Ultracentrifugation and Concentration of a Large Volume of Serum for HCV RNA During Treatment May Predict Sustained and Relapse Response in Chronic HCV Infection

J.G. McHutchison,1* L.M. Blatt,2 R. Ponnudurai,1 K. Goodarzi,1 J. Russell,2 and A. Conrad2

1Division of Gastroenterology/Hepatology, Scripps Clinic and Research Foundation, La Jolla, California
2National Genetics Institute, Los Angeles, California

The ability to predict accurately a sustained response during therapy in patients with hepatitis C virus (HCV) infection is unavailable. The aim of this study was to differentiate, during therapy, patients who would relapse from those with a sustained response by ultracentrifugation for residual serum HCV RNA. Sixty-one specimens (from 32 patients) collected during interferon therapy were assessed by ultracentrifugation. All were negative using a quantitative polymerase chain reaction (PCR) (detection limit ≤ 100 copies/ml). One-milliliter aliquots were ultracentrifuged at 23,000 × g (160 min), and then the nucleic acid pellet was extracted, precipitated, and resuspended. Qualitative PCR was carried out in quadruplicate using two separate 5’UTR primer sets (8 results/specimen). A specimen was positive if ≥ 1 gels was positive compared to controls. At weeks 12 and 24, 9/9 (100%) sustained response patients were negative by ultracentrifugation. In the 23 relapse patients at week 12, 7/12 specimens were positive; at week 24, 7/14 were positive. Earlier time points could not differentiate the patients’ eventual response to therapy. The predictive value of a positive ultracentrifugation test for relapse at week 12 or 24 was 100%. The predictive value of a negative test for sustained response was 62% and 50% at week 12 and 24, respectively. These preliminary results indicate that patients with an eventual sustained response will have no detectable serum HCV RNA by week 12 or week 24. A positive result is 100% predictive of relapse.

INTRODUCTION

The reported sustained biochemical and virological response rate observed with a standard 6- or 12-month course of interferon therapy is approximately 15–30% [Davis et al., 1989; Causse et al., 1991; Marcellin et al., 1991; Poynard et al., 1995, 1996; Hoofnagle and DiBisceglie, 1997]. Pretreatment variables reported to predict response to therapy in patients with chronic hepatitis C virus (HCV) infection are not consistent and the variables are predictive only in a population-based manner, providing little assistance for the management of individual patients [Davis et al., 1994; Martinot-Peignoux et al., 1995]. Published studies have shown that the presence of serum HCV RNA at early time points, particularly week 12 during treatment, accurately predicts nonresponse to therapy [Tong et al., 1997; McHutchison et al., 1998]. However, published studies suggest that the absence of serum HCV RNA early during therapy does not predict reliably, identify, and separate those individual patients who relapse after therapy from those with a sustained response [Kleter et al., 1993; Hino et al., 1995; Kohara et al., 1995; Orito et al., 1995; Hanley et al., 1996]. A test to separate these two groups would be advantageous for predicting the long-term outcome in sustained responders, and to potentially modify therapy in those likely to relapse.

In patients who are serum HCV RNA negative during therapy, we hypothesized that those who have an eventual sustained response will have no detectable serum HCV RNA by week 12 or week 24. A positive result is 100% predictive of relapse.

KEY WORDS: hepatitis C virus; treatment; response; HCV RNA RT-PCR; ultracentrifugation

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In such patients, measurement of HCV RNA in a concentrated, large volume serum specimen during therapy when conventional polymerase chain reaction (PCR) is negative should theoretically also be negative. By comparison, those individuals who relapse virologically should, according to this hypothesis, have some residual viral RNA detectable in a large volume serum sample, even when serum HCV RNA is negative by PCR during therapy.

The aim of this study was to attempt to differentiate those patients with a sustained response from those with a relapse response. This differentiation was accomplished by carrying out an ultrasensitive PCR for residual serum HCV RNA during therapy in patients who were, by conventional PCR, negative for serum HCV RNA.

