

## Different Hepatitis C Virus (HCV) RNA Load Profiles Following Seroconversion among Injecting Drug Users without Correlation with HCV Genotype and Serum Alanine Aminotransferase Levels

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**Hepatitis C virus (HCV) infection often persists in association with chronic hepatitis. Different factors have been proposed to determine the clinical outcome of HCV infection. The aim of this study was to examine three different factors of HCV infection among injecting drug users. Nineteen untreated HCV seroconverters were tested longitudinally for the presence of HCV RNA by reverse transcriptase (RT) PCR, and results were quantified by the branched-DNA (bDNA) assay. HCV genotypes were determined with the first sample taken after HCV seroconversion. To assess the natural course of infection, serum alanine aminotransferase (ALT) levels were measured at three stages in every individual. The concordance between bDNA and RT-PCR was 98.9%. Three distinct patterns were found, according to the HCV RNA load after seroconversion during a mean follow-up period of 5 years (range, 1 to 8 years). HCV genotype 1a was predominant (52.6%). There was a significant increase in serum ALT levels (mean 55.5 U/liter) in the early phase of HCV infection, compared with basal serum ALT levels before HCV seroconversion and at the end of the follow-up period. Three distinct HCV RNA load profiles were found, without apparent relationship to genotype and serum ALT levels in the first 5 years of HCV infection.**

Hepatitis C virus (HCV) is a major cause of parenterally transmitted acute hepatitis (5) and is widely spread among injecting drug users (IDUs) (25, 28). HCV is an important cause of chronic hepatitis and may eventually cause progressive liver disease, cirrhosis, and liver cancer (7, 10, 22). Chronic HCV infection is often silent, and clinical symptoms are absent or minimal unless the disease is severe or cirrhosis is diagnosed. Many attempts to identify the natural history, progression, and treatment of HCV infection have been made, but several aspects remain to be elucidated (13). In chronically infected individuals, viral load, genotype, and elevated serum alanine aminotransferase (ALT) levels may have clinical relevance (16, 18, 19). When parenchymal liver cells are damaged, aminotransferases leak from the liver into the blood, resulting in elevated levels of aminotransferases. Normalization of serum ALT levels after treatment with interferon (IFN) for 6 months is conventionally considered to indicate treatment efficacy (15), although half of the untreated patients with chronic HCV infections have normal or minimally elevated serum ALT levels (6). In the present study, we quantified HCV RNA in serum samples from 19 HCV seroconverters longitudinally and measured serum ALT levels at three different time points to assess the course of HCV infection. The HCV genotypes harbored by the 19 HCV seroconverters were determined with the first sample after seroconversion. The branched-DNA (bDNA) signal amplification assay (Quantiplex HCV RNA; Chiron Corp., Emeryville, Calif.) was used for quantification of

HCV RNA loads. This assay does not require sample preparation, is easy to handle, and is highly reproducible. It is unaffected by HCV genotype variability, because it is based on hybridization of HCV RNA to oligonucleotide probes that target the highly conserved 5' untranslated region and the 5' part of the core gene of the HCV genome (8).

In the present study, we investigated the course of HCV infection, the influence of HCV genotypes on viral loads, ALT levels during HCV infection, and the correlation between viral loads and ALT levels among IDUs in the first 5 years of HCV infection.

### MATERIALS AND METHODS

**Patients.** The study population consisted of IDUs living in Amsterdam and participating in the Amsterdam Cohort Studies on HIV and AIDS among IDUs, a collaboration between the Academic Medical Centre, the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, and the Municipal Health Service, Amsterdam, The Netherlands. The 358 IDUs in this study were recruited from a cohort started in December 1985 (27). We selected drug users who were monitored for at least 3 years and had at least 7 visits ( $n = 358$ ). Their serum and plasma samples were stored initially at 4°C and then frozen at -20°C within 24 h of collection and handling; ultimately, the samples were stored at -70°C. All subjects were screened at the first and last sampling times for HCV antibodies, and sequential samples of the seroconverters were subsequently tested to establish the approximate seroconversion point. The date of HCV seroconversion was determined by calculating the midpoint between the last seronegative sample and the first seropositive sample. Nineteen HCV seroconverters were identified; they were then studied longitudinally for HCV RNA levels. The mean follow-up period after HCV seroconversion was 5 years (range, 1 to 8 years), and none of the subjects had signs of HCV-related symptoms or were treated with IFN or other antiviral drugs.

