Natural history of hepatitis C virus among injecting drug users
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Chapter 4

Quantitative antibody responses to structural (core) and non-structural (NS3, NS4, and NS5) hepatitis C virus (HCV) proteins among seroconverting injecting drug users: impact of epitope variation and relationship to detection of HCV RNA in blood

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Hepatology in press.
Abstract

To gain insight in the natural history of HCV, 13 HVT seronegative injecting drug users were studied who seroconverted for HCV as determined by third generation ELISA, showed an ensuing antibody response to HCV, and were not treated with any antiviral drugs during follow-up. Subjects included 13 untreated HIV-negative individuals of whom 5 (38.5%) apparently cleared HCV and were PCR-negative in at least 3 consecutive samples, 3 (23.1%) showed transient viraemia and were PCR-negative in one sample during the study period, and the other 5 (38.5%) showed persistent viraemia. Viraemia was determined longitudinally by reverse transcription (RT) PCR and quantified by branched-DNA (bDNA). HCV genotypes were determined on serial samples during follow-up. Quantitative antibody levels to core, NS3, NS4, and NS5 were determined using the Chiron RIBA™ HCV-titering Strip Immunoblot Assay, which is based on HCV genotype 1. The antibody responses to core, NS3, NS4, and NS5 were erratic. In individuals infected with HCV genotype 1, significant higher median antibody responses to core ($P=0.02$) and to NS4 ($P=0.04$) were found as compared to those infected with other genotypes, showing a significant impact of HCV genotype-specificity of the assay. In groups infected with HCV genotype 1, significantly higher median NS3 antibody titers (2.61 RI vs 0.38 RI; $P=0.003$) were found in the individuals with persistent viraemia than in those with apparent resolution of HCV RNA in blood. In groups infected with genotypes other than genotype 1, significantly higher median NS3 antibody titers (0.89 RI vs 0.03 RI; $P=0.0004$) and NS5 antibody titers (1.86 RI vs 0.01 RI; $P=0.006$) were found in the individuals with persistent viraemia than in those with apparent resolution of HCV RNA in blood. Individuals with viral persistence had higher HCV RNA loads with higher antibody responses as compared to individuals with apparent viral clearance from blood. Apparent viral clearance from blood was observed in an unexpected high percentage (38.5%), associated with a significant decrease of antibodies to NS3, independent of HCV genotype, as compared to individuals with persistent viraemia ($P<0.005$). Apparent viral clearance from blood with gradual loss of antibodies to various HCV proteins, independent of HCV genotype, was observed in 4 of the 5 individuals within approximately 1 year after HCV seroconversion, whereas 1 of these individuals apparently cleared the virus from blood with complete seroreversion.
Introduction

Hepatitis C virus (HCV) is a major cause of acute hepatitis (1) and is widespread among injecting drug users, with up to 90 percent being positive for antibodies to HCV (2,3). HCV infection progresses with an indolent course and remains benign in the first few years (4), but the majority (>50%) of HCV-infected individuals eventually develop chronic HCV infection. Histologically, the chronic disease is commonly associated with liver necrosis, scarring, fibrosis, and cirrhosis. A further important sequela of HCV infection is the gradual progression to hepatocellular carcinoma in some patients (5-8).

Although several studies have been carried out to define features of the humoral immune response to HCV (9-16), little is known about the dynamics of HCV-specific antibody responses in humans, in relation to HCV genotype or persistence of viraemia. Multiple distinct episodes of acute hepatitis have been observed in injecting drug users (17), haemophiliacs (18), haemodialysed patients (19), in polytransfused thalassaemics (20). Lack of protective immunity against HCV, on rechallenge with homologous or heterologous HCV strains was also observed in chimpanzees (21). Mechanisms for maintaining viral persistence are unknown, but may be related to the presence of multiple variants, or quasispecies, that may occur both spontaneously and due to host immune pressure and escape immune surveillance. HCV genomes vary considerably not only from one isolate to another but also in the sequences derived over time from the same patient (22,23).

We therefore undertook a longitudinal study to quantify antibody responses to core, NS3, NS4, and NS5 HCV proteins and compared the resulting antibody profiles as to HCV genotype and persistence of viraemia in a cohort of HCV positive injecting drug users who remained HIV negative during the study period.

Participants and Methods

Participants

The injecting drug users were recruited from a cohort started in December 1985 (24) and consisting of drug users living in Amsterdam and participating in the Amsterdam Cohort Studies on HIV and AIDS. In March 1996, we selected individuals who had been followed for at least 3 years and seen at least 7 times (n=358) and 19 HCV seroconverters were identified as described elsewhere (25).

