

# Persistence of Viremia and the Importance of Long-Term Follow-up After Acute Hepatitis C Infection

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The purpose of this investigation was to prospectively characterize acute hepatitis C virus (HCV) infections and to evaluate the hypothesis that the outcome is affected by identifiable clinical or viral factors. One hundred forty-two people with a history of illicit drug use who were HCV antibody-negative in 1988 were followed semiannually through 1996. HCV seroconversion (second generation enzyme immunoassay and recombinant immunoblot assay) was recognized in 43 (30%) of the participants, who were followed up for a median of 72 months. HCV RNA was detected and quantified by polymerase chain reaction in a median of 10 specimens per participant and showed two distinct patterns of viremia: viral clearance was noted in 6 (14%) of the participants, and viral persistence was observed in 37 (86%) of the participants. Subjects with viral clearance were more likely to be white ( $P = .004$ ), have jaundice ( $P = .03$ ), and have lower peak viral titer ( $P = .003$ ). However, the outcome for a given person could not be predicted by clinical features, RNA level, or HCV subtype (as ascertained by analysis of core-E1 complementary DNA sequence). No acute infections were recognized by health care providers. At the time of seroconversion, HCV RNA was detectable in 81% of participants, and recombinant immunoblot assay (RIBA) was positive in 85% of participants. We conclude that approximately 85% of people with acute hepatitis C develop persistent viremia. However, acute infections are uncommonly recognized clinically, underscoring the importance of screening individuals at risk. Long-term follow-up, but no single laboratory test, is necessary to ascertain the outcome and in some cases make the diagnosis of acute HCV infection. (HEPATOLOGY 1999;29:908-914.)

In the United States, approximately 4 million people have chronic hepatitis C virus (HCV) infection, which is the leading reason for liver transplantation and results in an

estimated 10,000 deaths each year.<sup>1</sup> The onset of HCV infection generally does not cause symptoms, making it difficult to study early serological and virological events.<sup>2</sup> In look-back studies after transfusion of HCV-contaminated blood and after experimental inoculation of chimpanzees, HCV RNA was detected in recipient plasma within 2 to 4 weeks and HCV antibody within 6 to 8 weeks.<sup>3,4</sup> HCV antibody persisted for years in almost all patients, although HCV RNA was repeatedly, though sometimes intermittently, detected in 70% to 90% of persons receiving HCV-contaminated transfusions.<sup>2,5</sup>

Community-based investigations of acute HCV infection corroborate the high rate of RNA and antibody persistence.<sup>6,7</sup> However, these studies lack preinfection evaluations and are restricted to people with symptoms of acute infection (estimated to be less than 25%) who seek medical care. Posttransfusion acute HCV studies may not be representative, because less than 5% of acute infections in the United States follow transfusion.<sup>1</sup> Transfusion recipients also are generally older and receive a higher viral inoculum than persons infected in association with illicit drug use, the leading transmission route. In addition, many acute hepatitis C studies are limited by small numbers of cases<sup>3,8,9</sup> or short follow-up periods.<sup>10,11</sup>

Whereas some long-term studies of disease progression have shown that people with HCV subtype 1b, higher viral load, and elevated levels of alanine transaminase (ALT) were more likely to develop cirrhosis,<sup>12-14</sup> these and other possible cofactors have not been carefully examined with respect to viral persistence. In this investigation, the frequency of viral persistence was assessed in a community-based cohort of former and current injection drug users (IDUs), allowing us to test the hypothesis that this outcome is associated with viral load or subtype. In addition, recognition of acute HCV infection and the temporal pattern of HCV RNA and antibody detection were assessed in prospectively followed persons, irrespective of the occurrence of symptoms.

## PATIENTS AND METHODS

**Participants.** Eligible participants were IDUs from the Baltimore area who were enrolled in the AIDS Link to the Intravenous Experience study, a longitudinal investigation of the natural history of human immunodeficiency virus (HIV) infection.<sup>15</sup> Between 1988 and 1989, 2,921 people were enrolled in the study. All participants were at least 18 years of age, free of acquired immunodeficiency syndrome, and acknowledged a history of injection drug use in the preceding 10 years. Participants were interviewed and serum samples were collected at the initial visit and every 6 months thereafter.

Using a historical prospective study design, HCV seroconverters were identified in this cohort, as recently described.<sup>16</sup> Briefly, there were 142 participants who were followed for at least 1 year and were negative for antibodies to HCV (anti-HCV) at enrollment based on

Abbreviations: HCV, hepatitis C virus; ALT, alanine transaminase; IDU, injection drug user; HIV, human immunodeficiency virus; EIA, enzyme immunoassay; RIBA, recombinant immunoblot assay; OD, optical density.

