High Rate of Spontaneous Negativity for Hepatitis C Virus RNA after Establishment of Chronic Infection in Alaska Natives

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(See the editorial commentary by Lauer and Kim on pages 953–4)

Background. Hepatitis C virus (HCV) leads to chronic infection in 70%–85% of exposed patients. Spontaneous clearance of the virus after chronic infection is believed to occur rarely.

Methods. Alaska Natives who tested positive for HCV antibodies were enrolled in a prospective study that began in 1994 and were followed up on a regular basis. Individuals who tested positive for HCV RNA on 3 separate dates, each of which were at least 1 year apart, were included. Being negative for the virus was defined as having at least 1 negative HCV RNA test result after chronic viremia had been established.

Results. Of the 815 patients enrolled in the cohort, 139 met entry criteria and were observed for a mean period of 7.0 years. Eleven (8%) of the persons had at least 1 test in which HCV RNA was undetectable; 7 were classified as having either possible or probable clearance of the virus, corresponding to an annualized clearance rate of 0.74% per person-year (95% CI, 0.30%–1.53%). Of 9 patients who underwent subsequent HCV RNA testing, 5 (56%) had negative test results. A low HCV RNA level was significantly associated with spontaneous nondetectability of HCV RNA.

Conclusion. Spontaneous HCV RNA negativity during chronic HCV infection is a surprisingly frequent event and is associated with low HCV RNA titers. Knowledge of immunologic determinants of clearance may open up avenues of novel therapy.

An estimated 2.7 million persons in the United States and 170 million persons worldwide are infected with the hepatitis C virus (HCV), leading to significant mortality and morbidity. For most patients, infection is chronic; however, a minority (estimated to be 15%–30%) of persons experience clearance of HCV RNA shortly after onset of acute infection [1]. Until recently, it was assumed that patients infrequently or never experienced elimination of chronic HCV infection without receiving treatment. The present study of Alaska Natives with chronic HCV infection characterizes the rate of spontaneous viral clearance. This cohort was selected because of the existence of a serum bank dating back to 1969 and because of the relatively homogenous nature of the population, compared with other study groups.

MATERIALS AND METHODS

Subjects. Details of the cohort have been described elsewhere [3]. In brief, the Arctic Investigations Program Laboratory of the Centers for Disease Control and Prevention (CDC), in conjunction with the Alaska Native Medical Center (ANMC), created a serum repository for all Alaska Natives with documented cases of acute or chronic hepatitis in 1969, making possible a 30-year study period. Since 1992, the ANMC and the CDC have offered free HCV testing at all facilities serving Alaska Natives or American Indians. In 1994, ANMC and the University of Washington began a population-based longitudinal study to follow up persons...
infected with HCV to determine the disease outcome over time and to study the risk and virologic factors that influence disease progression. This study was approved by the University of Washington Institutional Review Board, the Alaska Area Native Institutional Review Board, the Indian Health Service National Institutional Review Board, the CDC Institutional Review Board, and 3 regional Alaska Native health corporations. Enrolled patients provided written and oral informed consent and were followed up on an annual basis. A study nurse interviewed patients and reviewed medical records for data regarding demographic characteristics, history of alcohol use, risk factors related to HCV transmission, and history of liver disease and previous liver biopsy and reviewed annually the field records of participants who did not return to a central clinic.

Between July 1992 and January 2003, a total of 1414 Alaska Natives and/or American Indians tested positive for HCV antibodies. 924 patients were enrolled, and 815 patients were found to have a positive test result for HCV RNA. Patients were eligible for this cohort study if they had evidence of chronic viremia, which was defined as having 2 positive RT-PCR–based test results for HCV RNA separated by at least 1 year plus having undergone a third HCV RNA test at a later time. Patients receiving antiviral treatment for HCV were excluded. Follow-up began when the second HCV RNA–positive specimen was obtained and ended when the last available specimen was tested. Of the 815 patients, the following patients were excluded: 215, because results of the initial test performed at our facility were negative for HCV RNA; 402, because they had undergone ≤2 tests; 4, because they had only 1 test result positive for HCV RNA prior to being negative for HCV RNA; 26, because they had received antiviral therapy for HCV; 3, because they were initially viremic but cleared HCV RNA within 1 year after their first positive test result; and 26, because they were initially positive for HCV RNA, subsequently became negative for HCV RNA, and did not undergo a follow-up test during the first year of follow-up.

