

Long-term Outcome (35 Years) of Hepatitis C After Acquisition of Infection Through Mini Transfusions of Blood Given at Birth

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Long-term follow up studies of hepatitis C virus (HCV) infection rarely exceed 20–25 yr. We studied the outcome of HCV infection in 35-yr-old adults infected at birth (1968) through mini transfusions of blood. A retrospective-prospective study was carried out. The cohort included 31 individuals who were given mini blood transfusions (21–30 ml) collected from a donor subsequently revealed to be HCV infected. At enrollment (1998), 18 of 31 (58.1%) recipients had anti-HCV antibody and 16 (88.9%) of them were HCV-RNA positive. All viremic recipients and the infectious donor had the same genotype 1b. Sequence analysis of E1/E2 and NS5b regions, coupled with phylogenetic analysis, indicated that HCV isolates from donor/recipients were linked. Eleven of the 16 viremic recipients gave consent to liver biopsy. Nine had no fibrosis or mild portal fibrosis and 2 had either discrete (Ishak's staging 3) or marked (Ishak's staging 4) fibrosis. During the prospective follow-up period (1998–2003), 2 patients were given therapy, one of whom achieved sustained clinical and virologic response. A second biopsy, performed in 5 patients at a 5 yr interval, revealed no substantial modifications in 4 cases and progression from absence of fibrosis to mild portal fibrosis in the fifth. In conclusion, taking into account the limited study sample, these findings suggest that HCV infection acquired early in life shows a slow progression and mild outcome during the first 35 yr of infection. (HEPATOLOGY 2004;39:90–96.)

Viral hepatitis type C has a high probability of evolving towards chronicity with a variable outcome over time. Different outcomes seem to be linked to the route of transmission, age at infection, and, possibly, to sex.^{1–9} Infections encountered in adulthood

carry a significantly higher probability of advancing to cirrhosis within 20 yr than those acquired by young individuals.^{4,10–18} Co-infections and comorbid conditions, which usually increase with age, may also be important contributors to the progression of hepatitis C infection.^{19–22}

The understanding of the natural history of hepatitis C virus (HCV) infection is hampered by the fact that primary infection is often asymptomatic, thus preventing a precise definition of the time of acquisition. In addition, disease progression may extend over several decades while most published prospective and retrospective studies rarely exceed 20 yr of follow-up.

During the 1960s in Italy, mini transfusions of blood or plasma represented a frequent treatment of underweight or preterm newborns. We previously postulated that these could represent a major source for HCV infections diagnosed today in Italian adults with a negative history of HCV exposure and no record of transfusions.²³

The identification of a cohort of 35-yr-old adults who acquired HCV infection shortly after birth through transfusion provides insight into the long-term outcome of HCV infection.

Abbreviations: HCV, hepatitis C virus; anti-HCV, antibody to hepatitis C virus; ALT, alanine aminotransferase; ELISA, enzyme-linked immunosorbent assay; LiPA, line probe assay; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase polymerase chain reaction; E1, envelope protein 1; E2, envelope protein 2; NS5b, non-structural 5b protein; HBsAg, hepatitis B surface antigen; anti-HBc, antibody to hepatitis B core antigen; HIV, human immunodeficiency virus; anti-HIV, antibody to human immunodeficiency virus; MU, mega unit.

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Table 1. Calendar of Mini-Transfusions Administered to Babies Born in 1968 From a Donor Who Was Subsequently Found HCV Infected and Rates of Anti-HCV Positivity in Recipients at the Moment of Enrollement

	Date of Infected Transfusion (year 1968)				Total
	February	April	June	December	
N babies transfused	11	12	10	10	43
N babies traced	6 (54.5%)	10 (83.3%)	8 (80%)	7* (70%)	31 (72.1%)
HCV status at enrollment:					
Anti-HCV positive	4 (66.7%)	3 (30%)	6 (75%)	5 (71.4%)	18 (58.1%)
Anti-HCV negative	2 (33.3%)	7 (70%)	2 (25%)	2 (28.6%)	13 (41.9%)

*3 infected subjects were twins.