Of these 19 HCV seroconverters, 10 remained HIV seronegative during the study period, whereas 3 seroconverted for HIV just before the end of follow-up. The average sampling interval among the 13 individuals during their HIV negative status was 4 months (range 1-12 months), with an average of 16 time-points per patient (range 6-26 time-points), and with an average follow-up of 61 months (range 16-114). Serum and plasma samples were stored initially at +4°C, then frozen at -20°C within 24 hours of collection and handling, and ultimately stored at -70°C. None of the subjects received antiviral therapy during follow-up.

Serological data

Sera were tested for the presence of antibodies to HCV by the third-generation Enzyme Immunoassay (EIA 3.0; Abbott Laboratories, Chicago, Illinois, USA). All positive EIA 3.0 assays were confirmed by the third-generation Strip Immunoblot Assay (SIA, RIBA™; Chiron Corporation, Emeryville, California, USA).

Antibodies to core, NS3, NS4, and NS5 proteins of HCV were quantified using the Chiron RIBA™ HCV titering Strip Immunoblot Assay which includes two levels of human IgG on each strip as internal controls. The lower limit of the assay was determined by calculating the mean of all negative measurements of each antibody plus 3 times the standard deviation.
The calculated lower limit for each antibody had a relative intensity (RI) of 0.07. The quantity of antibodies to HCV was expressed as relative intensity (RI) using the automated RIBA™ Processor System according to the manufacturer's manual.

Sera were tested for the presence of antibodies to HIV-1 with commercial Enzyme Immunoassay (EIA; Abbott Laboratories, Chicago, Illinois, USA) and confirmed by Western blot (Diagnostic Biotechnology, Herent, Belgium). All serological assays were performed according to the manufacturer's manual.

Detection of HCV RNA by RT-PCR
HCV RNA was isolated and detected using primers located in the highly conserved 5'-UTR region in a single reverse transcriptase (RT) PCR under conditions as earlier described elsewhere (25). For the PCR, the GeneAmp PCR carry-over prevention kit (Perkin Elmer Cetus, Branchburg, New Jersey, USA) was used to avoid contamination.

A pool of HCV-positive serum was quantified by the branched DNA (bDNA) technology (Chiron Corporation) to a level of 1.6 x 10^6 HCV RNA copies/ml and hundred-fold dilutions of the quantified serum pool were used as positive controls. As negative controls, we used a pool of commercially available serum (seronegative for HIV, HBV and HCV) and TE (Tris/EDTA). All positive and negative controls were tested in parallel with the test samples throughout the procedure, starting with RNA extraction. The test samples were considered reliable when all positive and negative controls gave expected results. The sensitivity of the single RT-PCR was evaluated by serial twofold dilutions of the quantified pool of serum, and found to be approximately 10^3 HCV RNA copies/ml (results not shown). The single RT PCR usually rendered good duplicates unless very low levels of HCV RNA were present in the samples. In such cases, the detection limit of the RT-PCR was reached, resulting in a plus/minus duplicate.

Viral RNA quantification
The HCV RNA load was determined longitudinally by the branched DNA (bDNA) signal amplification assay 2.0 (Quantiplex™ HCV RNA, Chiron Corp., Emeryville, CA, USA). All samples were tested in duplicate. The viral load, expressed as HCV RNA copies/ml, was determined by comparison with an external standard curve with a quantitation limit of 2.0 x 10^5 HCV RNA copies/ml. The bDNA assay was performed according to the manufacturer's manual.

Genotyping analysis
HCV genotypes were determined on serial samples either by the HCV LiPa protocol (Line Probe Assay, LiPa, Innogenetics, Ghent, Belgium) (26), according to the manual, or by direct-sequencing the products obtained by nested PCR of the 5'-UTR as previously described (25).

Statistical methods
The Mann-Whitney test was used for comparison of median values of HCV RNA levels and levels of all four quantified antibodies among various individuals and groups of individuals. Scatterplots were used to relate the distributions of HCV genotypes with the relative intensity of the four antibodies to HCV and HCV RNA load. A value of $P < 0.05$ was considered significant.
Results

Serial measurements of HCV antibody responses to core, NS3, NS4 and NS5
After determination of HCV seroconversions, quantitative HCV antibody responses to core, NS3, NS4 and NS5 proteins were measured from approximately 6 months before HCV seroconversion until the end of follow-up in 13 individuals. The average sampling interval among the 13 individuals during their HIV negative status was 4 months (range 1-12 months), with an average of 16 time-points per patient (range 6-26 time-points), with an average follow-up of 61 months (range 16-114). Antibody titers to all 4 proteins were measured on serial samples and plotted with HCV RNA levels as measured by PCR and bDNA.