Study procedures. Patients with a history of injection drug use were assigned to that risk exposure category, although many of these patients had other risk exposures and risk behaviors for HCV (e.g., blood transfusion, household member history of HCV infection, history of tattooing, history of intranasal cocaine use, >10 lifetime sexual partners, and a sexual partner with a history of injection drug use). Patients who were not injection drug users but who had received a blood transfusion were assigned to the blood transfusion risk exposure category. Patients who were neither injection drug users nor blood transfusion recipients but who had a history of one of the other behaviors of interest were assigned to the “other” risk exposure category.

The earliest obtained specimen positive for HCV antibodies and the specimen obtained at enrollment were tested for HCV RNA, and the HCV genotype was determined by restriction fragment–length polymorphism [4] at the University of Washington. HCV RNA testing was performed by the branched DNA assay (bDNA), version 2.0 (Bayer) and by Roche Amplicor RT-PCR (Roche) [5]. The sensitivity of the RT-PCR was 50 IU/mL. Quantitative HCV RNA levels were compared among participants by using the peak viral load and the most recent viral load (i.e., the viral load obtained using the last specimen found to be positive for HCV in patients who experienced clearance of HCV).

Participants who tested negative for HCV RNA were classified as having intermittent viremia, possible clearance of HCV, and probable clearance of HCV. Intermittent viremia was defined as an isolated negative result of a HCV RNA test followed by persistent positive results of HCV RNA tests. ”Possible” clearance was defined as only 1 negative HCV RNA test result during the follow-up period, after which subsequent tests were not performed. “Probable” clearance was defined as multiple negative results of HCV RNA tests. The estimated date of infection was defined as the date of first injection drug use or blood transfusion exposure (prior to July 1992) by correlating the historical risk factor data with the results of tests performed on stored serum samples, as described elsewhere [6].

Statistics. All comparisons were made using StatXact software, version 4.0 (Cytel Corporation). Continuous variables were analyzed by the exact Wilcoxon Mann-Whitney test, and categorical variables were analyzed by the Fisher’s exact test. All P values are 2-sided. P values <.05 were considered to be significant. To determine the HCV RNA negativity rate, the number of follow-up years was calculated for each person from the time at which the second HCV RNA–positive specimen was obtained to the time of at which HCV RNA clearance was achieved or, for those who did not clear spontaneously, to the date on which the last positive specimen was obtained. The time of HCV RNA clearance was estimated using the midpoint between the time at which the last HCV RNA–positive specimen and the first HCV RNA–negative specimen were collected.

Viral genome sequencing. Viral RNA was extracted from 160 μL of serum using the QIAamp Viral RNA isolation kit (Qiagen). cDNA was synthesized using oligonucleotide primers and Moloney murine leukemia virus reverse transcriptase, as described elsewhere [7]. PCR was performed using Advantage High Fidelity 2 DNA polymerase (BD Biosciences) with a nested PCR reaction, as described elsewhere [8]. Purified PCR products were directly sequenced using the second-round primers and the Applied Biosystems model 377 automated sequencer (Applied Biosystems).