Patients and Methods

In March 1998, we examined the clinical files of 1,688 children born at the Hospital of Legnano (Milan) in 1968. 166 infants (9.8%) received 385 blood transfusions from 84 donors who provided a total of 93 donations.

Thirty-six (42.9%) donors have been traced, 34 (94.4%) of whom were identified to be anti-HCV negative and 2 (5.6%) anti-HCV positive. One anti-HCV positive donor gave a single whole blood transfusion to a single infant who could not be traced. The second infected donor provided 4 blood donations that were subsequently divided and transfused to 43 infants.

Thirty-one of 43 recipients (72.1%) have been traced and were enrolled in the study, 10 recipients (23.3%) remained untraced, one refused participation and the last died at the age of 4 months from causes unrelated to liver disease.

Recipients

Of the 31 recipients (15 males, 16 females) included in this study, 11 received a single mini transfusion collected from the HCV positive donor. Twenty recipients were given 80 additional mini transfusions during the first 4 to 8 weeks of life. Indications for transfusion had been immaturity (birth weight <2500 g) in 26 (83.9%) infants and symptomatic disease (dyspepsia, gastroenteritis, eczema) in the remaining 5 (16.1%).

Each recipient was requested to provide information on the presence of additional risk factors for HCV infection, such as intravenous drug use, piercing, acupuncture, tattoos, and surgery, as well as a history of jaundice indicative of acute hepatitis. We also checked for liver damaging habits, such as alcohol abuse (mean daily intake \geq 50 gm) and use of medications and/or herbal remedies.

Thirty-two individuals (13 males, 19 females) born in the same yr and in the same hospital, who were given mini transfusions of blood collected from anti-HCV negative donors, gave their consent to entry in the study as a control group.

Donors

Altogether, 29 donors supplied 111 mini transfusions for the 31 traced recipients. Fifteen donors were traced, 14 of whom were anti-HCV negative and one anti-HCV positive.

The infected donor (index donor) was a periodic volunteer donor who was excluded from donation in 1987 (when measurement of transaminase levels in blood donors was introduced in Italy) due to persistently elevated ALT (alanine aminotransferase) levels. In 1991, when antibody assays became available, this donor was found to be anti-HCV positive.

During 1968, the index donor had supplied 4 blood units. Each 300 ml unit had been fractioned into 21- to 30-ml mini-units, for a total of 43 units, which were transfused to an equal number of newborn infants (Table 1).

Laboratory

Anti-HCV antibodies were assayed by a third generation enzyme-linked immunosorbent assay (HCV 3.0 ELISA; Ortho Clinical Diagnostics Systems, Raritan, NJ). Repeatedly reactive samples were analyzed using a supplemental recombinant immunoblot assay (RIBA 3.0, Ortho Clinical Diagnostics Systems).

HVC RNA was detected by qualitative and quantitative polymerase chain reaction (PCR) assays (COBAS Amplicor Hepatitis C Virus Test version 2.0 and COBAS Amplicor HCV Monitor Test version 2.0, Roche, Basel, Switzerland).

HCV genotype was determined using the Innogenetic Line Probe Assay (Inno-LiPA HCV II, Innogenetics, Ghent, Belgium).

HCV sequencing and phylogenetic analysis were performed. Extracted RNA was amplified by nested reverse transcriptase polymerase chain reaction (RT-PCR) using primers derived from the E1/E2 and NS5b regions of the HCV genome. In particular, for the E1/E2 region, after reverse transcription with primer HVR1305 (5'-GGTGTGGAGGGAGTCATTGCAGTT-3'), the first