Relationship between antibody responses and viral persistence
Serial samples for HCV antibodies and HCV RNA from 5 individuals with persistent viraemia, as determined by bDNA and PCR, were analyzed to evaluate their clinical course of HCV infection (Fig. 1a; 1114, 1123, 3059, 7030, and 7041). HCV RNA was consistently detected in all samples of these individuals. Antibodies to core, NS3, NS4, and NS5 were found in individuals 1114, 1123, and 7041. Viral persistence was erratic and not associated with HCV genotype. However, the presence and moment of appearance of antibodies to HCV after infection varied among individuals with persistent viraemia, as follows:

Individual 1114 raised antibodies to core, NS3, and NS4 within 7 months after infection with HCV genotype 1. After 18 months, HCV load dropped below the detection limit of bDNA assay for about 9 months but remained PCR-positive. Thereafter, the HCV load subsequently rose followed with an antibody response to NS5 that remained persistently detectable together with HCV RNA and antibodies to core, NS3, and NS4.

Individual 1123 had high levels of HCV RNA and raised antibodies to all 4 proteins within 4 months after being infected with HCV genotype 3a, and all remained persistently detectable.

Individual 3059, infected with HCV genotype 3a, had an initially antibody response to NS3 and NS4, but no antibodies to NS5 were detected during follow-up. Antibodies to NS4 dropped immediately after 4 months, whereas antibodies to NS3 remained present during follow-up. Antibodies to core were not detectable until 15 months after HCV seroconversion but thereafter remained detectable during follow-up.

Individual 7030, who was infected with HCV genotype 3a, revealed high levels of HCV RNA and antibody responses to core, NS3, and NS5, but antibodies to NS4 were not raised at all during follow-up. His HCV RNA level decreased between approximately 48 and 52 months after HCV seroconversion whereas levels of antibodies to NS3 increased, accompanied with an apparent HCV reinfection with genotype 1.

Individual 7041, who was initially infected with HCV genotype 3a, initially raised low levels of antibodies to NS3, NS4, and NS5. After approximately 13 months, these antibody responses rose and antibodies to core were found, apparently in response to HCV reinfection with genotype 1.

All 5 individuals remained HCV RNA positive regardless of the humoral immune response to core, NS3, NS4 and NS5, suggesting that humoral responses to the proteins tested or the lack of a humoral response to these HCV proteins does not influence viral persistence.
Figure 1a. Patterns of HCV viraemia and serological responses. Five individuals with persistent viraemia. HCV viraemia was determined by RT-PCR and bDNA. PCR results are shown as plus or minus, whereas bDNA values are indicated by the bold line, expressed as HCV RNA copies/ml. The dotted line represents the detection limit of the bDNA assay. The open bar indicates EIA 3.0 results and quantitative antibody levels to core (circle), NS3 (star), NS4 (diamond), and NS5 (triangle) are indicated and expressed as relative intensities.
Relationship between antibody responses to core, NS3, NS4, and NS5 and decline of HCV RNA levels in blood to undetectable levels

Eight individuals showed transient HCV viraemia or reduction of viral load in blood to undetectable levels: 0073, 0146, 1085, 1102, 1213, 3006, 3009, and 7095 (Fig. 1b). Systemic viral clearance in blood, or resolution of HCV infection, was assumed if 3 or more consecutive samples were HCV RNA negative, a finding in 5 (5 of 13 or 38.5 %) individuals: 0073, 0146, 1085, 3009, and 7095 (Fig 1b). Apparent resolution of HCV infection was not related to HCV genotype.

Individual 0073, who was infected with HCV genotype 1, showed transient HCV viraemia with low HCV RNA levels throughout follow-up, whereas high antibody responses to all 4 antigens was observed. Apparent reinfection with HCV genotype 1 occurred after 9 months and HCV RNA was detectable by PCR in the following 4 samples. Thereafter, HCV RNA was undetectable in serial samples for about 2.5 years. Subsequently, an apparently new infection occurred, again with HCV genotype 1. This was accompanied by HIV infection as indicated by HIV seroconversion between the last HCV RNA-negative sample and the next HCV RNA-positive sample.

Individual 0146, who was infected with HCV genotype 4, without measurable HCV RNA levels by bDNA throughout follow-up, showed antibody responses to core, NS3, and NS4 at low levels. After seroconversion, HCV genotype 3a was found transiently in the next 5 samples, taken over about 20 months. After this period, HCV RNA was undetectable by PCR in any sample for the next 4.5 years of follow-up, and only antibodies to core remained detectable.

Individual 1085, infected with HCV genotype 2, without measurable HCV RNA levels by bDNA throughout follow-up, seroconverted and antibody responses to core and NS4 were measurable at low levels. HCV genotype 1 was found in the next sample after seroconversion and apparent clearance of the virus was observed for the next 13 months of study period.

