MAJOR ARTICLE

Prospective Evaluation of Community-Acquired Acute-Phase Hepatitis C Virus Infection

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(See the editorial commentary by Busch and Page Shafer on pages 959–61)

Background. More than two-thirds of hepatitis C virus (HCV) infections in Western countries are caused by injection drug use, but prospective clinical data regarding the most common mode of HCV acquisition are rare, in part because acute-phase HCV infection is usually asymptomatic.

Methods. To characterize acute-phase HCV infection, 179 HCV antibody–negative injection drug users were prospectively evaluated; 62 (34%) of these patients had seroconverted. Twenty of the participants who seroconverted had long-term follow-up with consistent monthly sampling before and after seroconversion, allowing detailed study.

Results. The first indication of HCV infection was the presence of HCV RNA in serum, which preceded elevation of alanine transaminase levels and total bilirubin levels to ⩾2 times baseline in 45% and 77% of patients, respectively. No subjects had jaundice. The median time from initial viremia to seroconversion was 36 days (range, 32–46 days). In one instance, viremia was detected 434 days before seroconversion. However, in no other case was HCV RNA detected >63 days before seroconversion. In subjects with viral persistence, a stable level of HCV RNA in the blood was noted in some subjects within 60 days after the initial detection of viremia, but in others, it was not apparent until >1 year later. In subjects with long-term viral clearance, HCV became persistently undetectable as early as 94 and as late as 620 days after initial viremia.

Conclusions. These data underscore the importance of nucleic acid screening of blood donations to prevent HCV transmission and of long-term follow-up to ascertain whether there is viral persistence, at least among injection drug users.

The World Health Organization estimates that there are 170 million persons with hepatitis C virus (HCV) infection worldwide, and an estimated 4 million persons are infected with HCV in the United States [1, 2]. In most countries, HCV infection is found in 1%–2% of the general population and may cause cirrhosis or hepatocellular cancer [3–7]. In the United States, there are ~10,000 HCV-related deaths each year, and it is estimated that HCV-related mortality will double in the next decade [4, 8].

More than two-thirds of HCV infections in Western countries are associated with injection drug use. However, prospective clinical data regarding the most common mode of HCV acquisition are rare, in part because acute-phase HCV infection is usually asymptomatic. Most studies of acute-phase HCV infection have investigated infection resulting from transfusion or from needlestick exposure among health care workers [9–13]. Although these studies have provided valuable information about acute-phase HCV infection, they may not be representative of the most-common mode of acquisition because of the different size of the inoculum, as well as the age and underlying health status of the subjects. Longitudinal study of persons at highest risk for HCV infection also provides an opportunity to examine the full spectrum of acute-phase HCV infection, including asymptomatic infection and possibly transient, seronegative infection. The latter has been described in chimpanzees experimentally infected with small HCV inocula that may mimic what occurs among

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injection drug users (IDUs) [14]. However, the frequency with which this occurs in humans is unknown.

We previously assessed the frequency of viral persistence and the temporal pattern of HCV RNA and antibody detection in a community-based cohort of IDUs. However, this assessment was a retrospective evaluation that described changes after seroconversion, with follow-up appointments that occurred, at most, semiannually [5]. To perform a more detailed study of acute-phase HCV infection, we prospectively followed up high-risk IDUs who were HCV antibody negative with use of a protocol designed for monthly follow-up.

MATERIALS AND METHODS

Participants. The Risk Evaluation Assessment of Community Health prospective study of IDUs in Baltimore, MD, examined the incidence of and risk factors for HCV infection, as described elsewhere [15, 16]. Participants eligible for the study were HCV antibody negative, aged 15–30 years, and acknowledged use of injection drugs. Participants who consented to co-enroll in a substudy of acute-phase HCV infection had blood samples obtained for isolation of serum, plasma, and peripheral blood mononuclear cells in a protocol designed for monthly follow-up. At each visit, participants were provided counseling to reduce the risks of injection drug use. The study protocols were approved by the institutional review boards of the Johns Hopkins Schools of Medicine and Hygiene and Public Health.