Nucleotide sequences were optimally aligned using the CLUSTAL W program [9]. Phylogenetic analysis was performed using programs from the Phylogeny Inference Package, version 3.5c [10]. Nucleotide distances were estimated by generating a
RESULTS

There were 139 patients who met entry criteria and who were observed for a mean period of 7 years (range, 1 month–18.5 years), for a total follow-up of 943 person-years. Of the 135 patients, 11 (8%) had at least 1 negative HCV RNA test result (figure 1). Demographic and clinical characteristics of these 11 patients are detailed in table 1. The rate of spontaneous HCV RNA negativity among patients with established viremia was 1.15 cases per 100 persons per year (95% CI, 0.57–2.06 cases). Seven of these 11 patients had probable or possible clearance of infection, for a clearance rate of 0.74 cases per 100 persons per year (95% CI, 0.30–1.53 cases). Nine patients who had a negative HCV RNA test result underwent a follow-up HCV RNA test. Of these 9 participants, 5 (56%) continued to have negative HCV RNA test results (95% CI, 24%–83% of patients), and 4 (44%) had positive follow-up test results. Figure 2 depicts representative courses of disease for patients with probable clearance, possible clearance, and intermittent viremia.

Demographic and virologic characteristics of patients with probable clearance are compared with those of patients with persistent viremia in table 2. Patients who probably experienced clearance of HCV RNA were infected at a younger age than patients who did not (mean age at the estimated date of infection, 22.4 years vs. 28.8 years; \( P = .01 \)). Patients who experienced clearance were significantly younger at the start of follow-up \( (P = .04) \) than patients who did not. All 5 of the patients who probably experienced clearance were women, compared with 75 (59%) of 128 patients in the chronically infected group who were women \( (P = .16) \). All patients for whom a history was available reported injection drug use as the risk factor for HCV transmission, compared with 57% of the nonclearers \( (P = .22) \). There was no difference in the estimated duration of HCV infection between the 2 groups. Patients who probably experienced clearance of HCV had a lower median alanine aminotransferase level than that of patients who did not \( (38 \text{ vs. } 83 \text{ patients}; P = .005) \). However, for all but 1 patient, these lab values were obtained after clearance occurred.

Quantitative HCV RNA levels were significantly lower among patients who probably experienced clearance than among patients who did not \( (>1 \log \text{ difference}; P < .01) \). This relationship was found whether viral load was measured using the last specimen found to be HCV positive or the maximum viral load measured among all specimens. Four patients had HCV genotype 1, and genotype could not be determined for 1 person. Of the 11 patients who experienced spontaneous HCV RNA negativity, patient 3 had the most remarkable clinical course. She had >1 year of established chronic viremia and then experienced a spontaneous disappearance of HCV RNA that was followed by a reappearance (at a level of 7,344,000 IU/mL). She experienced an episode of symptomatic hepatitis characterized by a peak alanine aminotransferase level of 2311 U/L that occurred concurrently with the reappearance of HCV RNA. The genotype remained the same \( (1a) \) for both viremic episodes. She subsequently experienced clearance of HCV RNA that was demonstrated by negative results of 2 qualitative tests for HCV RNA during the next 2 years. The patient continued to use injection drugs throughout this time period.

Three subjects (patients 2, 3, and 4 in table 1) had adequate specimens available for nucleic acid sequence analysis to determine whether the same quasispecies population was present in serum at successive time periods during the study. The phylogram in figure 2 demonstrates the persistence of related HCV quasispecies variant populations over a minimum of 1 year in all 3 patients, as indicated by the phylogenetic clustering of sequential isolates. This experiment also clearly demonstrated HCV superinfection during the chronic phase of hepatitis C in patients 2 and 4. In patient 2, related quasispecies were detected in 1985 and 1986 (both genotype 1b), whereas in 1987, a genetically distinct variant (genotype 1a) was detected in this patient’s serum. Likewise, patient 4 was infected by the same genetically related quasispecies population in 1985 and 1991 \( (1a) \) and by a genetically distinct variant population \( (1b) \) in 1994 and 1995. Interestingly, patient 4 had a negative HCV RNA test result between genotype 1a and 1b infections and had another negative HCV PCR test result >1 year after the genotype 1b infection \( (figure \ 1) \). Finally, closely related quasispecies populations \( (1a) \) were detected in patient 3’s serum in 1985 and 1986, although, on the basis of hypervariable region-1 sequencing, the genotype 1a quasispecies detected in serum in 1984 was less obviously related. Based on our previous research in the renal dialysis population, the degree of genetic divergence in patient 3’s hypervariable region between 1984 and 1985 is most consistent with a jump in envelope diversity within the same quasispecies population, as opposed to superinfection with a closely related genotype 1a isolate \[7\].