round of the nested PCR used primers sense HVR949 (5'-CGCATGGCGTGGGACATGATG-3') and anti-sense HVR1305, and the second round the primers sense HVR955 (5'-GCTTGGGATATGATGATGAACTG-GTC-3') and antisense HVR1262 (5'-TGCCACCT-GCCATTGGTGTT-3'). The complementary DNA (c-DNA) synthesis of the NS5b region was performed with primer Hep102 (5'-AGCATGATGTTATCA-GCTCC-3') and then amplified by PCR with primer sense Hep101 (5'-ATACCCGCTGCTTTGACTC-3') and primer antisense Hep102 for the first round, and primers sense Hep101 and antisense Hep105 (5'-ATA-CCTAGTCATAGCCTCCGTGA-3') for the second round. The PCR products of 308 bp (nt 1295–1603) for the E1/E2 region and of 381 bp (nt 8258–8638) for the NS5b region were directly sequenced using an automated sequencer (ABI Prism, Applied Biosystems, Foster City, CA). The phylogenetic analysis compared 17 sequences obtained from the index donor and 16 HCV-RNA positive recipients involved in the study, with 9 sequences (genotype 1b) originating from unrelated patients attending the same hospital, and 9 sequences of genotype 1b sampled from GenBank. For both the genomic regions, the sets of sequences were resampled by bootstrapping 1,000 times and the distances between sequences estimated by the Kimura 2-parameters method as in DNA-DIST of the PHYLIP package (version 3.5c). Consensus phylogenetic trees were then built using NEIGHBOR (UPGMA) and CONSENSE of the same package.

To prevent possible cross-contaminations between the samples, highly stringent procedures were applied for nucleic acid extraction and amplification.²⁴

Hepatitis B surface antigen (HBsAg) and anti-core antibody (anti-HBc), were detected by commercially available assays (Hepanostika HBsAg Uni-Form II Microelisa System and Hepanostika anti-HBc Uni-Form Microelisa System, Organon Teknika, Boxtel, The Netherlands).

Anti-HIV antibody was detected by EIA (Vironostika HIV Uni-Form II Ag/Ab Microelisa System, Organon Teknika-Boxtel, The Netherlands).

Liver function tests were performed according to routine methods.

Liver biopsy was recommended for all HCV RNA positive recipients. Specimens were scored for activity of hepatitis (grading 0–18) and fibrotic changes (staging 0–6) according to the Ishak scoring system.²⁵ Samples were read by a pathologist who was blinded to clinical data.

All anti-HCV positive individuals were clinically followed from 1998 until 2003. Liver function tests were performed at 3-month intervals and virologic determinations were performed every 6 months.

Interferon treatment was offered to all patients with HCV infection and persistently increased serum ALT.

The study protocol was approved by the Institutional Review Committee of the Hospital of Legnano.

Statistical Analysis

Student's *t*-test and Fisher tests were used to compare mean values and frequencies; a *P*-value less than .05 was considered statistically significant.

Results

Clinical and Virologic Features of Donors and Recipients

None of the 31 individuals enrolled in the study was aware of having been transfused during the first weeks of life and none reported a history of jaundice or had signs or symptoms of hepatitis.

Eleven of the 31 (35.5%) recipients were given a single mini transfusion of blood collected from the HCV infected index donor. Twenty (64.5%) recipients received an additional 80 mini-units (mean number of transfusions per newborn: 4 ± 1.86 , range 1–7) collected from an additional 28 donors.

At enrollment, 18 of 31 (58.1%) recipients had anti-HCV antibody and 16 of these (88.9%) were HCV RNA positive. All 16 viremic individuals, as well as the index donor, displayed the same viral genotype: 1b. Rates of infection were similar in single unit recipients and in those transfused with blood collected from additional donors (5/11 or 45.5% vs. 13/20 or 65%; *P* = not significant). Rates of infection ranged between 30% and 75% in all 4 donations given by the infectious donor (Table 1). No difference was noted between infected and uninfected recipients regarding gestational age and type of delivery, as well as number and size of mini transfusions received after birth (Table 2).

Seventeen of 18 (94.4%) infected recipients had no additional risk factors for HCV infection, while 1 (5.6%) was an intravenous drug use. No cases of chronic alcohol abuse or of treatment with liver damaging drugs or herbal medications were reported; hepatitis B markers and anti-HIV were negative in all recipients.