**MATERIALS AND METHODS**

**Patient Selection**

The group of 32 patients evaluated in this study had documented chronic HCV infection and had undergone a standard course of therapy with 3 million units three times weekly of interferon alpha 2b for 6 months. All patients had a sustained or relapse virologic response as defined below. The protocol was approved by the Human Subjects Ethics Committee and all patients gave informed consent. Chronic HCV infection was documented using standard criteria, including abnormal alanine aminotransferase (ALT) values for at least 6 months and biopsy-proven histological changes consistent with chronic hepatitis C. All patients tested repeatedly reactive for anti-HCV with the Ortho HCV second-generation enzyme-linked immunosorbent assay (ELISA) (Ortho Diagnostics, Raritan, NJ), and had detectable serum HCV RNA by reverse transcription-PCR (RT-PCR). Other etiologic factors for liver disease were excluded in all subjects by serological testing, liver histology, and clinical history. All patients had compensated liver disease detected biochemically and clinically. The patients were followed throughout treatment and for a 6-month follow-up period. ALT values were assessed monthly throughout this time. Patients' serum was stored in 1.0 ml aliquots at -70°C within 2 hours of collection: at baseline, during treatment at weeks 1, 2, 4, and 12; at end of treatment week 24; and follow-up week 48. A total of 66 specimens from these 32 patients were thus available for testing by ultracentrifugation.

Biochemical response (BR) was defined using standard ALT criteria. A sustained response (SR) was defined when ALT values normalized during treatment and remained normal during follow-up; a response with relapse (RR) when ALT values normalized during therapy, but became abnormal during follow-up; and nonresponse (NR) when ALT values did not return to normal during treatment and follow-up. A virological response was defined as sustained when serum HCV RNA became undetectable during treatment by a multiplex PCR assay and continued through follow-up week 48; virological response with relapse when serum HCV RNA became undetectable during therapy but reappeared during follow-up.

**Detection of Serum HCV RNA**

HCV RNA was detected in serum samples in a blinded duplicate manner using a multicycle quantitative PCR assay as described previously [Tong et al., 1997]. The sensitivity of this assay is $\leq 100$ copies/ml with an intrasample coefficient of variation of 26%. Ultracentrifugation of samples was then undertaken only if this assay was negative, and adequate serum was available.

**Detection of Serum HCV RNA by Ultracentrifugation**

Viral RNA was concentrated from the 1.0 ml patient serum aliquots by ultracentrifugation at 23,000 x g for 160 minutes at 16°C and extracted using a guanidine thiocyanate-phenol-chloroform mixture followed by ethanol-ammonium acetate precipitation [Chomczynski and Sacchi, 1987]. The precipitated RNA was centrifuged and the resulting pellet dried in a Centrivap console (Labconco, Kansas City, MO). The dry pellet was then resuspended in 30 μl of an Rnasin (Promega Corp., Madison, WI), dithiothreitol, and diethylpyrocarbonate-treated water mixture. The samples were kept at or below -20°C until RNA RT-PCR.

RT was carried out on the prepared RNA in eight separate reactions using random hexadeoxynucleotide primers (Pharmacia Biotech, Piscataway, NJ) (100 ng/μ) for cDNA synthesis. The mixture was heated to 70°C to denature RNA secondary structure and then cooled to room temperature to allow random primers to anneal to the RNA templates. Twenty microliters of a mixture containing M-MLV reverse transcriptase (USB, Cleveland, OH) and standard buffer components were then added and RT carried out at 40°C for 0 minutes. The cDNA was then heated to 90°C to inactivate reverse transcriptase, cooled to room temperature, and used immediately as template for the PCR. RT conditions were as follows: 3.3 U/μ reverse transcriptase, 5 mM MgCl$_2$, 50 mM KCl, 2.5 mM DTT, 1 mM dNTPs, 0.1% Triton X-100, 10 μg/ml acetylated bovine serum albumin (BSA), and 10 mM Tris-HCl, pH 9.0 for 1 hour at 40°C.