From 1997 through 2002, 179 participants were enrolled in the study, and 62 (34.6%) developed anti-HCV antibodies (i.e., seroconverted). Antibody responses, HCV RNA levels, and liver function test results were studied in greater detail for a subset of 20 subjects who seroconverted, were assessed frequently during the 6-month period before and after infection, and had long-term follow-up. These 20 subjects were evaluated at a combined total of 498 visits. In addition, from the 117 subjects who did not experience seroconversion, a subset of 31 patients with the highest-risk injection practices were also assessed for seronegative infection.

Drug use risk. Patients were interviewed at each visit regarding drug use behavior. An index of risk associated with drug use was established by integrating self-report of the frequency of injection drug use and sharing of needles and other drug-use paraphernalia. Highest risk was attributed to persons acknowledging daily injection use with sharing of drug-use paraphernalia.

HCV testing protocol. Serum or plasma samples obtained at monthly follow-up visits and stored at −80°C were tested for the presence of HCV-specific antibodies with use of the Ortho ELISA, version 2.0 or version 3.0 (Ortho Clinical Diagnostics). Specimens in which antibodies were detected were retested in duplicate, as were the participants' previous seronegative samples, to identify patients who had seroconverted.

For HCV seroconverters, HCV RNA testing was performed using serum or plasma samples obtained before seroconversion until a negative result was obtained and using samples obtained after seroconversion to evaluate the outcome of infection (viral persistence vs. clearance) with use of the quantitative and, if levels were undetectable, qualitative HCV RNA tests that are described below.

HCV RNA Assays

Qualitative. For detection of HCV RNA, we used the Cobas Amplicor HCV test, version 2.0 (Roche Molecular Systems). The Amplicor assay uses RT-PCR technology to detect HCV RNA. The manufacturer reports a limit of detection of 1.7 $\log_{10}$ IU/mL at >95% detection for this assay. HCV RNA detection was also performed at Chiron Corporation (Emeryville, CA) with use of the Procleix dHCV assay, which uses the transcription-mediated amplification technology (Chiron). This assay is also available as the Versant HCV transcription-mediated amplification qualitative assay (Bayer). Transcription-mediated amplification was performed as described elsewhere [17, 18]. The sensitivity of the assay is reported as 37 IU/mL at >99% detection [18].

Quantitative. To determine concentrations of HCV RNA in serum, we used a quantitative RT-PCR assay (Cobas Amplicor HCV Monitor, version 2.0; Roche Molecular Systems). This assay has a lower limit of quantitation of 2.8 $\log_{10}$ IU/mL.

HCV genotyping. Genotype was determined by performing phylogenetic analysis on sequences of the Core-E1 region of HCV obtained from the first viremic specimen. For most specimens, sequences were obtained from cDNA clones generated with a long amplicon RT-PCR method that has been described elsewhere [19]. For other specimens, genotype was determined by direct sequencing of RT-PCR products from the same Core-E1 region as previously described [20]. Sequences were aligned using ClustalX [21], trimmed to equal length using BioEdit software, version 5.0.7 [22]. The GTR+I+G analytical model (with parameters $pinvar = 0.1663$ and $\alpha = 0.8091$) was selected using the AIC criterion as implemented in ModelTest, version 3.06 [23], and PAUP*4b10 (Sinauer Associates). Phylogenetic trees were estimated using the neighbor-joining algorithm implemented in PAUP*, and robustness of clustering was tested by boot-strapping [24]

Biochemistry tests. Alanine transaminase (ALT) and total bilirubin levels were determined in The Johns Hopkins Hospital laboratory (Baltimore, MD).

Viral recovery. HCV clearance was defined as the presence of anti-HCV antibody with HCV RNA levels undetectable by the Cobas Amplicor qualitative assay in serum or plasma specimens obtained during ⩾2 consecutive visits ⩾300 days after the initial detection of viremia. Persistence was defined by detection of HCV RNA by the qualitative or quantitative Cobas

Amplicor assay in serum or plasma specimens obtained ⩾300 days after initial viremia.