The clinical and virologic features of the 16 viremic recipients at enrollment are summarized in Table 3.

ALT levels were within the normal range in 9 (56.2%) participants, slightly abnormal (<1.5 the upper normal limit) in 5 (31.3%), and more than 1.5 the upper normal limit in 2 (12.5%) participants.

Liver biopsy was performed in 11 of 16 (68.8%) viremic participants, while the remaining 5 participants (all with normal liver function tests) refused biopsy. Among the biopsied individuals, the mean Ishak scores were

Table 2. Correlation Between Obstetric Factors, Number and Size of Mini-transfusions and HCV Infection of Blood Recipients

Characteristic	Recipients			P Value
	Total N = 31	Anti-HCV Negative N = 13	Anti-HCV Positive N = 18	
Gender M/F	15/16	7/6	8/10	0.722
Birth weight (kg)*	2.267 ± 0.627	2.215 ± 0.653	2.309 ± 0.624	0.688
Delivery				
Gestational week at childbirth				
28–31	8 (25.8%)	1 (7.7%)	7 (38.9%)	0.095
32–36	12 (38.7%)	7 (53.8%)	5 (27.8%)	0.262
>37	11 (35.5%)	5 (38.5%)	6 (33.3%)	1.000
Mode of delivery				
Eutocic	20 (64.5%)	10 (76.9%)	10 (55.6%)	0.275
Dystocic	7 (22.6%)	3 (23.1%)	4 (22.2%)	1.000
Caesarian	4 (12.9%)	-	4 (22.2%)	0.120
Recipient's age at infected transfusion (days)*	25 ± 18	27 ± 18	23 ± 16	0.520
Volume (mL) of transfused blood and HCV status:*				
Anti-HCV positive	27.9 ± 2.7	27.9 ± 1.4	27.8 ± 3.4	0.929
Anti-HCV negative	53.4 ± 54.9	47.9 ± 52.8	57.3 ± 57.6	0.646
Anti-HCV nt	49.3 ± 73.9	58.5 ± 99.4	42.6 ± 50.7	0.565
Total	130.5 ± 103.8	134.3 ± 123	127.8 ± 91.3	0.866
Total N° of mini-transfusions:*				
Anti-HCV positive	31 (1 ± 0)	13 (1 ± 0)	18 (1 ± 0)	
Anti-HCV negative	46 (1.5 ± 1.6)	19 (1.5 ± 1.7)	27 (1.5 ± 1.6)	0.948
Anti-HCV nt	34 (1.1 ± 1.2)	14 (1.1 ± 1.2)	20 (1.1 ± 1.3)	0.940
Total	111 (3.6 ± 2.4)	46 (3.5 ± 2.6)	65 (3.6 ± 2.4)	0.937

*Values are expressed as mean ± SD.

Abbreviation: nt, not tested.

4.6 ± 0.8 for liver activity grading and 1.4 ± 1.1 for fibrosis staging (Table 3).

Inflammatory activity was minimal (grading 3) in 1 subject and mild (grading 4–6) in 10 (90.9%) subjects. No fibrosis or mild portal fibrosis (staging scores 0 or 1)

were present in 9 (81.8%) participants and either discrete (staging score 3) or marked (staging score 4) fibrosis were observed in 2 (18.2%) subjects. No cases of cirrhosis, either complete or incomplete (staging scores 5 or 6), have been observed.

All 32 individuals of the control group were anti-HCV antibody and HCV RNA negative, with normal ALT levels.

Table 3. Clinical and Virological Characteristics of Adults Who Appeared Viremic at the Moment of Their Enrollment (n = 16)

Characteristic	Number of Patients (%)
Sex	
M	7 (43.8)
F	9 (56.2)
ALT levels*	
Normal	9 (56.2)
≤1.5 upper normal value	5 (31.3)
≥1.5 upper normal value	2 (12.5)
HCV load	
Copies/mL†	9.4 ± 13.2 × 10 ⁵
Liver biopsy	
Number of patients	11 (68.8)
ALT value at biopsy*	
Normal	4 (36.4)
≤1.5 upper normal value	5 (45.4)
>1.5 upper normal value	2 (18.2)
Ishak grading†	4.6 ± 0.8
Ishak staging†	1.4 ± 1.1

*ALT normal value = within 60 U/L.