**Laboratory tests.** Antibodies to HCV were assayed by the third-generation Enzyme Immunoassay (EIA 3.0; Abbott Laboratories, Chicago, Ill.) according to the manufacturer's manual. All positive EIA 3.0 assays were confirmed by the third-generation Strip Immunoblot Assay (RIBA; Chiron Corp.) according to the manufacturer's instructions.

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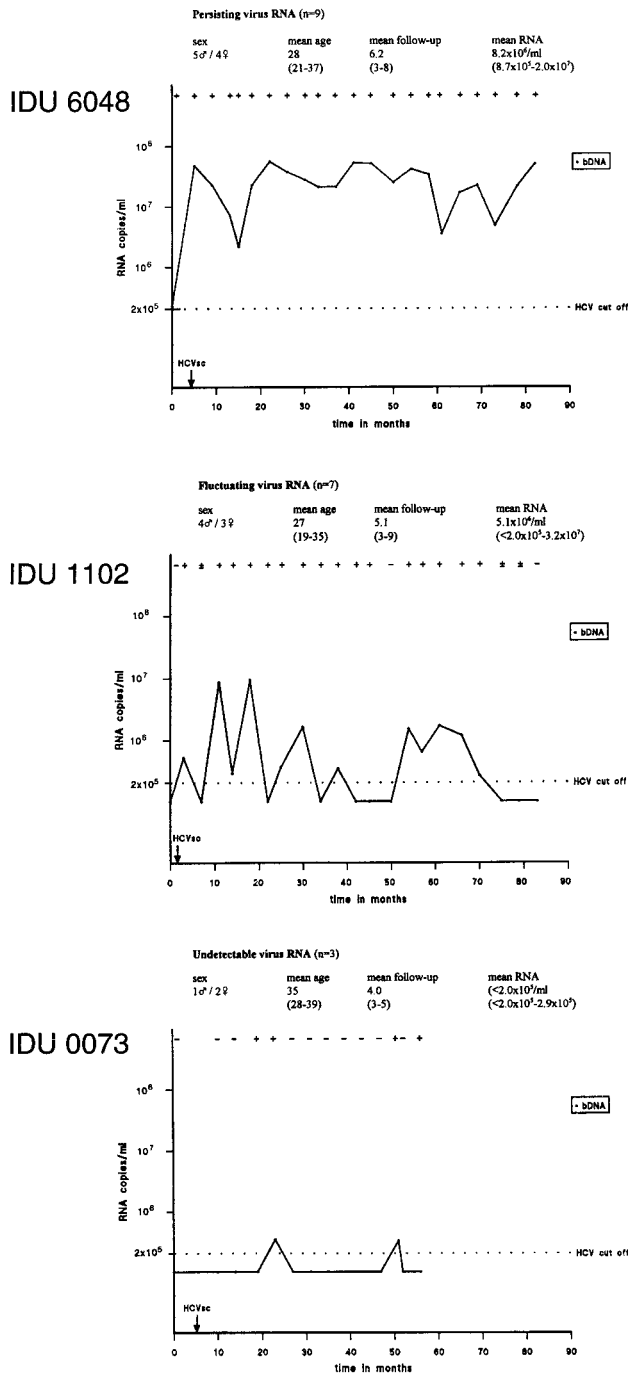


FIG. 1. Three examples of viral load profiles found in 19 HCV seroconverters among IDUs. HCV RNA loads were measured longitudinally by bDNA assay. The detection limit of the bDNA assay is shown as a dotted line. Characteristics of each group are indicated at the top of each panel. RT-PCR results in duplicate are indicated by +, ±, or -. SC, seroconversion.