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Received August 7, 1998; accepted October 29, 1998.

Supported by grants DA-04334, DA-08004, AI-40035, and DA-023201 from the U.S. Public Health Service, and grant 5M01RR00052 from the Outpatient General Clinical Research Center.

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second generation enzyme immunoassay (EIA). Among these 142 IDUs, the most recent serum sample available as of May 1996 was tested by EIA, and those who were positive underwent testing of intermediate time points such that the majority of seroconversions could be identified within a 7-month interval. HCV RNA was quantitatively assessed in serum samples collected just before EIA seroconversion and at all available time points thereafter. Samples from the visit of EIA seroconversion were also tested by second generation recombinant immunoblot assay (RIBA). True HCV seroconverters were defined as those participants who developed a positive serum EIA during the follow-up period, which was confirmed by detection of serum HCV RNA or by positive RIBA. Hepatitis C seroconverters were later interviewed and examined for signs of liver disease by one investigator.

**Laboratory Testing.** All serum samples were stored at  $-70^{\circ}\text{C}$ , and all had undergone two or three freeze-thaw cycles before testing. Anti-HCV was assessed by EIA with the second generation Ortho HCV 2.0 enzyme immunoassay (Ortho Diagnostic Systems, Raritan, NJ) according to the manufacturer's specifications. Negative antibody results were repeated at least once. Seroconversions were confirmed by repeated EIA testing at later visits, HCV RNA testing, and second generation RIBA (Chiron RIBA HCV 2.0 strip immunoblot assay; Chiron Corporation, Emeryville, CA).

HCV RNA was initially detected with a commercially available quantitative reverse transcriptase polymerase chain reaction assay (AMPLICOR HCV MONITOR Test Kit; Roche Diagnostic Systems, Branchburg, NJ). The linear range of this assay in this and other laboratories was 500 to 500,000 copies per mL.<sup>17,18</sup> All samples with original values above 500,000 copies per mL were diluted 1:100 with phosphate buffered saline and repeated. The repeated measure was multiplied by 100 to generate the final result. Samples below the linear range of the quantitative assay were assigned a value of 250 copies per mL, and, when possible, were tested again with one of two qualitative HCV RNA assays: a commercially-available qualitative reverse transcriptase polymerase chain reaction assay (AMPLICOR HCV Detection Kit, Roche Diagnostic Systems) or an in-house nested polymerase chain reaction assay using universally conserved primers from the 5' noncoding region of the HCV genome.<sup>19</sup> With these assays, HCV RNA was detected in diluted samples of a reference strain (Hutchinson) to levels of approximately 100 copies per mL.

HCV genotyping was performed by analysis of HCV RNA nucleotide sequences as follows: extracted RNA was amplified by nested reverse transcriptase polymerase chain reaction using primers (donated by J. Bukh, National Institutes of Health) directed to the core-envelope 1 segment of the genome.<sup>20</sup> The complementary DNA was then analyzed with an automated sequencer (Perkin-Elmer; Foster City, CA). Sequence data from major HCV genotypes and subtypes were obtained from GenBank and aligned with those of the study participants. A phylogenetic tree was constructed using PHYLIP version 3.572 programs DNADIST and FITCH.<sup>21</sup> HCV serotyping was also performed on all patients (Chiron RIBA HCV Serotyping strip immunoblot assay, Chiron Corporation).

After January 1995, ALT measurements were performed on the day of the visit by a commercial laboratory.

**Statistical Analysis.** Date of patient seroconversion was estimated as the midpoint between the last visit that tested negative and the first visit that tested positive for anti-HCV. Duration of follow-up refers to the time between the date of patient seroconversion and the latest visit (as of May 1996) from which serum was available for testing. Between outcome groups, categorical variables were analyzed by using Fisher's exact test, and continuous variables were compared with the Mann-Whitney rank sum test. All statistical tests were two-tailed.

## RESULTS

**Patterns of Viremia.** Of 142 anti-HCV-negative participants, there were 43 (30%) patients who were HCV seroconverters.

For each participant, the quantity of HCV RNA was assessed at a median of 10 separate time points, representing 72 months of follow-up (range 14 to 93 months), and two distinct patterns were noted: viral clearance and viral persistence.