†Values are expressed as mean ± SD.

Molecular Analysis

The aligned sequences of 308 bp (nt 1295–1603), encompassing the E1/E2 region, and of 381 bp (nt 8258–8638) encompassing the NS5b region, obtained from the 16 HCV RNA positive individuals and from unrelated HCV RNA positive patients, were resampled by bootstrapping 1,000 times and phylogenetic trees based on the UPGMA method were obtained. As shown in Fig. 1, recipients (denoted as Rcp 1–16) and the infected index donor (denoted as BD) form a distinct cluster in the E1/E2 region from both the prototypes and the other unrelated patients, although the bootstrap value of 432 supporting this cluster is not high. However, no further grouping of the subjects can be observed inside the cluster, thus supporting the hypothesis that all recipients were infected from the same donor. Recipients and donor cluster again when the NS5b region trees are considered (Fig.

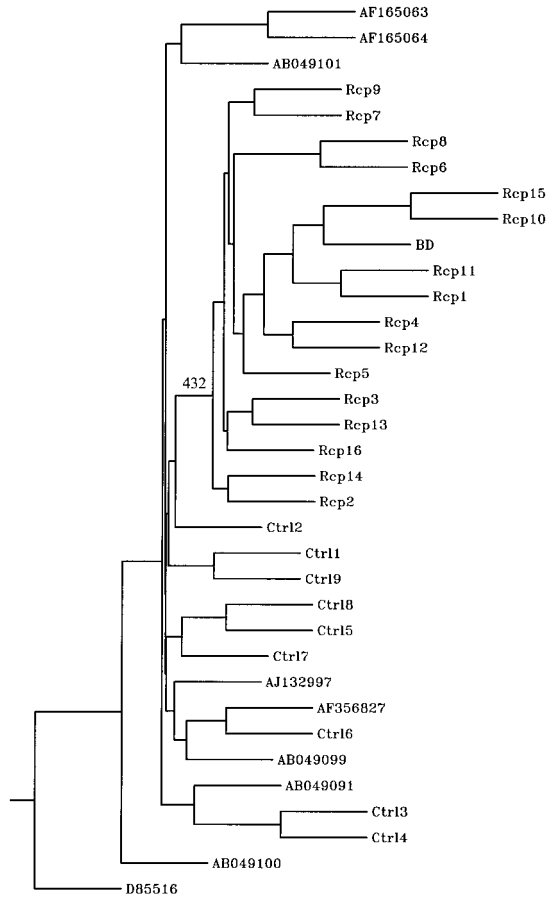


Fig. 1. Phylogenetic tree based on the E1/E2 region of the HCV genome. The analysis compares 17 sequences obtained from donor (BD) and 16 recipients (Rcp 1–16) with 9 sequences, all of genotype 1b, obtained from unrelated controls (Ctrl 1–9) and 9 sequences sampled from GenBank (AF356827, AF165063, AF165064, AB049099, AB049100, AB049091, AJ132997, D85516, AB049101). HCV sequences included in this study were submitted to the GenBank database. The accession numbers are: from AY338828 to AY338853.

2). In this region, the cluster is more robust, being supported by a bootstrap of 637. Inside the cluster, no further subdivision is evident, confirming the interpretation of a common source of HCV infection.

Prospective Follow-up (1998–2003) of HCV Infected Individuals

The 2 anti-HCV positive recipients who were HCV-RNA negative at enrollment remained persistently non viremic with normal ALT levels and no clinical evidence of liver disease throughout the follow-up period.