**Detection of HCV RNA by RT-PCR.** HCV RNA was isolated from 100-μl serum or plasma samples, according to the method of Boom et al. (3), and was immediately used in reverse transcriptase (RT) PCR experiments or stored at -70°C. One-fifth (10 μl) of the isolated RNA was applied to reverse transcription and a single PCR with primers located in the highly conserved 5' noncoding region described previously by Attia et al. (2). For reverse transcription, 10 μl of RNA was incubated with 25 ng of antisense primer (nucleotides [nt] 319 to 324) 5'-ACCTCC-3' for 5 min at 65°C and then cooled to 42°C. Finally, 14 μl of the reverse transcription mixture, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl,

0.1% Triton X-100 (Packard Instrument Co., Inc., Downers Grove, Ill.), 6 mM MgCl<sub>2</sub>, 0.6 mM (each) deoxynucleoside triphosphates, 20 U of RNase inhibitor (Promega, Madison, Wis.), and 100 U of SuperScript II (Life Technologies, Gaithersburg, Md.), was added. The mixture was incubated for 30 min at 42°C, and 12.5 μl was used for PCR in duplicate. For PCR, the GeneAmp PCR carryover prevention kit (Perkin-Elmer Cetus, Branchburg, N.J.) was used to avoid contamination. PCR was performed in a 50-μl volume containing 100 ng of sense primer (nt 47 to 68) 5'-GTGAGGAAGTCTTCTTACAG-3', 100 ng of antisense primer (nt 292 to 312) 5'-ACTCGCAAGCACCCTATCAGG-3', 2.5 U of AmpliTaq polymerase (Perkin-Elmer Cetus), 50 mM Tris-HCl (pH 8.3), 20 mM KCl, 1.2 mM MgCl<sub>2</sub>, 2.5 μM (each) deoxynucleoside triphosphates, 25 μM dUTP, and 0.5 U of uracil N-glycosylase (Perkin-Elmer Cetus). A type 480 thermal cycler (Perkin-Elmer Cetus) was programmed as follows: 5 min at 95°C followed by 40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and then incubation of samples for 8 min at 72°C. PCR products were subjected to electrophoresis in 2% agarose containing ethidium bromide and were visualized under UV. A pool of HCV-positive serum, quantified by bDNA technology (Chiron Corp.) to a level of 1.6 × 10<sup>6</sup> HCV RNA copies/ml, and a 100-fold dilution of this quantified serum pool were used as positive controls. The sensitivity of RT-PCR was evaluated by serial twofold dilutions of the quantified pool of serum and was approximately 10<sup>3</sup> HCV RNA copies/ml (results not shown). As negative controls, we used a pool of commercially available serum (seronegative for human immunodeficiency virus, hepatitis B virus, and HCV) and Tris-EDTA. All positive and negative controls were tested in parallel with test samples throughout the entire procedure, starting with RNA extraction. The results of the single PCR usually rendered good duplicates, unless very low levels of HCV RNA were present in the samples. In such cases, the detection limit of RT-PCR was reached, resulting in a plus-minus duplicate.

**HCV RNA quantification.** The HCV RNA load was determined longitudinally by the bDNA signal amplification assay, version 2.0 (Quantiplex HCV RNA; Chiron Corp.), according to the manufacturer's manual. All samples were tested in duplicate, and the mean values from the duplicate tests were used for data analysis. Viral load, expressed as the number of HCV RNA copies per milliliter, was determined by comparison with an external standard curve with a quantitation limit of 2.0 × 10<sup>5</sup> HCV RNA copies/ml.