Of the 43 seroconverters, HCV RNA was only transiently detectable in 5, and was never detected in another, who nonetheless developed antibodies to three of four recombinant HCV antigens by RIBA (Fig. 1). Thus, 6 (14%) of the 43 seroconverters had clearance of viremia after acute infection. Among the 5 patients with detectable virus, the median duration of viremia after seroconversion was 19 months (range 14 to 45 months). HCV RNA then remained undetectable for a median of 37 months (range 27 to 73 months), as determined by at least four and up to eight separate measurements per person. For each of the 6 participants, at least two samples taken more than 1 year apart during the period of clearance were also tested with the more sensitive commercial

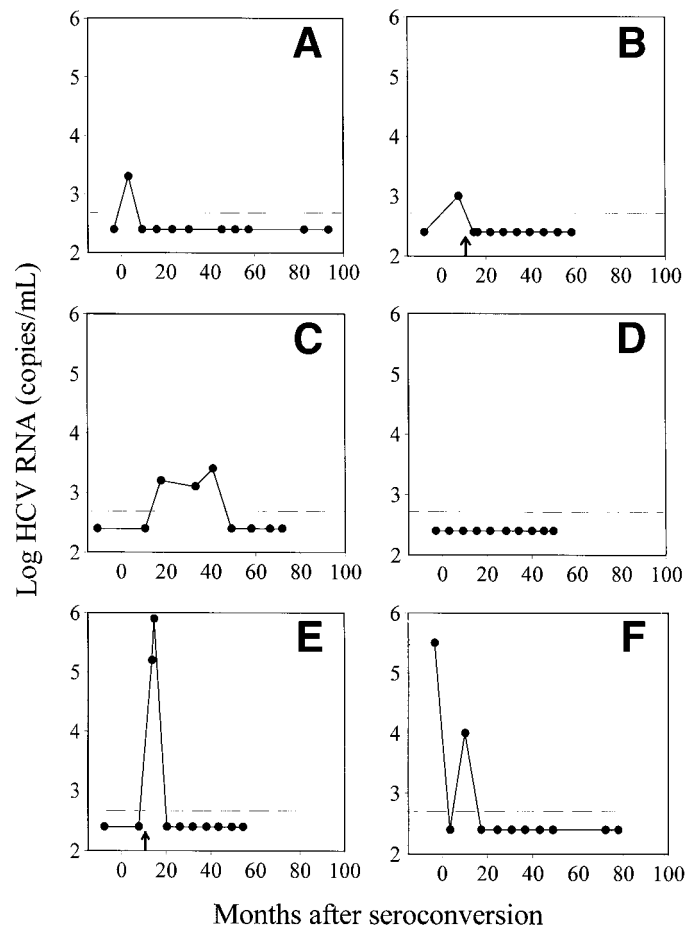


FIG. 1. HCV RNA levels among six individuals with clearance of viremia after acute infection. Dashed lines indicate the lower limit of detection with the quantitative assay (500 copies per mL). HCV levels not detected by this assay were plotted at 250 copies per mL. In patient D, HCV RNA was never detectable, despite repeated testing and seroconversion confirmed by immunoblot assay. In patients E and F high levels of viremia were found before clearance; samples from their seroconversion visits had been stored for 55 and 89 months, respectively, versus a median of 74 months for all six individuals. Patient F was found to have HCV RNA present at first visit, when he was still HCV antibody negative, and is presumed to have been in the process of seroconversion at enrollment. Patients B and E also acquired HIV infection; time of HIV seroconversion is shown by arrows.

TABLE 1. Characteristics of 34 HCV Seroconverters According to Pattern of Viremia\*

Characteristic	Viral Clearance (n = 6)	Viral Persistence (n = 28)	P*
Median age at seroconversion, yr (range)	27 (25-39)	30 (23-44)	>0.2†
Median duration of follow-up, mo (range)	68 (50-93)	78 (45-93)	>0.2†
Median sample storage time, mo‡ (range)	74 (55-101)	82 (54-99)	>0.2†
Sex, n (%)			
Male	6 (100)	22 (79)	
Female	0 (0)	6 (21)	>0.2
Race, n (%)			
White	4 (67)	2 (7)	
Black	2 (33)	26 (93)	0.004
HIV positive at time of HCV seroconversion, n (%)	0 (0)	2 (7)	>0.2
Acquired HIV infection during follow-up period, n (%)	2 (33)	7 (25)	>0.2
Use of injection drugs after seroconversion, median % visits	61	87	>0.2†
Sharing of needles after seroconversion, median % visits	23	23	>0.2†

\*Unless otherwise noted, *P* values were derived by using a 2-tailed Fisher's Exact test. Data from 9 of 43 HCV seroconverters are not shown because follow-up was insufficient (less than 45 months) to ascertain the outcome.

†*P* value derived using a Mann-Whitney rank sum test.