Statistical analysis. Descriptive statistical methods were used to analyze the data. To guide interpretation, statistical inferences were made with use of the χ^2 or Fisher's exact tests for categorical variables and with use of nonparametric rank-sum tests for continuous variables. A P value of <.05 was considered to be statistically significant. The HCV incidence rate was calculated as the number of new HCV infections per 100 person-years of follow-up, with 95% CIs obtained by a normal approximation to the Poisson distribution.

RESULTS

Participants

Despite counseling to prevent HCV infection, 62 (34.6%) of the subjects seroconverted during the course of the study. The participants contributed 228.3 person-years of follow-up time, resulting in an overall incidence rate of 27.2 HCV seroconversions per 100 person-years (95% CI, 26.6–44.4). Of those patients who seroconverted, 58% were male. The ethnic distribution of those who seroconverted was 63% white, 31% black, and 6% other. At the time of analysis, 40 subjects had sufficient follow-up, defined as ⩾2 specimens available ⩾300 days after initial viremia, to determine the outcome of infection (viral persistence or clearance). In this subset, 31 (78%) remained persistently viremic, 8 (20%) of the subjects cleared viremia, and 1 (2%) of the subject cleared viremia but became persistently viremic after reinfection. There were no significant differences detected between those who cleared viremia and those who were persistently infected in duration of follow-up, age at seroconversion, sex, race, or genotype.

Twenty subjects with defined outcomes of infection were chosen for detailed study, because they had samples obtained on a consistent, monthly basis during the period surrounding HCV seroconversion. The mean duration of follow-up, the mean age at seroconversion, the genotypes, the sex, and the racial composition of this subset are shown in table 1 and did not differ from those of the subjects with less-frequent assessment during the period surrounding seroconversion.

Table 1. Characteristics of 20 patients with seroconversion who had follow-up visits before and after seroconversion.

Characteristic	Value
Age at seroconversion, median years (range)	23 (18–30)
Duration of follow-up, median months (range)	36 (21–48)
Sex	
Male	9 (45)
Female	11 (55)
Race	
White	15 (75)
Black	4 (20)
Other	1 (5)
Genotype	
1a	17 (85)
3a	3 (15)
ALT level, mean baseline IU/L (range)[a]	15 (7–26)
Total bilirubin level, mean baseline mg/dL (range)[a]	0.2 (0.1–0.4)

NOTE. Data are no. (%) of patients, unless otherwise indicated. ALT, alanine transaminase.

[a] Baseline indicates the results of liver function testing of samples obtained ⩾1 month before detection of HCV RNA.

Time of Infection and Seroconversion

Infection was estimated to occur midway between the last visit at which the subject was HCV RNA negative and the first visit at which the subject was HCV RNA positive, and development of antibody response was estimated to occur midway between the last visit with no detectable antibody and the first visit at which antibody was detectable. On the basis of these estimates, the mean time from infection to seroconversion was determined to be 36 days (range, 32–46 days).

Viremia and liver biochemistry. Baseline ALT levels were <26 IU/L in all subjects at the visit before viremia was detected. At first detection of viremia, levels of HCV RNA ranged from 3.4 $\log_{10}$ IU/mL to 6.4 $\log_{10}$ IU/mL. This represented the peak of viremia for 10 (50%) of the subjects, and no subject had subsequent HCV RNA levels >1.4 $\log_{10}$ higher than the initial value. At the initial detection of viremia, the mean increase in ALT level from baseline was 4-fold (range, baseline to 11 times baseline). Viremia preceded elevation of ALT and total bilirubin levels (elevation, ⩾2 times baseline) in 45% and 77% of patients, respectively. The median ALT levels at baseline, at initial viremia, and at 1 month after the first detection of HCV RNA were 14, 35, and 159 IU/L, respectively. In some instances of persistently detectable HCV RNA, ALT levels decreased to less than the laboratory upper limit of normal but remained greater than the baseline level.