DISCUSSION

In this study, we have demonstrated that, among Alaska Natives with chronic HCV infection, a significant percentage (8%) of them will have a spontaneous negative HCV RNA test result, corresponding to a rate of 1.15 cases per 100 persons per year. Approximately one-half of these negative HCV RNA test results are durable (based on subsequent results of sensitive [<50 IU/mL] assays over many years) and support the assertion that
Figure 1. Hepatitis C virus (HCV) RNA levels in Alaska Natives with chronic HCV infection who subsequently had a negative HCV RNA test result, by year since study enrollment; POS, positive qualitative HCV RNA test result. POS/NEG, positive and negative qualitative HCV RNA test results in the same year. *Patient had test results negative for HCV RNA by the branched DNA assay, version 2.0 (Bayer), which has a lower limit of detection of 200,000 IU/mL.

viral resolution probably did occur in these 5 patients. However, it is possible that patients simply had very low levels of HCV RNA that were not detectable by this test. Castillo et al. [12] have demonstrated recently that it is possible to have HCV RNA levels in serum that cannot be detected by a very sensitive RT-PCR test and to find evidence of HCV RNA in liver tissue. Similarly, Pham et al. [13] used a highly sensitive PCR–nucleic acid hybridization assay to study 16 patients who experienced resolution of viremia spontaneously or by means of treatment and found that 15 of 17 samples had detectable HCV in serum.
Table 1. Characteristics of patients with intermittent negative results of tests for hepatitis C virus (HCV) RNA and of patients with possible and probable clearance of HCV.

<table>
<thead>
<tr>
<th>Patient</th>
<th>HCV RNA clearance status</th>
<th>Sex</th>
<th>Genotype</th>
<th>Risk Factor</th>
<th>ALA level, mean IU/L</th>
<th>Age at time of negative HCV RNA test result, years</th>
<th>HCV RNA level prior to clearance, IU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Probable</td>
<td>F</td>
<td>1a</td>
<td>IDU</td>
<td>169</td>
<td>32.5</td>
<td>800</td>
</tr>
<tr>
<td>2</td>
<td>Probable</td>
<td>F</td>
<td>1b</td>
<td>IDU</td>
<td>18</td>
<td>37.2</td>
<td>500</td>
</tr>
<tr>
<td>3</td>
<td>Probable</td>
<td>F</td>
<td>1b</td>
<td>IDU</td>
<td>248</td>
<td>33.5</td>
<td>7,344,000</td>
</tr>
<tr>
<td>4</td>
<td>Probable</td>
<td>F</td>
<td>1a</td>
<td>IDU</td>
<td>25</td>
<td>31.8</td>
<td>829,000</td>
</tr>
<tr>
<td>5</td>
<td>Probable</td>
<td>M</td>
<td>NA</td>
<td>IDU</td>
<td>42</td>
<td>34.3</td>
<td>1,333,000</td>
</tr>
<tr>
<td>6</td>
<td>Possible</td>
<td>M</td>
<td>1a</td>
<td>NA</td>
<td>16</td>
<td>32.8</td>
<td>483,000</td>
</tr>
<tr>
<td>7</td>
<td>Possible</td>
<td>F</td>
<td>2b</td>
<td>IDU</td>
<td>33</td>
<td>44.5</td>
<td>800</td>
</tr>
<tr>
<td>8</td>
<td>Intermittent</td>
<td>M</td>
<td>1b</td>
<td>IDU</td>
<td>145</td>
<td>34.3</td>
<td>468,000</td>
</tr>
<tr>
<td>9</td>
<td>Intermittent</td>
<td>M</td>
<td>1b</td>
<td>INC</td>
<td>66</td>
<td>45.0</td>
<td>590,000</td>
</tr>
<tr>
<td>10</td>
<td>Intermittent</td>
<td>M</td>
<td>1b</td>
<td>Household</td>
<td>80</td>
<td>62.9</td>
<td>384,000</td>
</tr>
<tr>
<td>11</td>
<td>Intermittent</td>
<td>M</td>
<td>1a</td>
<td>IDU</td>
<td>NA</td>
<td>29.8</td>
<td>305,000</td>
</tr>
</tbody>
</table>