‡Time interval between date of seroconversion visit and date of HCV RNA testing.

and in-house qualitative assays, and HCV RNA was undetectable in sera in all instances by both methods.

In the remaining 37 (86%) participants, HCV RNA was detectable at the latest visit, after 14 to 93 months (median 74 months) of follow-up. For 32 of the 37 participants, HCV RNA was continuously detectable, while for 5 participants HCV RNA levels were undetectable in at least one interval visit, as determined by both quantitative and qualitative commercial assays. Nine of these individuals were followed for less than 45 months after seroconversion (the maximum

duration of viremia found among those with viral clearance). Although HCV RNA was still detected at their last visits, the possibility of ultimately clearing viremia could not be ruled out, and these 9 individuals were excluded from subsequent comparative analyses. Considering only those patients who were seroconverters followed beyond 45 months, there were 6 (18%) patients with viral clearance and 28 (82%) patients with viral persistence.

**HCV Persistence Versus Clearance.** Individuals with viral clearance were of similar age and gender as those with persistent viremia, although a greater percentage were white (Table 1). There were no significant differences between the groups regarding the duration of sample storage, prevalence of HIV infection at seroconversion, or acquisition of HIV infection during follow-up. Members of both groups described similar patterns of injection drug use in the time period after seroconversion.

An aggregate view of viral dynamics was constructed using the median values of HCV RNA levels before and within each 3-month interval after patients underwent seroconversion for each of the two groups (Fig. 2). Peak HCV RNA levels were significantly lower among patients with viral clearance compared with patients with viral persistence ( $P = .003$ , Fig. 3). This difference was evident 24 months after seroconversion and was independent of the number of assessments per member of each group. Although individuals who initially had low levels of HCV RNA were more likely to go on to have clearance of viremia, the ultimate course of infection could not be predicted by a single quantitative assessment.

ALT measurements were available from samples collected after January 1995, when patterns of viremia had been established. Of those patients still in follow-up, 29 patients were at least 45 months beyond seroconversion. Each participant had a median of three separate ALT measurements (range, 1-4). Although the peak ALT values among those participants with viral persistence varied widely (and without relation to the level of viremia), there were no abnormal ALT values measured in those participants with viral clearance (Fig. 3). At the latest visit, 43% of patients with viral persistence had abnormal ALT values (data not shown).

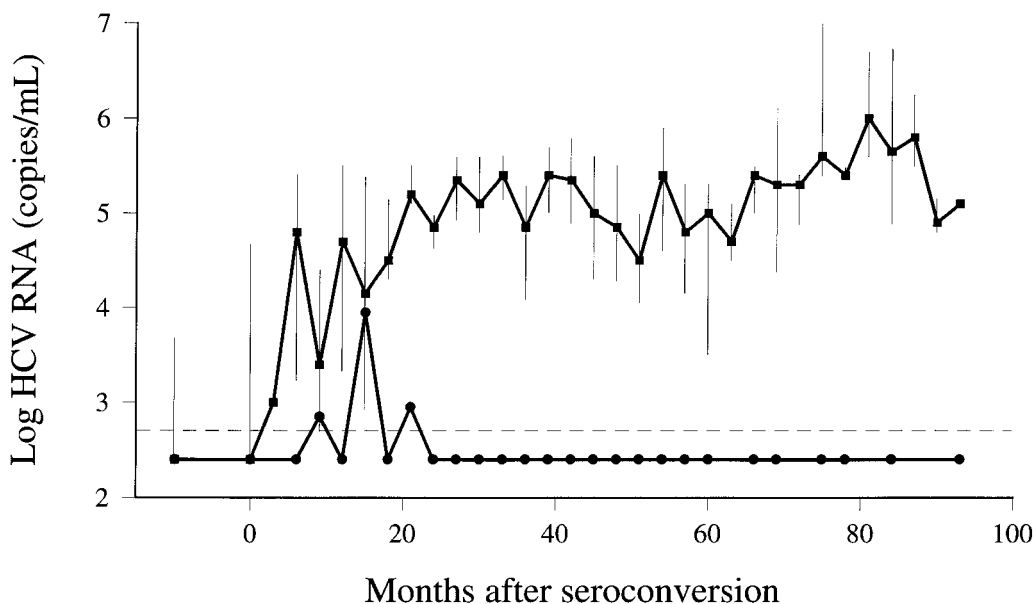


FIG. 2. HCV RNA levels: persistence vs. clearance. Shown are median HCV RNA levels for each 3-month interval relative to time of seroconversion among 28 individuals with viral persistence (■) and 6 individuals with viral clearance (●). All members of a group were considered if they were evaluated at that interval. Vertical lines represent the 25th to 75th percentile of values at each interval for those with viral persistence. The dashed line indicates the lower limit of detection with the quantitative assay (500 copies per mL). Undetectable HCV RNA levels were plotted at 250 copies per mL.