**Genotyping.** Genotypes were determined either by the HCV line probe assay protocol (LiPa; Innogenetics, Ghent, Belgium) (24), according to the manufacturer's manual, or by direct sequencing of the products obtained by nested PCR on 1/10 of the initial in-house single PCR products. Nested PCR (encompassing the same region used in the HCV LiPa protocol) was performed under the same conditions as the single PCR but for 25 cycles, with sense primer (nt 74 to 91) 5'-AGCGCTAGCCATGGCGT-3 and antisense primer (nt 243 to 260) 5'-TACCAAGGCCCTTTCGC-3', which were extended with the -21 M13 primer

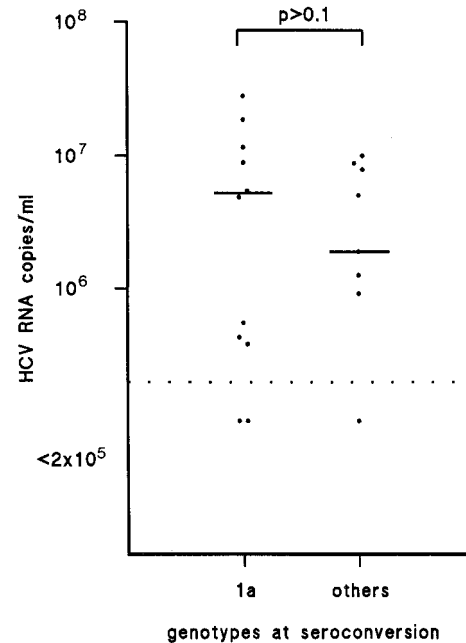


FIG. 2. Comparison of HCV RNA loads with genotypes in the first sample after HCV seroconversion. The horizontal dotted line indicates the detection limit of the bDNA assay. The data points are from the first samples after seroconversion. Horizontal bars represent the median value of each genotype. Statistical analysis was performed with the Mann-Whitney test.