NOTE. ALA, alanine aminotransferase; IDU, injection drug use; INC, intranasal cocaine use; NA, not available.

and that 9 of 12 samples did not have detectable HCV in PBMCs. However, these findings are contradicted by 2 recent studies of patients who had sustained virologic responses after receiving IFN therapy. In liver biopsy specimens and PBMCs obtained from patients who had completed treatment 5–10 years previously, no HCV sequences were detected [14, 15].

A low viral load was associated with viral clearance, a finding that is both new and intuitive from a viral-host interaction perspective. Data from studies of the response to IFN therapy demonstrate that lower viral loads are correlated with a higher rate of therapy-induced viral clearance [16, 17]. There was also a trend towards younger age at onset of HCV infection and at spontaneous HCV resolution, and all 5 patients with sustained negative HCV RNA test results were females. Previous studies have found that a younger age at acquisition of HCV infection (<40 years) is predictive of a slower progression to fibrosis [18]. Additionally, the unique racial background of participants may explain the unexpectedly high clearance rate.

In a recent study from Japan [2], researchers found a lower overall rate of clearance (16 [3.7%] of 435 patients) and a lower rate of annualized clearance (0.5 % person-years). The follow-up period for the 2 studies was similar (~7 years). Researchers used surrogate tests to determine the presence of chronic liver disease and found that low results of a zinc sulfate turbidity test and no evidence of liver or spleen abnormalities on ultrasound (both of which are suggestive of milder liver disease) were independent predictors of clearance. These authors did not document low viral HCV RNA levels or female sex as predictors of spontaneous clearance. Other studies similarly document loss of detectable virus, but available data make it difficult to ascertain the timing of resolution and whether subjects had acute or chronic HCV infection [19, 20].

Our study, like the study from Japan, found that none of the patients who experienced clearance had a history of blood transfusion as a risk factor. It is possible that patients with blood transfusion as a route of infection are less likely to experience HCV clearance after developing chronic infection, possibly because they receive a larger initial inoculum of virus than do persons who are injection drug users. Similarly, we observed 1 patient (an active injection drug user) who had an acute flare of hepatitis that was temporally associated with clearance of HCV RNA. A vigorous Th1 cytopathic immune response might be involved in HCV clearance, because persons who recover after acute HCV infection are found to have a highly activated cytotoxic T cell response, including a CD8 T cell response [21].

Several case reports have described loss of HCV RNA in chronically infected patients as being associated with special circumstances, including the development of hepatocellular carcinoma, withdrawal of immunosuppression after transplantation, gastrectomy for gastric cancer, and pregnancy, which may explain the occurrence of viral elimination in these instances [22–25]. Withdrawal of immunosuppression could conceivably have resulted in a boost in anti-HCV immune responses, leading to viral clearance in the transplant recipient. Likewise, it is possible that hepatocellular carcinoma, pregnancy, and gastrectomy facilitated viral clearance by nonspecific stimulation of immune responses that either enhanced or triggered an antiviral response. However, as in our study, mechanisms of HCV clearance were not addressed in these isolated case reports.

It is possible that the clearance of HCV RNA could have been underestimated in our study, because most persons who experienced clearance of HCV RNA were injection drug users, and because we used a conservative definition of chronic viremia (a duration of 1 year). Using ≥6 months as our definition.
of chronic infection would have caused the inclusion of another 3 patients in our study. Finally, an additional 18 patients were identified who tested positive for HCV RNA at least 12 months after their estimated date of infection and who subsequently tested negative for HCV RNA by PCR. However, none of these cases were included, because demonstration of chronicity by estimated date of infection is not as reliable as demonstration by molecular detection of viral RNA in successive specimens. Inclusion of these additional 21 patients in our definition of chronically infected subjects would have significantly increased the calculated rate of spontaneous negative HCV RNA test results during chronic infection.