Seventy-five microliters of PCR mix was added to the entire RT reaction volume (26 μl) to a final MgCl$_2$ concentration of 1.65 mM in a total volume of 101 μl. A layer of mineral oil was added to prevent evaporation during thermocycling.

The PCR cycle consisted of annealing for 60 sec, extension for 60 sec, and denaturation for 60 sec, at 55°C, 74°C, and 94°C, respectively. After thermocycling, samples were submitted to a final 74°C final extension for 10 minutes. The thermocycling efficiency was assessed by satisfactory amplification of known copy number RNA standards included in each run. Two primer sets were used for the amplification, both from...
the 5′ untranslated region of the HCV genome [Oka-
moto et al., 1990]. Both of these primer sets have been
tested in our laboratory and have shown that they are
highly conserved and will detect all known subtypes of
HCV. Primer set 1: upstream 5′-GTG GTG TGC GGA
ACC GGT GAG T-3′, downstream 5′-TGC ACG GTC
TAC GAG ACC TC-3′ which produced a 196-bp prod-
uct. Primer set 2: upstream 5′-CTG TGA GGA ACT
WCT GTC TTC -3′, downstream 5′-CCC TAT CAG
GCA CTA CCA CAA-3′, which produced a 256-bp prod-
uct.
Both primer sets used in the ultrasensitive RT-PCR
assay were shown to detect all of the known genotypes
of HCV, as confirmed by comparisons to cloned HCV
isolates from each of the different genotypes. The corre-
sponding correlation coefficients (rho) for quantita-
tion of known-quantity HCV clones from different
genotypes including 1a, 1b, 2a, 2b, 3a, 4a, 5a, and 6a were .9875, .9290, .9967, .9983, .9997, .9949, .9960, and
.9988 respectively (P < .001 for all correlations) indi-
cating a high level of quantitative efficiency for each
HCV genotype.
The amplified cDNA was then electrophoresed in 3%
agarose gel and detected by Southern blotting and im-
umnostaining using a nonradioactive digoxigenin-
labeled complementary DNA probe made in our lab-
ratory. These procedures were carried out using au-
tomated instruments for PCR thermocycling, agarose gel
electrophoresis, vacuum-transfer Southern blot, hy-
bridization, and immunostaining. An internal control
RNA was added to each sample at the first stage of the
nucleic acid extraction. Amplification of this internal
control ensures successful RNA extraction and con-
firms the sample does not contain PCR inhibitors. To
minimize the possibility of post-PCR contamination,
the pre- and post-PCR laboratories were located in
separate buildings to ensure total segregation of per-
sonnel, equipment, and supplies. In each laboratory,
ultraviolet (UV) lights were used to control contamina-
tion by depurination and/or cross-linking of any air-
borne or surface-bound PCR products. As a routine
practice, all reagents used in the laboratory were pre-
pared in single-use portions to prevent contamination of
stock solutions. In addition, we included both re-
agent blanks and negative controls in each PCR run.
The negative controls were of several types. All nega-
tives were co-prepared with the samples and accompa-
nied the samples through the entire PCR process. From
prior experiments, the sensitivity of this assay is 13
copies/ml (95% CI of 8–18 copies/ml). This qualitative
PCR was carried out in quadruplicate on four separate
3-μl aliquots. This yielded a total of eight individual
results per specimen. A result was considered positive
if one or more of the eight resulting gels was positive.

**HCV Genotype Determination**

HCV genotyping was carried out on biotin-labeled
PCR products by hybridization to oligonucleotides di-
rected against the variable region of the 5′UTR, and
then immobilized as a parallel line on membrane strips
early time points at weeks 1, 2, and 4 that revealed persistence of detectable virus by ultracentrifugation. However, by week 12, 7 of the 12 responder relapser specimens tested were positive and 5 were negative, and at week 24, 7 of the 14 responder relapser specimens tested were positive and 7 were negative as shown.

