2A–2C). Thus, all 4 RT-PCR procedures produced identical results in 97.8% of all samples tested. None of the 641 HCV RNA–positive serum and plasma samples were associated with an HCV RNA–negative whole-blood specimen from the same sample set, with the exception of 1 whole-blood specimen, which was obtained 20 weeks after the end of treatment and analyzed with the LightCycler (figure 2C). Specimens obtained from all patients with a sustained virologic response had the same HCV RNA detection profile for each of the PCR methods (figure 2D).

Predictive value of whole-blood testing. None of the 12 patients with whole-blood specimens that tested positive but serum and plasma specimens that tested negative for HCV RNA showed a sustained viral response (figure 1). Thus, the results of whole-blood analysis, when combined with those of serum and plasma analysis, were highly predictive of relapse (\( P = .0002 \); table 2).

Whole-blood testing identified only 3 patients who became HCV RNA negative within the first 3 months after the onset of therapy (12–24 weeks before the end of treatment), 2 of whom continued to have negative test results 3 years after the end of treatment (figure 2A). This was in contrast to the high number of subjects (\( n = 14 \)) who had nondetectable HCV RNA levels in serum and plasma specimens. Although the number of subjects is too small for firm conclusions to be drawn, these data suggest that differences in early clearance of HCV RNA from whole blood versus serum and plasma 4–12 weeks after the start of therapy may provide additional information for predicting sustained virologic response.

On the basis of data available at the end of treatment, detection of HCV RNA in whole-blood specimens obtained from end-of-treatment responders had a positive predictive value of 100% for viral relapse (\( P = .0002 \)), with a specificity of 100% (\( P = .06 \)) and a sensitivity of 86% (\( P = .0056 \)). Negative results of a whole-blood HCV RNA test had a negative predictive value of 67% for a sustained virologic response (\( P = .23 \)) (table 2).

Monitoring of end-of-treatment responders. By the end of treatment, serum and plasma specimens had tested negative for HCV RNA in 18 patients. In contrast, during the same period, 12 of these patients had whole-blood specimens that tested positive for HCV RNA. Results of serum and plasma tests became positive 4 and 8 weeks after the end of treatment in 8 and 11 patients, respectively, which indicated treatment failure. The twelfth patient experienced viral relapse 20 weeks after the end of treatment (figures 1 and 2).
Figure 2. Specimen-specific hepatitis C virus (HCV) clearance in 18 end-of-treatment (ETx) responders, which demonstrates the concordance of results for 4 different PCR methods. A–C, Time-dependent clearance of HCV RNA in 18 ETx responders; striking differences in the sensitivity of analyses performed before ETx are evident between whole-blood specimens and serum and plasma specimens. D, Complete course of all 4 patients with a sustained HCV RNA response up to 96 weeks after ETx; all specimens obtained from these patients showed the same profile for each of the 4 PCR methods.

formation. However, the sensitivity of the RT-PCR system is not the only important variable, because the type of specimen used for RNA isolation may also substantially influence the efficiency of detection. Several reports [11–15, 17] show that whole-blood analysis is more sensitive than serum and plasma analyses for detection of HCV RNA, although serum and plasma samples have served as the primary end point in most clinical studies of IFN/ribavirin treatment [1–4, 22, 23, 28].

HCV is primarily found in hepatocytes, but evidence is growing that viral RNA is also associated with peripheral blood cells [5–8], lipoprotein complexes and immunoglobulin complexes [29], and precipitates of immune complexes and cryoglobulin complexes [9–11]. Although a recent study failed to demonstrate superior analytical sensitivity of whole-blood analysis versus serum and plasma analyses [18], we believe this was due to the low sensitivity of the methods that were used to detect HCV RNA. Among samples with low levels of HCV RNA, whole-blood testing was superior to serum and plasma testing. At high viral concentrations, the sensitivity of whole-blood and serum and plasma assays were comparable. Explanations of how whole blood yields more HCV RNA than serum and plasma focus not only on HCV residing in or attached to peripheral blood cells [12–17] but also refer to the association of HCV with cryoglobulin complexes and rheumatoid factor complexes [9–11], both of which are lost (in addition to all peripheral blood cells) during conventional preparation of serum and plasma specimens.

A strength of this study is the inclusion of a large number of patients who did not respond to initial IFN monotherapy (i.e., a “worst-case scenario”). In 18 (32%) of the 56 such patients in our study, combination therapy (ribavirin and an increased dosage of IFN [10 MU every 2 days]) resulted in a transient complete virologic response in serum and plasma up to 5 months before the end of treatment. However, a sustained virologic response was achieved in only 4 (7%) of 56 patients. This may be explained by the high percentages of patients with HCV genotype 1 (87%) and genotype 4 (6%) in our study (table 1), because both strains are known to be generally less
responsive to antiviral treatment [1–4, 22, 23, 28], and by the fact that the study population had not previously responded to IFN monotherapy.

The large number of specimens analyzed and the confirmation of PCR results by 4 different RT-PCR approaches (analytical agreement, 97%) strongly support the validity of our study. A total of 755 sample sets of serum, plasma, and whole-blood specimens obtained from 56 patients were analyzed, 268 of which were tested by all 4 techniques. Furthermore, none of the 641 serum and plasma samples that tested positive for HCV RNA were associated with whole-blood samples that had negative test results (with 1 exception, which was detected by means of the LightCycler) (figure 2C).

One limitation of the data is that very few patients with a sustained virologic response \( (n=4) \) were evaluated (table 2 and figure 2D). Thus, the statistical power of the negative predictive value (67%) of whole-blood HCV RNA testing is limited, and further investigations to test this finding are underway. Nevertheless, the high positive predictive value (100%) of detecting HCV RNA in whole-blood specimens among individuals with negative results of serum and plasma HCV RNA tests is impressive. These data suggest that whole-blood analysis offers the possibility to tailor HCV combination therapy [3, 4]. Our study clearly demonstrated that HCV RNA analysis of cell- and precipitate-free blood specimens (serum and plasma specimens, respectively) significantly underestimates the “true” viral load in the circulation, resulting in overestimation of virologic response rates. Our data recommend that early clearance of HCV RNA from whole blood, compared with serum and plasma, may provide a new tool for predicting a sustained virologic response. Further studies addressing early time points for viral clearance in patients with a sustained virologic response may identify new strategies to improve the prediction of sustained virologic response, based on a combination of serum and plasma and of whole-blood HCV RNA testing.

An important limitation of studies with a virologic end point is the sensitivity of the tests used to detect HCV RNA [30]. Preliminary results from a pegylated-IFN/ribavirin study [28] show that viral loads in whole-blood samples are up to 10 times higher (median, 2–3 times higher) than in simultaneously obtained serum samples, thus strongly supporting our findings of a generally increased sensitivity associated with analysis of whole-blood specimens by standard regimens.

Our study is the first to demonstrate the benefit of whole-blood testing in patients who represent the “worst-case scenario” (i.e., individuals who did not respond to primary IFN monotherapy), >90% of whom were infected with HCV genotype 1a and/or 1b or genotype 4. It will be of considerable interest to see the results of whole-blood HCV RNA testing, which is now under investigation, for patients treated with current standard-of-care pegylated-IFN/ribavirin.

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Potential conflicts of interest. All authors: no conflicts.

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