Two viremic patients with persistently elevated ALT levels and with fibrosis staging 1 and 4, respectively, were assigned to treatment. An initial 12-month course of interferon alfa (3 MU three times per week) yielded no response in 1 patient, while the other showed a virologic relapse after a period of therapeutic response. Both par-

ticipants were switched to combined therapy with interferon (5 MU three times per week) and ribavirin (1,000 mg daily). The 1st patient remained a nonresponder after 3 months of treatment and the 2nd achieved sustained clinical and virologic response after a course of 48 weeks of therapy.

Among the remaining 14 untreated patients, 5 (35.7%) had persistently normal ALT levels, 6 (42.9%) had fluctuating levels ranging between normal and less than 1.5 times the upper normal value, and 3 (21.4%) had ALT fluctuating between normal and more than 1.5 fold.

Liver biopsy was repeated 5 yr from the first histologic evaluation in 5 patients. No substantial modifications of the initial histologic picture was seen in 4 patients, while the 5th showed mild portal fibrosis (staging score 1) that was absent at the time of the first evaluation.

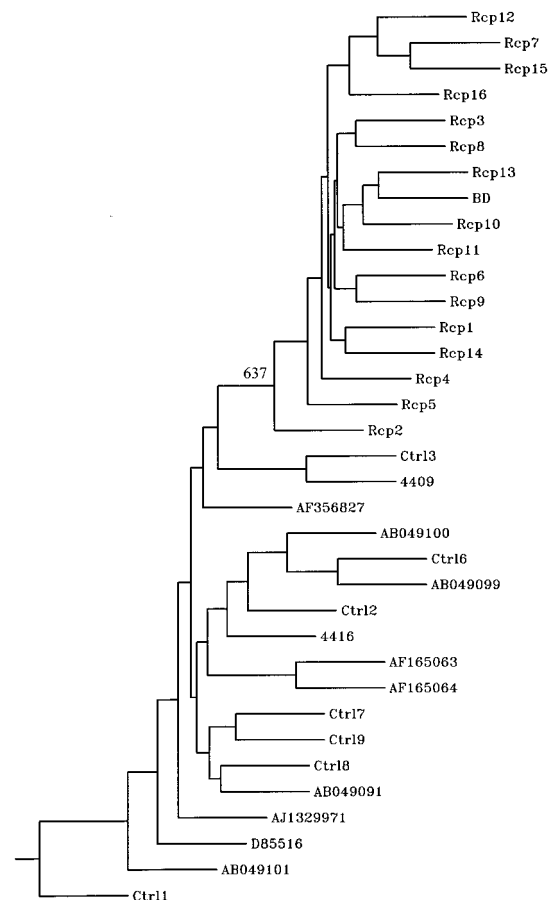


Fig. 2. Phylogenetic tree based on the NS5b region of the HCV genome. The analysis compares 17 sequences obtained from donor (BD) and 16 recipients (Rcp 1–16) with 9 sequences, all of genotype 1b, obtained from unrelated controls (Ctrl 1–9) and 9 sequences sampled from GenBank (AF356827, AF165063, AF165064, AB049099, AB049100, AB049091, AJ132997, D85516, AB049101). HCV sequences included in this study were submitted to the GenBank database. The accession numbers are: from AY338877 to AY338902.

Discussion

The administration of small volumes of blood to premature infants, as a treatment for poor weight gain or simply to maintain a given hemoglobin concentration, is a common practice in neonatal care.²⁶ Nowadays, the blood of one donation is reserved for a single recipient in order to restrict the number of donors to which an infant is exposed. In contrast, in past yr, the primary blood pack, divided into multiple small aliquots, would be administered to several infants.²⁷ Hence, before the implementation of anti-HCV blood donor screening strategies, a single infected donation could transmit infection to multiple recipients.

In this study, 31 individuals given mini transfusions of HCV-infected blood as newborn infants were enrolled 30 yr later.

Thirteen (41.9%) recipients had no marker of HCV infection. The remaining 18 (58.1%) recipients were anti-HCV antibody positive, and 88.9% of these presented HCV viremia.