We then analyzed the virological response at week 48 as predicted by the week 12 ultracentrifugation results (Table II). Seven specimens were positive at week 12 by ultracentrifugation and all 7 patients were relapsers at week 48. Thirteen specimens were negative at week 12, 8 of whom were sustained responders at week 48 and 5 relapse responders. The predictive value of a positive ultracentrifugation test at week 12, or its ability to predict relapse, was thus 100% with a sensitivity of 58% and specificity of 100%. The predictive value of a negative test in predicting sustained response was 62%, and the overall accuracy was 75%. The likelihood ratio was 9.60 ($P = .002$). The evaluation of response at week 48 using week 12 ultracentrifugation data revealed that 7 patients were positive by ultracentrifugation at week 24, and all were relapsers at week 48. The predictive value of a positive test in predicting relapse was thus 100%, with 100% specificity but only 50% sensitivity. Fifteen samples were negative, 8 of which were eventual sustained responders at week 48. The predictive value of a negative test was thus 50%, with an overall accuracy of 65% (Table II). The likelihood ratio was 7.54 ($P = .006$).

We set out subsequently to determine whether the negative ultracentrifugation results observed in relapse patients might be a function of the volume of serum tested. Two milliliters of sera from eight relapse patients and two sustained response patients during week 12 or week 24 of therapy were retested by ultracentrifugation. Both samples from sustained responders were also negative when this larger volume of serum was retested by this methodology. In the relapse patients, four of the eight results were negative but not interpretable, due to strong PCR inhibition within the samples as assessed by internal controls. Of the remaining four evaluable specimens, three (75%) did have detectable serum HCV RNA. The age of the serum specimen could not be correlated with negative ultracentrifugation results. In addition, we found no correlation between the results with the ultracentrifugation test and HCV genotype. Equal numbers of patients and tests were either negative or positive for each genotype.

### DISCUSSION

This preliminary study assessing the ability of ultracentrifugation of a large volume of serum to detect residual HCV RNA, suggests that at week 12 and week 24 of therapy, we were able to detect residual serum HCV RNA in 50–60% of patients. These patients were negative during therapy by conventional serum PCR, but were eventual virological relapsers. The preliminary results suggest that the most appropriate time to perform such an assay is week 12 or 24 of therapy. Using this technique, all patients who were positive by ultracentrifugation at week 12 or 24 were classified as relapsers at 6 months follow-up, and all sustained responders were negative by ultracentrifugation at these points.
same time points. In addition, some relapse patients retested had detectable virus by ultracentrifugation when 2 ml, but not 1 ml, of serum was tested.

The potential to identify and separate relapse responders from sustained responders may have therapeutic implications. In these situations alternative therapies such as dose alteration or addition of other antiviral agents may be used in individual patients in the future, when proved effective in these clinical situations.

It has been suggested that patients with HCV who relapse after therapy have residual viral RNA too low to be detected by conventional assays. The results agree with this concept and indicate that residual serum HCV RNA can be detected in more than half of the relapse patients. However, some relapse patients were persistently negative when 1 ml of serum was tested by ultracentrifugation. There are a potential number of factors to explain these negative results in this patient population. Assay sensitivity and volume of serum tested could explain such negative results, and our preliminary findings with 2 ml of serum do indicate that the ability to detect residual virus is partially a function of assay sensitivity and/or volume of serum tested. Degradation of serum HCV RNA in stored specimens or varied assay sensitivity according to HCV genotype could also explain such negative results, but we found no correlation between the age of the specimens, HCV genotype, and these negative results. Finally, intracellular viral reservoirs (such as peripheral blood mononuclear cells) or actual degradation of RNA during ultracentrifugation are potential reasons for negative results that require further investigation.

These preliminary results require further expansion, verification, and prospective study in a larger cohort of patients with chronic HCV infection undergoing therapy. We do not advocate that this assay be used currently in a clinical setting, but the results obtained so far support our original hypothesis and require further careful study.

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REFERENCES