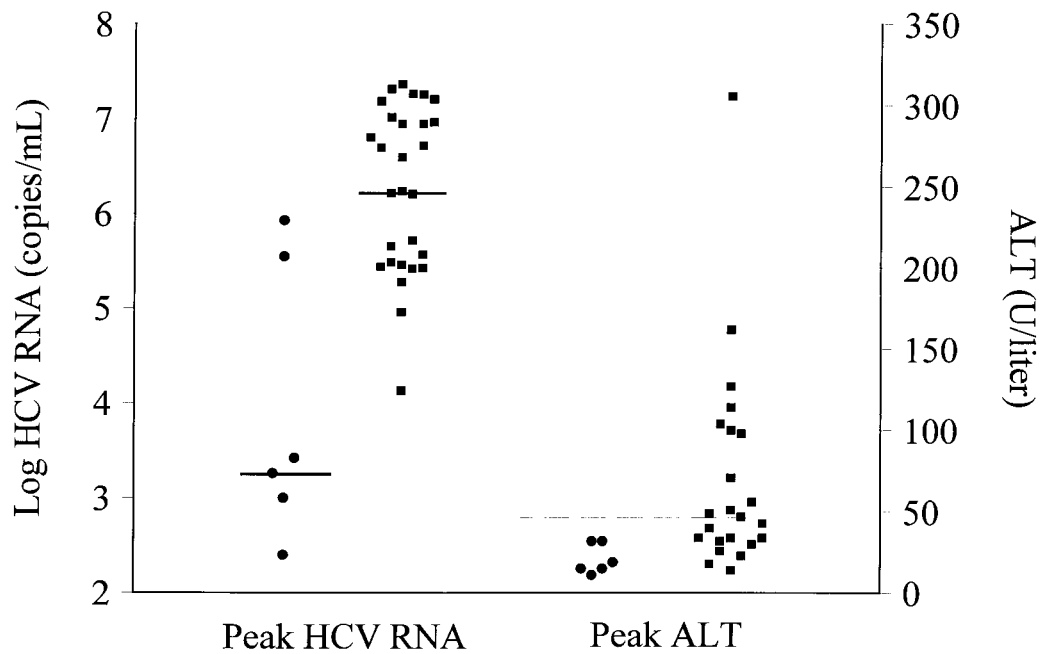


FIG. 3. Peak levels of HCV RNA and ALT by pattern of viremia. Shown are peak HCV RNA levels (n = 34) and peak ALT values (n = 29) for patients with HCV who are seroconverters with viral persistence (■) and viral clearance (●). Solid lines indicate the median peak HCV RNA levels in each group. The one individual from the viral clearance group in whom HCV RNA was not detected was assigned a peak value of 250 copies/mL (see text). The dashed line indicates the upper limit of normal ALT values. All ALT measurements from those with viral clearance were taken during periods of undetectable HCV RNA.

**Antibody-RNA Relationship.** For each of the 43 HCV seroconverters, anti-HCV was assessed by EIA at a median of five separate time points after the date of seroconversion. A rapid and sustained antibody response was noted (Fig. 4). Thirty-nine individuals had maximum optical density (OD) values at their first anti-HCV positive visit, and this antibody level was maintained in all but three participants who had a single subsequent measurement that was below maximum. Only one participant had an OD value below maximum at the latest visit tested (60 months after seroconversion). The four participants with submaximal OD values at the first positive visit had maximum values at the next visit and remained so until the last visit tested.

Among the 42 participants with detectable viremia, HCV RNA was first detected at the seroconversion visit in 20 people (48%), before seroconversion in 14 people (33%, median interval of 3.8 months before the estimated date of

seroconversion), and not until after seroconversion in 8 people (19%, median interval of 15.3 months after the estimated date of seroconversion). Among the latter 8 individuals, 7 had remaining serum from the seroconversion visit. Testing with the in-house nested polymerase chain reaction assay confirmed the absence of detectable HCV RNA despite the presence of anti-HCV and detection of RNA at later visits. Notably, 3 of these 8 patients had submaximal EIA OD values at this visit (Fig. 4). The temporal relationship of detection of HCV antibody and RNA was not significantly different in those with persistent versus cleared viremia ( $P > .2$ ), nor was there an association with age, race, duration of sample storage, or HIV status (data not shown).