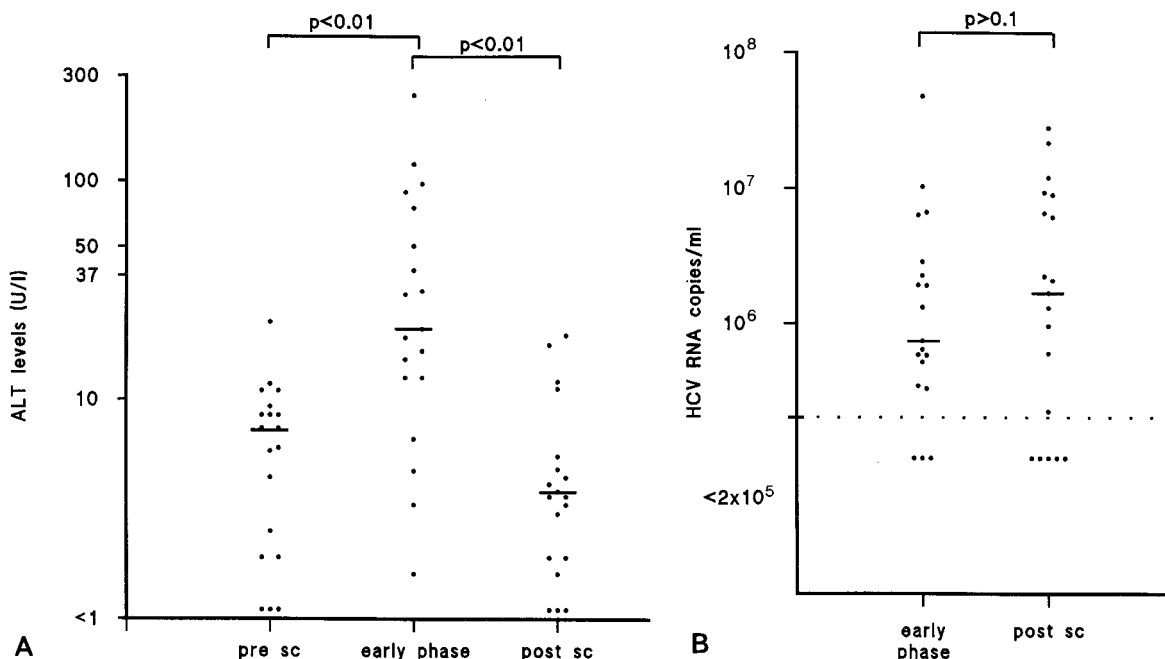


FIG. 3. (A) Differences among serum ALT levels at three different time points in HCV infection. Horizontal bars represent the median value at each time point. Each data point represents the ALT level from one seroconverter. The upper limit of the normal range for the ALT assay is 37 U/liter. Statistical analysis was performed with the Mann-Whitney test. (B) Differences among HCV RNA loads in 19 HCV seroconverters in the early and chronic phases of HCV infection. Each data point represents the HCV RNA level from one seroconverter. Horizontal bars represent the median value of HCV RNA copies per milliliter. The horizontal dotted line depicts the detection limit of the bDNA assay. Statistical analysis was performed with the Mann-Whitney test. The mean preseroconversion period was 9 months (range, 2 to 38 months). The early phase had a mean duration of 6 months (range, 1 to 23 months), and the postseroconversion (post sc) period was 60 months (range, 14 to 91 months).

(5') and the reverse M13 primer (3'), respectively. The Thermo-Sequenase cycle sequencing reaction was performed according to the manufacturer's manual by the dye-primer method (Amersham Life Science, Buckinghamshire, England).

**Serum ALT levels.** Serum ALT levels were measured with a Hitachi 747 analyser at 37°C, according to the manufacturer's protocol (reagents BM 300683 and BM 1127799 [pyridoxal-phosphate enhanced]; Boehringer GmbH, Mannheim, Germany). The upper limit of the normal range for this assay was 37 U/liter. Serum ALT levels were measured at three different time points for all 19 HCV seroconverters: before HCV seroconversion (without HCV RNA present), in the first sample after HCV seroconversion (to assess the early phase of HCV infection), and during the chronic phase of HCV infection.

**Computer sequence analysis.** PCR products were directly sequenced with an ABI automated sequencer, model 373A, using 1.2.0 software. The direct sequences were assembled by the Sequence Navigator program (ABI) and were further optimized manually.

**Statistics.** Scatterplots were used to review the distributions of HCV RNA and ALT concentrations, and log-transformed HCV RNA concentrations were used for analysis. Samples negative by bDNA were assigned a value of  $1.0 \times 10^5$  HCV RNA copies/ml. We used the Mann-Whitney test for comparison of the different parameters. A *P* value of  $<0.01$  was considered to indicate statistical significance.

## RESULTS

**Evaluation of the course of HCV infection in 19 HCV seroconverters.** The natural course of HCV infection was investigated in 19 HCV seroconverters who were exposed to HCV by injecting drugs and sharing needles. None were treated with IFN, and none mentioned complaints or had signs of HCV-related symptoms.

Detection of HCV RNA by the bDNA assay was highly dependent on viral RNA titers, which varied considerably among our subjects. HCV RNA was, nevertheless, detected at various time points after seroconversion in all 19 HCV seroconverters. Three patterns of HCV seroconverters were defined according to their HCV RNA loads (Fig. 1). The first group consisted of nine (47.4%) individuals having high HCV RNA loads (mean,  $8.2 \times 10^6$  HCV RNA copies/ml) through-

out the infection, and the second group, seven (36.8%) individuals, had fluctuating HCV RNA loads (mean,  $5.1 \times 10^6$  HCV RNA copies/ml). The third group consisted of three (15.8%) individuals having low HCV RNA loads (mean,  $<2.0 \times 10^5$  HCV RNA copies/ml) throughout the infection. Mean HCV RNA copies per milliliter were calculated by counting all viral loads above the lower limit of  $2.0 \times 10^5$  HCV RNA copies/ml and dividing by the total number of visits after HCV seroconversion. Samples negative by bDNA were assigned a value of  $1.0 \times 10^5$  HCV RNA copies/ml.