Patterns of viremia. Figure 1 shows the patterns of viremia and ALT levels over time for 12 representative subjects. Of the 8 subjects not shown in figure 1, 6 had patterns similar to that of subject 29, 1 had a pattern like that of subject 20, and 1 had a pattern resembling that of subject 21. Patterns of viremia varied substantially among subjects. For those with viral persistence (figure 1*A*), development of a stable level of HCV RNA in the blood (i.e., a viral set point) often occurred within 60 days after the detection of viremia (e.g., subjects 15, 29, and 31) but did not occur in some subjects until >1 year after initial viremia (e.g., subject 17). Among the subjects with viral clear-

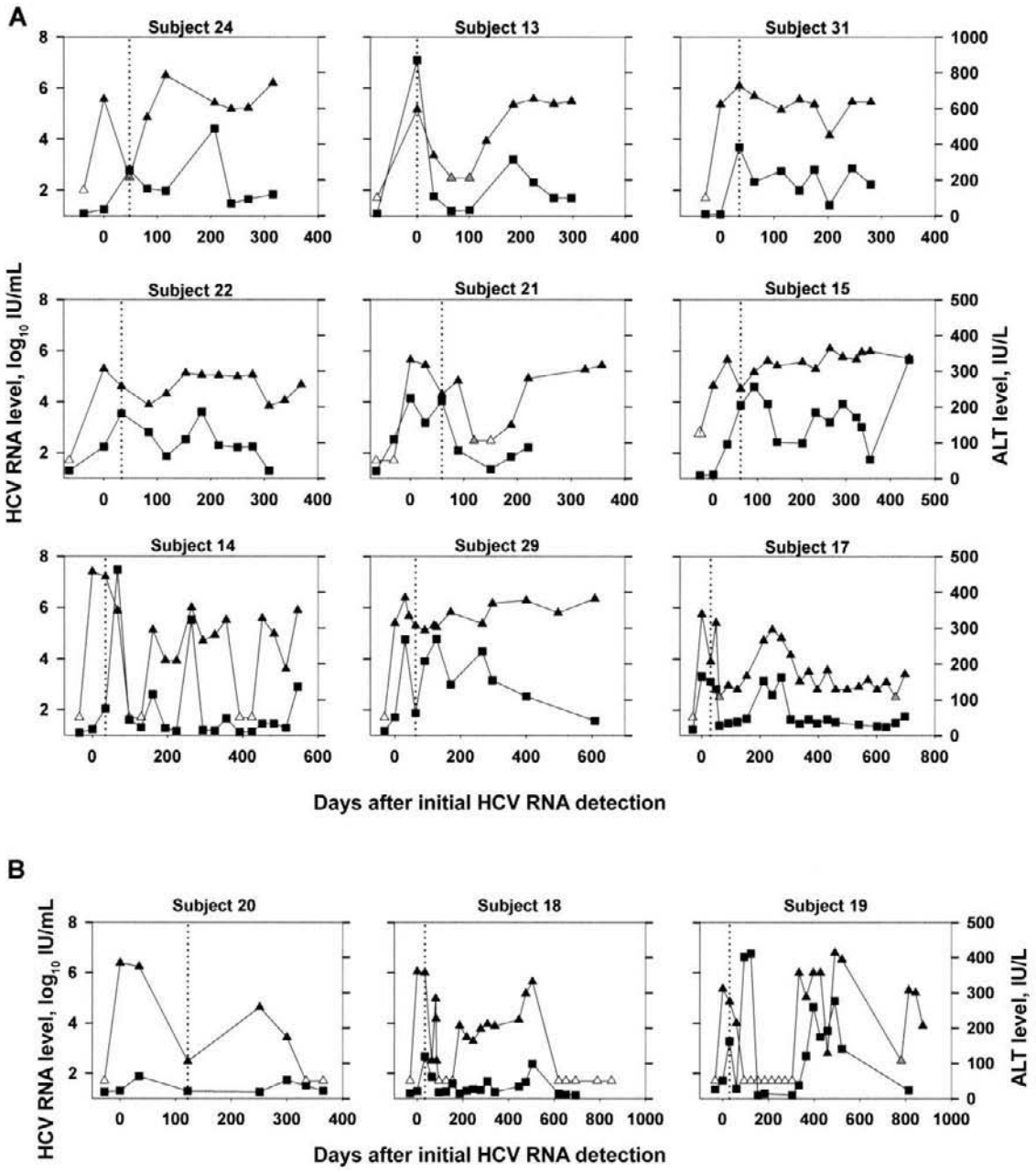

Figure 1. Analysis of HCV RNA levels *(triangles)* and alanine aminotransferase (ALT) levels *(squares)* over time for 12 subjects representing all the patterns seen in the 20 subjects studied. Open triangles represent time points at which HCV RNA could not be detected by qualitative assay. Gray triangles represent time points at which HCV RNA was detected but not quantifiable. Black triangles represent HCV RNA levels as measured by quantitative RNA assay. The dotted vertical line represents the first time point at which HCV antibodies were detected. Day 0 represents the visit date at which HCV RNA was first detected. *A,* Patterns of viremia for subjects who were persistently infected with HCV. *B,* Patterns of viremia representing viral clearance for subjects 18 and 20 and clearance with subsequent reinfection resulting in persistence for subject 19.

ance (figure 1*B*), HCV became persistently undetectable as early as 94 days after and as late as 620 days after initial viremia.

For subjects 13, 18, 19, and 21, periods of viremia were separated by intervals during which HCV RNA could not be detected. To assess the possibility that the viremia following the period of undetectable HCV RNA represented reinfection, the envelope sequence was examined at the visits immediately before and after the RNA-negative interval (figure 2). In every subject except subject 19, the isolates clustered together, indicating persistent infection. In subject 19, the isolate detected 333 days after the onset of viremia differed as much from those detected in the first 60 days of viremia as from isolates obtained from other subjects, suggesting reinfection. After a 10-month drug-free hiatus, the subject had resumed injection drug use