A limitation of our study is the inability to demonstrate chronic persistence of genetically related HCV variants over time in all study patients, because of limited sample availability. However, sequence analysis of the HCV envelope 2 gene hypervariable region demonstrated the persistence of related quasispecies populations for at least 1 year in all 3 patients with adequate sample availability. However, 2 of these 3 patients also showed evidence of either coexistent infections or superinfections with a different HCV genotype. In one very interesting case, the data suggest spontaneous clearance of sequential chronic infections of different genotypes (the first was infection with genotype 1a, and the second was infection with genotype...
Table 2. Comparison of persons with chronic hepatitis C virus (HCV) infection with persons with probable HCV RNA clearance.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Persons with probable clearance (n = 5)</th>
<th>Persons with chronic infection (n = 128)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female sex</td>
<td>5 (100)</td>
<td>75 (59)</td>
<td>.16</td>
</tr>
<tr>
<td>Age at estimated date of infection, mean years</td>
<td>22.4</td>
<td>28.8</td>
<td>.20</td>
</tr>
<tr>
<td>Age at start of follow-up, mean years</td>
<td>28.8</td>
<td>37.7</td>
<td>.04</td>
</tr>
<tr>
<td>Risk exposureab</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection drug use</td>
<td>5 (100)</td>
<td>72 (57)</td>
<td>.22</td>
</tr>
<tr>
<td>Blood transfusion</td>
<td>0</td>
<td>27 (21)</td>
<td></td>
</tr>
<tr>
<td>Otherab</td>
<td>0</td>
<td>28 (22)</td>
<td></td>
</tr>
<tr>
<td>Duration of HCV infection, median years</td>
<td>17</td>
<td>15</td>
<td>.37</td>
</tr>
<tr>
<td>HCV RNA level, 1 million IU/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>On testing of last positive specimen</td>
<td>0.762</td>
<td>3.668</td>
<td>.01</td>
</tr>
<tr>
<td>Maximum</td>
<td>0.828</td>
<td>6.761</td>
<td></td>
</tr>
<tr>
<td>Genotypec</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4 (100)</td>
<td>85 (66)</td>
<td>.44</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>31 (24)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>12 (9)</td>
<td></td>
</tr>
<tr>
<td>Duration of follow-up, mean years</td>
<td>11.7</td>
<td>7.1</td>
<td>.12</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. (%) of patients, unless otherwise indicated.

a Risk exposure and/or behavior information was unavailable for 1 person who experienced clearance of HCV and for 1 person who had chronic infection.

b Includes persons with no risk exposures or behaviors and those with no blood transfusions or history of injection drug use but who reported other risk behaviors.

c Genotype was untypable for 1 person who experienced clearance of HCV.

1b). The data not only suggest that coinfection and/or superinfection with unique HCV quasispecies populations may be a common event in this repeatedly exposed population, but they also, for the first time, raise the possibility of cross-genotype protective immunity against chronic hepatitis C infection.

In conclusion, our study demonstrates that spontaneously negative HCV RNA test results occur more frequently (1.15 cases per 100 persons per year) than has been previously recognized. One-half of patients with negative HCV RNA test results subsequently had positive results, and, thus, follow-up HCV RNA testing on an annual basis may be necessary. It is not known whether patients with spontaneously negative HCV RNA test results have better long-term outcomes; this question requires further study. Immunological studies of persons who experience clearance of HCV RNA after chronic infection are needed to investigate the role of immunity in these events and to compare responses in persons who experience resolution of chronic infection with responses that occur in persons who experience clearance of HCV viremia during the acute phase. Exploitation of these events may reveal new avenues of treatment.

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