Samples from the seroconversion visit were available for RIBA in 40 patients; 34 patients (85%) were RIBA positive and 6 patients were indeterminate. Among those who did not have detectable HCV RNA at the seroconversion visit, 4

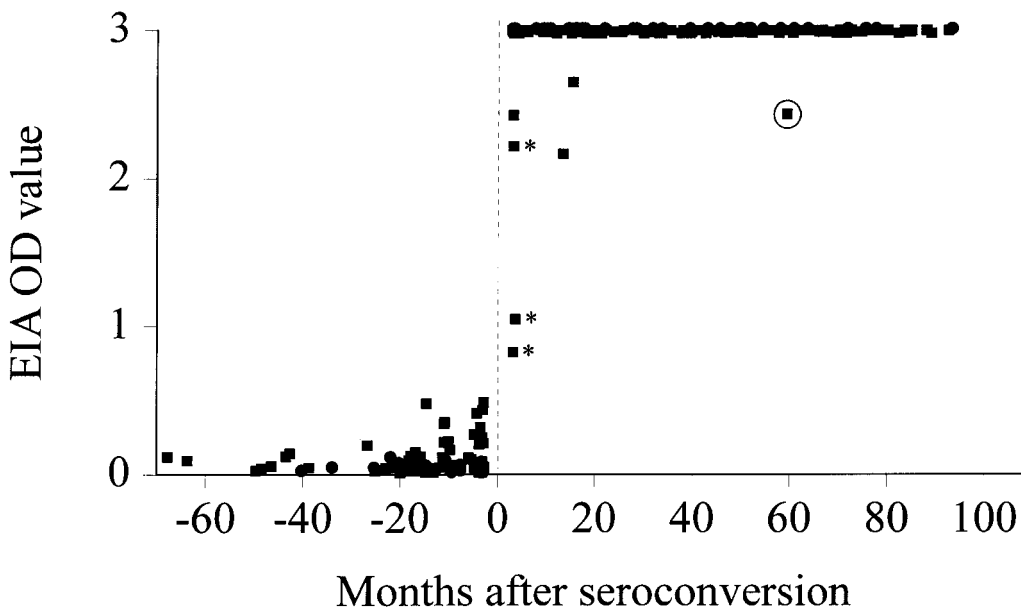


FIG. 4. EIA OD values for HCV seroconverters with viral persistence (■) and viral clearance (●) relative to date of seroconversion. Maximum OD value was 3.0. All four individuals with submaximal OD values at their first anti-HCV positive visit had maximal values at their next visit, and three were also HCV RNA negative at that visit (\*). The one participant with an OD value below maximum at the last visit tested is circled.