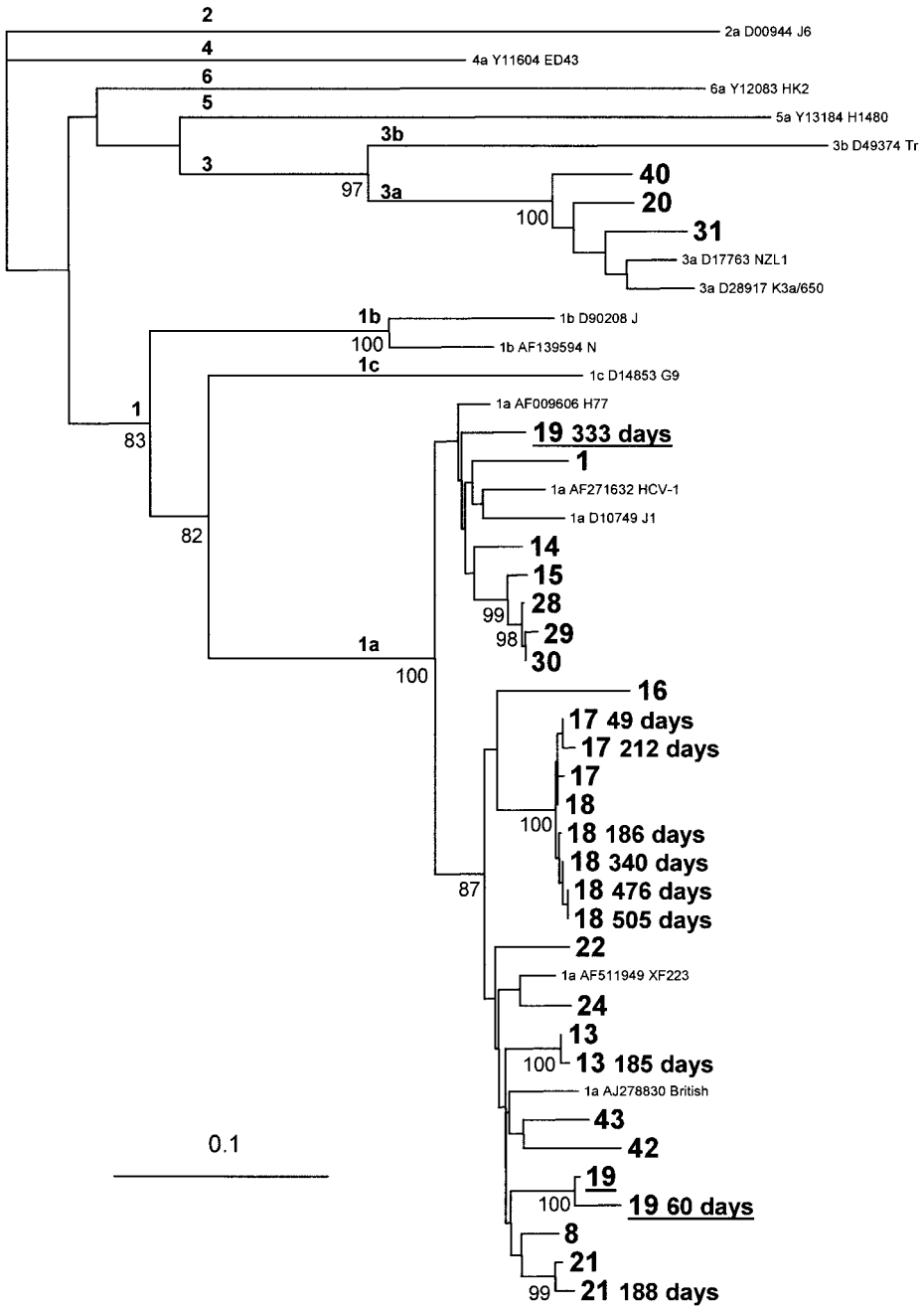

Figure 2. Phylogenetic analysis of HCV core/E1 region sequences obtained from serum or plasma samples during asymptomatic acute-phase infection. Sequences labeled in boldface are from this study, obtained from the first viremic specimen. Additional visits from subjects 13, 17, 18, 19, and 21 are presented, with sampling time indicated as days after detection of viremia. Reference sequences were obtained from GenBank and labeled with subtype, GenBank accession number, and isolate name. Numbers in boldface above internal branches indicate genotype (single digit) and subtype. Numbers below internal branches indicate the proportion of 1000 permuted trees that supported the indicated node during boot-strap analysis, when support was >80%.

within days of onset of the second course of viremia, supporting reinfection. In some cases, sequences from different subjects clustered together, raising the possibility of amplicon contamination. However, contamination was excluded by taking numerous precautions [25] and finding consistent results from multiple visits amplified and sequenced on separate days. Among IDUs from Baltimore, epidemiological linkage is not unlikely.

Seronegative infection. To address the hypothesis that early HCV infection is characterized by low-level intermittent vire-

mia that precedes the ramp-up and plateau phases [26], seroconverters were assessed for HCV RNA before seroconversion. From 28 seroconverters, 101 samples, obtained a mean of 208 days (range, 11–1194 days) before seroconversion, were tested for the presence of HCV RNA with use of Procleix dHCV assay (for 53 samples) or Cobas Amplicor qualitative assay (for 48 samples) (table 2). Visits were selected according to availability. HCV RNA was detected in only 1 subject (subject 31) >63 days before seroconversion. HCV RNA was detected by Procleix dHCV assay (but not the Cobas Amplicor qualitative assay) in a serum sample collected 434 days before seroconversion. HCV RNA was not detected in serum samples obtained from the same subject collected 539, 483, 462, or 64 days before seroconversion.