The concordance between bDNA and the qualitative single-step in-house RT-PCR was 98.9%. In only 4 of 356 serum samples did we find repeated positive results, with bDNA just above the detection limit, accompanied by repeated negative RT-PCR results.

**HCV genotypes in relation to HCV RNA load at seroconversion.** We determined the HCV genotypes for all 19 subjects, using the first sample taken after HCV seroconversion, and compared the genotypes with the HCV RNA load in the same sample. Genotyping revealed that HCV genotype 1a was the predominant genotype (52.6%), followed by genotypes 3a (36.8%) and 1b (5.3%). One double infection (5.3%) with genotypes 1a and 3a was found. Figure 2 shows that there was no statistically significant difference ( $P > 0.1$ ) between HCV genotypes and HCV RNA loads in the first sample after seroconversion. Moreover, there was no apparent relationship between genotypes and HCV RNA load profiles (results not shown).

**Relationship between HCV RNA loads and ALT levels.** To address the relationship between serum ALT levels and the development of HCV infection, serum ALT levels were measured at three different time points during follow-up in the 19 HCV seroconverters. The first measuring point was before

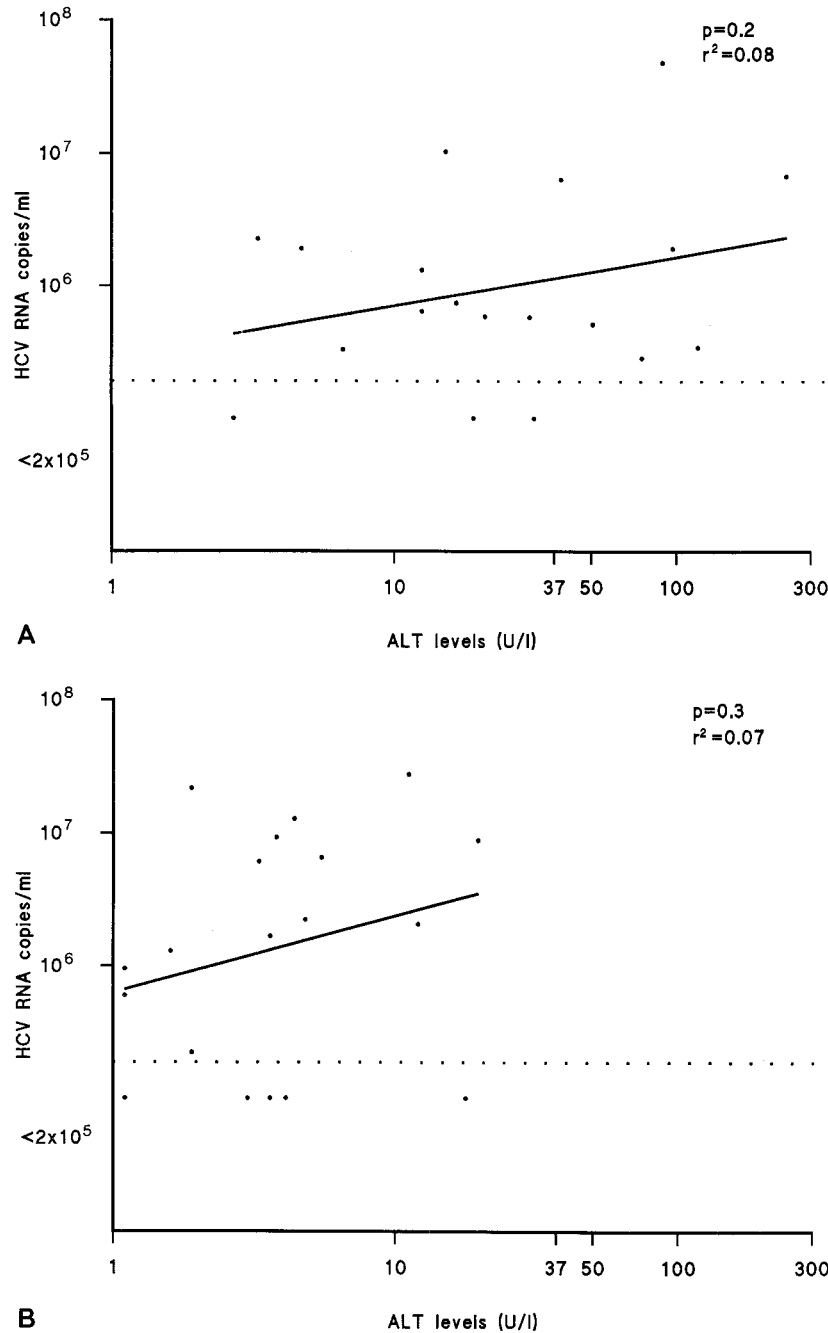


FIG. 4. (A) Correlation between HCV RNA loads and serum ALT levels in the early phase of HCV infection. Each point represents data from one seroconverter. (B) Correlation between HCV RNA loads and serum ALT levels in the chronic phase of HCV infection. Each point represents data from one seroconverter. The horizontal dotted lines show the detection limit of the bDNA assay. Statistical analysis was performed with the Mann-Whitney test.

seroconversion, with a mean period of 9 months (range, 2 to 38 months) without detectable HCV RNA, to determine a basal serum ALT level. The second measuring point was just after seroconversion, with a mean period of 6 months (range, 1 to 23 months), to assess the early phase of HCV infection. Finally, to assess the chronic phase of HCV infection, serum ALT levels were measured at a mean period of 60 months (range, 14 to 91 months) after seroconversion. The serum ALT levels before seroconversion and in the chronic phase of HCV infection were, in all 19 seroconverters, below the upper limit of the