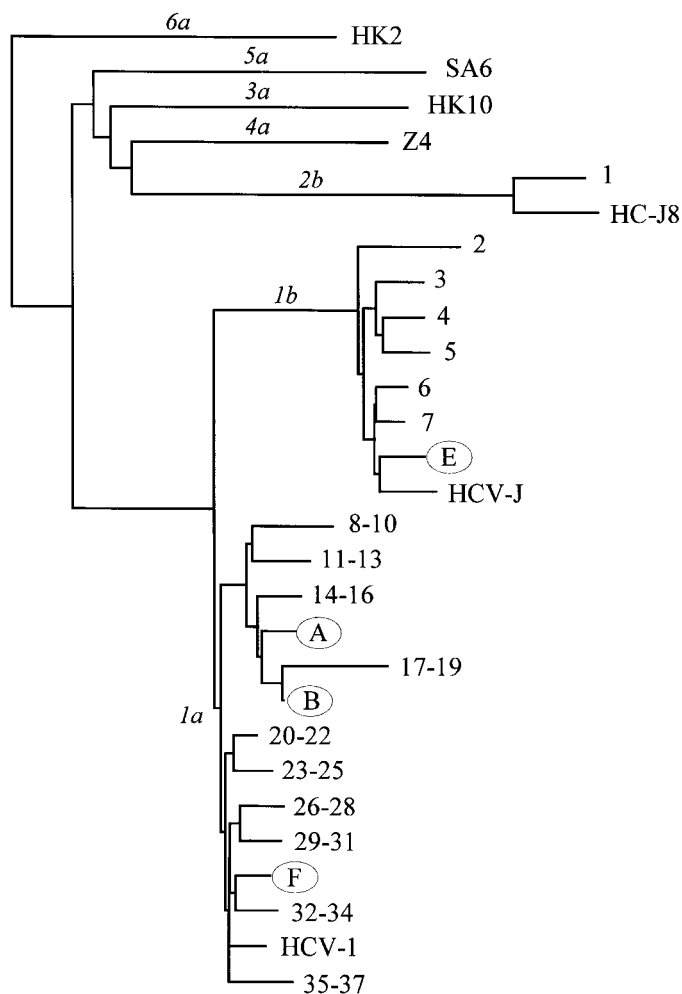


Fig. 5. Phylogenetic tree of 41 HCV isolates based on the C-E1 genes. Genotype designations of HCV isolates are indicated in *italics*, and a representative (published) sequence of each genotype is shown. Numbers represent clinical isolates from individuals with viral persistence; those of genotype 1a are shown in groups of three genetically similar isolates. *Circled letters* represent isolates from individuals with viral clearance; *letters* refer to panels in Fig. 1.

patients were RIBA positive and 3 patients were indeterminate. All of the indeterminate results had detectable bands corresponding to the c33c recombinant antigen, and at later visits all patients ultimately developed additional bands making them RIBA positive. All 43 patients who were seroconverters remained RIBA positive at their latest visit.

**Phylogenetic Analysis.** HCV subtype was determined for all 37 individuals with persistent viremia and for 4 of 6 individuals with viral clearance as follows: 1a ( $n = 33$ ), 1b ( $n = 7$ ), and 2b ( $n = 1$ ). Three of the patients with viral clearance had HCV genotype 1a (Fig. 1A, B, and F), and one had type 1b (Fig. 1E). No genetic relationship was detected among the 41 viral strains according to the pattern of viremia (Fig. 5). Of the 2 patients in which genotype could not be determined, 1 was found to have serotype 1 (Fig. 1D) and 1 had serotype 2 (Fig. 1C). In the remaining 41 patients, genotype and serotype were identical in 38 (93%).

**Clinical Characteristics.** At each semiannual visit, participants were asked about jaundice, hepatitis, and any medical treatments. During the 6 months before or after the sero-

conversion visit, 6 individuals (14%) reported jaundice or hepatitis. Three (50%) of the 6 participants with viral clearance reported jaundice or hepatitis, compared with 3 (8%) of the 37 cases with viral persistence ( $P = .03$ ). There was no significant difference in reporting of jaundice or hepatitis between white and black individuals (data not shown). Thirty-two participants (74%) acknowledged contact with medical providers during this seroconversion period. The majority of these encounters were for routine health maintenance or various acute medical illnesses other than hepatitis.

As of April 1997, when seroconverters were evaluated by a study investigator, 6 of the 43 seroconverters had died (of causes unrelated to liver disease), and another 6 patients had been lost to follow-up for over 2 years. Among those participants still in follow-up, 19 were interviewed and examined (4 from the viral clearance group and 15 from the viral persistence group). Only 1 participant was symptomatic (recent jaundice) and was found to have stigmata of liver disease on examination (hepatomegaly and scleral icterus). This man was a member of the viral persistence group who acknowledged long-term heavy alcohol use. One member of the viral persistence group stated that he had received a 3-month course of treatment with interferon alfa that occurred approximately 4 years after he became HCV seropositive. No other participants had been treated for hepatitis C.

#### DISCUSSION

A recent National Institutes of Health Consensus Panel recommended that a positive EIA test be confirmed by either HCV RNA testing or RIBA.<sup>22</sup> It is generally assumed that HCV RNA is detectable before antibody, as has been shown in well-characterized posttransfusion panels and after experimental infection of chimpanzees.<sup>3,8</sup> However, in these studies HCV RNA occasionally was transiently undetectable. At the time of seroconversion, we found that HCV RNA was not detected in 19% of participants at the time of patient seroconversion using commercially available assays (both quantitative and qualitative) and an "in house" test. In addition, HCV RNA was transiently undetectable in 13% of subjects who ultimately had persistent viremia. Processing and storage of serum samples are known to be of critical importance in the recovery of HCV RNA, and multiple freeze-thaw cycles can decrease the HCV RNA titer.<sup>23</sup> We cannot rule out the possibility that some samples would have been HCV RNA positive if they were tested immediately after they were drawn, or that RNA would have been detected before antibody in all our participants if testing were done at more frequent intervals. Nevertheless, it is concerning that virological testing at a single clinical assessment could have led to the erroneous exclusion of HCV infection or misclassification of ongoing infection as self-limited. These data emphasize the importance of clinical follow-up in the diagnosis and management of HCV infection.