HCV RNA level was also assessed at multiple time points for 31 IDUs with high-risk drug use practices who did not experience seroconversion. Fifty specimens obtained during periods of high-risk behavior and 27 specimens obtained during periods of low-risk behavior were tested for HCV RNA with use of the Procleix dHCV assay. Despite the fact that samples were obtained during periods of daily injection drug use and sharing of drug-use paraphernalia, no HCV RNA was detected in any of the 77 samples tested.

DISCUSSION

In this prospective investigation of acute-phase HCV infection, viremia was the earliest marker of acute-phase HCV infection, preceding detection of HCV-specific antibodies by 5–6 weeks, and in 1 case, by >12 months. Liver enzyme levels were uniformly elevated in patients with acute-phase infection. However, liver enzyme levels did not correlate closely with HCV RNA levels or viral persistence. These data are uniquely representative of most HCV infections, because the mode of ac-

Table 2. Qualitative hepatitis C virus (HCV) RNA test results before HCV seroconversion for 28 patients with acute-phase HCV infection.

	Test result(s), by days before HCV seroconversion												
Subject	>540	540–496	495–451	450–406	405–361	360–316	315–271	270–226	225–181	180–136	135–91	90–64	63–1
7	…	…	…	…	…	…	…	…	…	−, −	−	…	+
11	…	…	…	…	…	…	…	…	…	…	…	…	−, +
12	…	…	…	…	…	…	…	…	…	…	…	…	−, +
13	…	…	…	…	…	−	…	−	−	−	…	−	+
14	…	…	…	…	…	…	…	…	…	…	…	−	+
15	…	−	…	…	…	−	−	…	…	…	−	…	+
16	−, −, −	…	…	…	…	…	…	…	−	…	…	…	+
17	…	…	…	…	…	…	−	…	…	…	…	−	−, +
18	…	…	…	…	…	…	−	−	−	…	…	−	+
19	…	…	…	…	…	…	…	…	−	…	…	−	+
20	…	…	…	…	…	…	−	…	…	−	…	…	+
21	…	…	…	…	…	…	…	…	…	…	−	−	−, +
22	…	…	…	…	…	…	…	…	…	…	−	…	+
23	…	…	…	…	…	…	…	…	−	…	−	…	+
24	…	−	…	−	…	…	…	…	…	…	…	−	+
25	…	…	…	…	…	…	−	…	…	−	…	…	+
26	…	…	…	…	…	…	…	…	…	…	−	…	+, +
29	…	…	…	…	…	…	…	…	…	…	−	−	+, +
30	…	…	…	…	…	…	…	…	…	…	…	−	+
31	…	−	−, −	+	…	…	…	…	…	…	…	−	+
32	…	…	…	…	…	…	…	…	…	…	−, −	−	−, +
33	…	…	…	…	…	…	…	…	−	−	−, −	…	+, +
34	−	…	…	…	…	…	…	…	…	…	…	…	+
35	…	…	…	…	…	…	…	…	…	…	−	−	−, +
36	…	…	…	…	…	…	…	…	…	…	…	−	+
37	−, −, −	…	…	…	…	…	…	…	…	…	…	…	+
38	…	…	…	…	…	…	…	…	…	…	…	−	+
39	…	−	…	−	…	…	…	…	…	…	−	…	+

NOTE. RNA testing was done with use of Procleix dHCV assay (for 53 smaples) or Roche qualitative Amplicor assay (for 48 samples). Visits were selected according to availability. Multiple symbols in a single analytical field indicate that separate samples collected at >1 time point in that time period were tested.

quisition for these subjects represents the most common mode of HCV infection in the Western world and because the subjects were studied prospectively without regard to symptoms before, during, and after infection. The results significantly expand the available data on the dynamics of hepatitis C viremia preceding seroconversion.

The findings were supportive of some, but not all, prior studies. Busch et al. [26] described evidence that early-phase HCV infection includes a period of very low-level intermittent viremia that precedes the ramp-up and plateau phases. This low-level intermittent viremia was detected by Procleix dHCV assay in plasma donors who subsequently developed antibodies to HCV [26]. We detected HCV RNA with use of the Procleix dHCV assay before the previously described ramp-up and plateau phases of viremia that precede seroconversion in 1 patient with seroconversion at a single time point. Further study is needed to ascertain whether HCV RNA would be detected more often with more-frequent sampling in the weeks immediately preceding the ramp-up phase, as was done in the study by Busch et al. [26].