normal range, 37 U/liter. There was a significant correlation ( $P < 0.01$ ) between increased serum ALT levels and the early phase of HCV infection (Fig. 3a). The serum ALT levels we found in the early phase of HCV infection had a mean value of 55.5 U/liter (range, 2.7 to 246.1 U/liter), with a median value of 21.1 U/liter. The intervals among the values of serum ALT levels in this phase reflect the long sample intervals ( $\pm 2$  years) in five subjects around the time of HCV seroconversion.

Comparison of the relationship between HCV RNA load in the early and chronic phases showed no significant difference



( $P > 0.1$ ) between the two time points (Fig. 3b). A trend of HCV RNA levels in the three groups paralleling the serum ALT levels can be seen, although it is statistically not significant. This trend can best be illustrated by individuals having HCV RNA loads above the detection limit of the bDNA assay (range,  $3.8 \times 10^5$  to  $1.2 \times 10^7$  copies/ml) with serum ALT levels ranging from 1.0 to 246.1 U/liter, regardless of their load profiles ( $P > 0.1$ ) (Fig. 4).

## DISCUSSION

Since HCV infection often persists in association with chronic hepatitis, it is appropriate to consider the natural history of HCV. We must determine what factors determine the clinical outcome of HCV infection and whether normalization of liver enzymes, with or without the presence of HCV RNA, is of any importance. The diagnosis and monitoring of HCV infection have become easier and more accurate with the quantitative and qualitative amplification techniques now available. However, the clinical relevance of the presence of HCV RNA, in correlation with serum ALT levels and HCV genotypes, is still not fully understood (18, 19).