The second option recommended by the National Institutes of Health Consensus Panel for confirmation of a positive HCV EIA was RIBA. In this study, 34 (85%) of 40 patients were RIBA positive at the seroconversion visit. The remaining 6 patients (15%) were RIBA indeterminate, although all were positive for c33c and later became positive for other recombinant antigens. The correlation of c33c-positive, indeterminate RIBA tests with positive RNA results has also been reported in other studies.<sup>24,25</sup> The changes in

the third generation RIBA that is used in Europe may decrease the frequency of indeterminate results.

The favorable performance of antibody testing in this study may relate to the study design, in which we characterized patterns of HCV viremia in participants who were known to be anti-HCV negative at entry into the study and anti-HCV positive at their last visit. It is possible that some of the remaining individuals who were initially anti-HCV negative acquired HCV infection either before or during the study but had only a transient antibody response. Such patterns have been reported, although they appear to be rare when using second generation (and later) anti-HCV assays.<sup>6,26-28</sup>

Although this is among the largest studies of acute HCV infection, the only factors that differed significantly between the patients with viral clearance and those with persistent viremia were race and viral titer. The more frequent detection of viral clearance among white participants could reflect host factors or suggest a common viral strain transmitted among this epidemiologically linked group. Although phylogenetic analysis of core-E1 sequences showed no differences between viral strains associated with each outcome group, it does not exclude the possibility that specific HCV sequences from this or another genomic region might be associated with clearance of viremia.

Participants whose HCV RNA levels became undetectable showed lower peak HCV RNA levels during the course of infection, and had no evidence of biochemical liver disease after clearance of viremia. Multiple freeze-thaw cycles may have decreased serum HCV RNA levels and possibly explain the lack of RNA detection at some visits.<sup>23</sup> However, because sample processing and testing was similar in both groups, this effect would be unlikely to cause significant differences in RNA levels between groups or explain the repeated absence of detectable viremia among those with viral clearance.

Although ALT values were not available early in the study, it is notable that approximately half of those patients with persistent viremia had repeatedly normal ALT values later in the course of infection, confirming that chronic hepatitis C is notoriously difficult to follow with biochemical testing alone. Although repeatedly low HCV RNA measurements suggested eventual clearance, high levels were not exclusively detected among those with viral persistence. Thus, in clinical practice, virologic outcome must be determined by long-term follow-up, not a single HCV RNA level.

Consistent with studies after transfusion, the minority (at most 14%) of IDU-related acute HCV infections was associated with jaundice. The similarity in the frequency of jaundice after illicit drug use and transfusion suggests that the quantity of viral inoculum does not dramatically affect the acute clinical presentation, although a direct comparison of large numbers of cases would be necessary to detect subtle differences.

Remarkably few cases of acute hepatitis C infection were recognized. Because the majority (74%) were evaluated by medical providers during the seroconversion interval, this low frequency of recognition does not appear to relate to restricted access to health care but other factors, such as low severity of symptoms and possibly lack of awareness of the disease by providers.

Individuals who ultimately cleared viremia were more likely to have had jaundice or been diagnosed with hepatitis near the time of seroconversion. This suggests that, as with hepatitis B infection, a more brisk immune response early in

the course of infection may affect the natural history of this disease.<sup>29</sup> In addition, these data indicate that studies based on symptomatic or otherwise clinically evident cases of acute hepatitis C may underestimate the rate of viral persistence.

We do not know if spontaneous clearance of viremia implies eradication of infection or ongoing hepatic viral replication that is undetectable in serum. Haydon et al.<sup>30</sup> found HCV RNA in liver tissue from 10 of 12 untreated patients in whom viremia was not detected in at least three visits over 18 months. Thus, the viral clearance described in the present investigation may refer to suppression of viremia rather than complete viral clearance. On the other hand, HCV RNA is frequently not detected in liver tissue of patients who clear viremia after interferon therapy, and liver histology improves markedly.<sup>31,32</sup> Because detection of HCV RNA remains subject to significant interlaboratory and interassay variability, determination of true viral clearance and its clinical implications requires vigilant monitoring and the use of the most sensitive testing methods.<sup>33,34</sup>

Further investigation will be necessary to ascertain the long-term prognosis of patients with HCV antibody but repeatedly undetectable HCV RNA. In the meantime, the results of this investigation emphasize the importance of long-term follow-up of HCV-infected persons. In addition, the improved treatment outcomes that have been recently reported<sup>35</sup> and the infrequent recognition of HCV infection in this and other cohorts, suggests that patients with HCV risk factors should regularly be screened for infection.

**Acknowledgment:** The authors thank Karen Gutekunst of Roche Molecular Systems for generously providing the kits for HCV RNA testing and Jacquie Astemborski for assisting with data analysis.

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