We could not detect HCV RNA in a subset of our cohort who never seroconverted (despite daily injection drug use and sharing of drug-use paraphernalia in some of these subjects). Given the small number of samples tested, the failure to detect HCV RNA does not rule out the possibility that low-level exposure resulting in viremia occurs, but it does suggest that it is infrequent. In experiments in which chimpanzees were sequentially exposed to increasing doses of HCV, HCV infective doses as low as 1–10 RNA virions induced detectable cellular immune responses without consistently detectable viremia or persistent seroconversion. One chimpanzee developed cellular immune responses to HCV without ever having detectable levels of HCV RNA [14]. Studies involving humans have also demonstrated that low-level exposure to HCV can induce and/or boost HCV-specific T cell responses in the absence of antibody production or detectable levels of HCV RNA [27, 28].

The detection of cellular responses in some seronegative persons exposed to HCV suggests that intermittent viremia below the limit of detection of the available HCV RNA assays may stimulate HCV-specific cellular immune responses without inducing antibody production. Additional experiments comparing the cellular responses of IDUs in our cohort before and after initiation of high-risk injection practices could confirm exposure without seroconversion or detectable viremia in this population. The presence of cellular immune responses in the absence of HCV-specific antibodies and detectable viremia would support that exposure to HCV without persistent viremia is more common than current screening methods suggest.

The prospective nature of this study permitted comparison of liver function test results before and after HCV infection. Serum bilirubin levels increased by 2.5–11-fold over baseline in all but 1 subject (93%). However, the maximum peak bilirubin level for all subjects was only slightly above normal, at 1.7 mg/dL. Similarly, the mean peak ALT level in those with baseline ALT levels obtained was 360 IU/L. This peak was 9-fold above the clinical laboratory's upper limit of normal, but it represented a mean increase of 26-fold over the subjects' baseline values. None of the subjects who acquired HCV infection in this study had jaundice or developed symptoms that lead the subject to seek medical attention, consistent with existing literature showing that acute HCV infection is usually asymptomatic [4, 29].

The clinical and pathophysiological correlation of elevations in the ALT level in HCV infection is not understood. In this study of acute-phase infection, the degree of ALT level elevation was highly variable among individuals and was not explained by the level of HCV RNA, the outcome of infection, the HCV genotype, or the sex or race of the subject. Likewise, in chronic HCV infection, there is substantial variation in the ALT levels among persons and within a given person, despite fairly stable levels of HCV RNA in blood [30, 31]. That viremia frequently precedes ALT level elevation supports the hypothesis that HCV is not a hepatotoxic virus and that factors that come into effect later, such as the immune response, are responsible. It is also interesting that, in some subjects who appeared to have given samples very close to the time of infection, an initial mild increase in ALT level preceded the peak. Our sampling was too infrequent to exclude the possibility that these levels were merely the up-slope of a single phase. However, it is also possible that these levels reflect 2 stages of HCV infection: innate immune responses to initial expansion within the liver, followed by adaptive immune responses.

It is important to note that these early increases in ALT level preceding seroconversion—and in some cases, increases during the acute phase after seroconversion—would not have been noticed without earlier testing of baseline levels, because the results were still within the normal range reported by the laboratory. Infection would have been missed if ALT testing alone had been employed. This finding contributes to the growing literature questioning the clinical relevance of what is reported as normal by clinical laboratories.

In this investigation, there was an alarming incidence of HCV infection noted among these young IDUs despite ongoing efforts to reduce risky practices. This finding underscores the importance of novel efforts to reduce the societal and individual influences that contribute to illicit drug use and the importance of ongoing efforts to develop vaccinations to prevent chronic hepatitis C in this population.

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