No difference in medical or transfusion history that could account for a greater risk of HCV infection was noted between anti-HCV positive and anti-HCV negative recipients. During the 30 yr elapsed from transfusion to enrollment, all recipients but 1 reported no additional risk factors for HCV exposure. None of the individuals belonging to the control group resulted infected. These findings strongly support the epidemiologic link of HCV infection to the mini transfusions administered.

All 16 HCV RNA positive recipients, as well as the index donor, were infected with the same 1b HCV genotype. Sequence analysis of both E1/E2 and NS5b viral genome regions, coupled with phylogenetic analysis of HCV derived from the index donor and recipients, clearly indicated that these isolates were linked, demonstrating the common source of infection. Phylogenetic analysis, performed on 2 different regions of the viral genome, gave the same indications, although with different degrees of confidence. The bootstrap value for the node leading to the cluster of recipients was higher for the analysis based on the NS5b coding region than the one based on the hypervariable E1/E2 region, presumably because the NS5b region is subjected to a lower selective pressure than the E1/E2 region.

Two of 18 (11.1%) anti-HCV positive recipients were repeatedly HCV RNA negative with normal ALT values and were thus deemed to have spontaneously recovered from infection.

We do not know whether the 13 recipients found to be anti-HCV and HCV RNA negative were resistant to the infection or whether they lost HCV markers over time.

Absence of serologic and molecular markers of HCV infection in subjects with documented exposure to HCV has been reported by others.^{12,18,28} Loss of such markers during follow-up of HCV infected patients has been documented as well.^{11,29} Takaki et al.³⁰ showed that, in women accidentally exposed to HCV-contaminated human Rh immunoglobulin, levels of anti-HCV antibody may decrease and eventually disappear 18 to 20 yr after recovery. It is therefore possible that the rate of spontaneous remission of HCV infection may be even higher than is usually estimated by the detection of anti-HCV positivity in the absence of HCV RNA and with normal ALT levels.

During the 5-yr prospective follow-up, 2 viremic recipients underwent monotherapy with alfa-interferon treatment without success. One of these achieved a sustained clinical and virologic response after a course of combined treatment with interferon plus ribavirin.

A benign course of HCV infection was observed in viremic cases despite infection with the 1b genotype, considered the most aggressive HCV genotype.³ Most patients (87.5%) had normal or slightly fluctuating ALT levels. No cases of cirrhosis, either complete or incomplete, were observed. Only 3 (27.3%) of 11 participants who underwent liver biopsy had histologic signs of progressive liver damage. A second biopsy, performed in 5 participants at a distance of 5 yr, showed no substantial modification of the initial histologic picture in 4 cases and progression from absence of fibrosis to mild portal fibrosis in the 5th case.

Thus, in our study sample, the course of chronic hepatitis C was mild with a low rate of progression even after 35 yr of infection. This outcome is more benign than what has been reported in studies on subjects acquiring infection in adulthood, in whom a more rapid evolution of liver disease has been observed, with development of cirrhosis in approximately 20% of cases within 20 yr.^{4,11}

Our data is similar to that reported in children who acquired HCV infection after cardiac surgery, and in young women accidentally exposed to HCV-contaminated immunoglobulins, confirming that patients infected at a young age are at lower risk for progressive liver disease.^{5,6,16} There is evidence that the presence of additional risk factors, such as elevated alcohol intake (>50 gm per day), or co-morbid conditions like HBV or HIV coinfections, is associated with fibrosis progression.^{3,4,19-22} The absence of these factors in our cohort, coupled with the very young age at the time of infection, may have favorably influenced the long-term outcome.

Histologic studies performed in HCV-infected children show that fibrosis may progress with increasing age and duration of infection, but no data is currently avail-

able as to whether severe manifestations of liver disease can appear at time intervals longer than 2 decades from the moment of infection.

The findings in this study indicate that a 35-yr span of HCV-infection has not, in itself, been the cause of significant liver disease. Whether HCV infection acquired early in life may reach a type of life-time steady state, or whether fibrosis may have an abrupt acceleration with aging, requires further investigation.

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