Our study shows a very high concordance, 98.9%, between the bDNA assay and our single-step RT-PCR. Both assays target the same highly conserved region of HCV. The bDNA assay is highly reproducible and is unaffected by the genotypic variability of HCV (8); it is therefore a useful tool for monitoring HCV RNA levels throughout the course of disease. Our longitudinal monitoring of serum samples by bDNA and RT-PCR of 19 untreated HCV seroconverters revealed three distinct HCV RNA load profiles. In the groups with high and fluctuating HCV RNA loads, the mean HCV RNA load did not significantly differ. It is quite remarkable that HCV RNA levels can fluctuate significantly without treatment, as was earlier described for HCV with a comparable frequency (33%) in untreated hemodialysis patients (26). This natural fluctuation of HCV RNA levels may be confusing in the treatment of HCV-infected individuals, because testing of a single sample during treatment probably does not accurately reflect the antiviral effect of therapy. Long-term monitoring of patients for decreases in HCV RNA levels, along with serum ALT levels, is needed to evaluate the true efficacy of therapy.

The correlation between HCV genotype and HCV RNA load remains controversial (11, 12, 17). Various HCV genotypes have been identified (23) and seem to be associated with either benign or severe disease and sensitivity to IFN treatment (9). In this study, however, the different genotypes were distributed randomly among HCV RNA profiles in the 19 seroconverters, suggesting that the three HCV RNA profiles are not influenced by the genotypes we found after seroconversion.

Considering a direct relationship between HCV RNA load and increased hepatocellular damage (1, 26, 29), together with an increase in serum ALT levels to reflect inflammatory activity in the liver (4), one might expect a direct correlation between HCV RNA load and serum ALT levels. Therefore, it is important to know that the HCV RNA loads measured in serum reflect the HCV RNA loads present in the liver. Idrovo et al. (14) found a strong correlation between HCV RNA loads present in serum and liver biopsy samples from the left and right lobes. However, they found no significant correlation between the severity of hepatic damage and HCV RNA loads present in serum or liver samples in patients with chronic hepatitis. A recent study describing liver fibrosis progression in patients with chronic HCV also showed that virological factors in HCV infection are minor prognostic markers for liver dis-

ease progression compared to male sex, excessive alcohol consumption, and age at infection (21).

In our study, serum ALT levels differed significantly between the early and chronic phases of HCV infection; they rose in the early phase and fell below the upper limit of the normal range in the chronic phase. This probably points to active inflammation of the liver in the early phase, with normalization of liver functions, as measured by ALT levels, at the end of the follow-up period. Of the 16 subjects belonging to groups having high or fluctuating loads, all but 2 still had high HCV RNA loads (mean,  $1.0 \times 10^7$  copies/ml), together with normalized serum ALT levels, at the end of the follow-up period. Alter et al. (1) noted that serum ALT and HCV RNA peaks were not simultaneous in some acute cases. They found an HCV RNA peak prior to peak serum ALT levels. For our study, this could mean that we might have missed serum ALT peaks in some individuals; however, we did find a correlation between peak serum ALT levels and the early stage of HCV seroconversion. In one chronic case, Alter et al. (1) found a parallel fluctuating pattern of both HCV RNA and serum ALT levels, suggesting a direct relationship between viral replication and liver damage in some patients. On the other hand, it is known that cellular immune responses, particularly those mediated by CD8<sup>+</sup> cytotoxic T lymphocytes, are important in the pathogenesis of HCV. Nelson et al. (20) pointed out that in patients exhibiting cytotoxic T-lymphocyte activity, lower HCV RNA levels and more active liver disease were reflected by histological abnormalities and serum ALT levels. This might indicate immunologic control of HCV during infection by elimination of virus-infected cells, resulting in damaged liver tissue and increased ALT levels in serum. Thus, these elevated levels probably reflect active liver inflammation mediated by the immune system. Therefore, controversy remains regarding the importance of normalized serum ALT levels as a marker of normalization of liver function and the relationship of serum ALT levels to persistent HCV RNA loads during HCV infection.

In summary, three distinct HCV RNA patterns were obtained, and a peak serum ALT level was found in the early stage of HCV infection. The predictive value of these patterns remains to be evaluated by long-term follow-up.

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