Individual 3009, infected with HCV genotype 3a, had antibodies to core and NS3 at low levels that dropped gradually. Apparent viral clearance was observed, and HCV reinfection occurred with genotype 1. HCV RNA was then detectable for 4 months but thereafter, HCV RNA was undetectable for 17 months. Seroreversion occurred, and antibodies to HCV disappeared for approximately 40 months. In this antibody-undetectable period, a reinfection was detected by PCR and bDNA, but no HCV antibody response was seen. A second HCV seroconversion occurred due to either reactivation or reinfection with HCV genotype 1, leading to antibody responses to core, NS3, and NS4.

Individual 7095, who had a double infection with HCV genotypes 1 and 3a, raised antibodies to NS3 and NS4 and apparently cleared both viruses within 10 months. Reinfection with HCV genotype 1 (or its reactivation) occurred after 15 months, and HCV RNA was detectable by PCR in 1 sample and was undetectable for the next 18 months in serial samples. Thereafter, reinfection or reactivation of HCV genotype 1 occurred, antibodies to core and NS5 were raised and both HCV RNA load and antibodies to NS3 and NS4 increased.
Figure 1b. Patterns of HCV viraemia and serological responses. Eight individuals with transient and apparent viraemia. HCV viraemia was determined by RT-PCR and bDNA. PCR results are shown as plus or minus, whereas bDNA values are indicated by the bold line, expressed as HCV RNA copies/ml. The dotted line represents the detection limit of the bDNA assay. The open bar indicates EIA 3.0 results and quantitative antibody levels to core (circle), NS3 (star), NS4 (diamond), and NS5 (triangle) are indicated and expressed as relative intensities.
Relationship between HCV-specific antibody responses and HCV genotypes

Among the 13 HCV seroconverters, no relation was found between HCV genotype and the initial presence or absence of antibody response to particular HCV genotypes. Considering antibody responses to HCV to be genotype-specific and acknowledging that the antigens used in the Chiron RIBA™ HCV titering Strip Immunoblot Assay are derived from HCV genotype 1, antibody responses were compared in samples of individuals infected solely with HCV genotype 1 versus samples of individuals infected with other HCV genotypes.

In the 13 HCV seropositive individuals, 4 individuals were infected with genotype 1 (0073, 1102, 1114, and 3006), whereas 8 individuals were infected with other single genotypes (0146, 1085, 1123, 1213, 3009, 3059, 7030, and 7041) and 1 individual (7095) had a double infection with genotype 1 and 3a. For statistical analyses, we calculated the median antibody responses to all 4 HCV proteins in samples with known HCV genotype of every HCV-infected individual. All median antibody titers to core, NS3, NS4, and NS5 were higher in individuals infected with HCV genotype 1 as compared to median antibody titers found in individuals infected with other HCV genotypes, but only median HCV antibody responses to core ($P=0.02$) and NS4 ($P=0.04$) in individuals infected with HCV genotype 1 were significantly higher as compared to individuals infected with other HCV genotypes (Fig. 2).

Figure 2. Scattergram of antibody responses to core, NS3, NS4, and NS5 in individuals initially infected with HCV genotype 1 in comparison to individuals infected with other HCV genotypes. Median values are indicated by short horizontal bars. Statistical analysis was done using the Mann-Whitney test for comparison of groups. A value of $P<0.05$ was considered to be significant.
This suggests more variation in immunogenic core and NS4 B-cell epitopes relative to NS3 and NS5. To confirm that immunodominant epitopes, previously published by others (27-34), were indeed type-specific in all 4 proteins used in the Chiron RIBA™ HCV titering Strip Immunoblot Assay, we performed amino acid alignments of all 4 proteins for different HCV genotypes. The short synthetic peptides of the NS4 (Fig. 3a) and core (Fig. 3b) region used in the assay show, especially in NS4, significant epitope variability between different genotypes. Conversely, NS3 (Fig. 3b) and NS5 (Fig. 3c), which show high sequence diversity among HCV subtypes and HCV genotypes, displayed a higher degree of cross-reactivity than both core and NS4 between heterologous genotypes. This finding may reflect the presence of recombinant proteins of NS3 and NS5 in the RIBA™ assay, which cover approximately the whole region of both NS3 and NS5 containing multiple epitopes.

Figure 3a. Comparison of amino acids of HCV genotypes 1 to 3 in NS4. Identical residues are indicated by -. The proteins used in the RIBA™ assay are indicated by the box. The immunogenic epitopes and regions of published sequences are indicated.
Figure 3b. Comparison of amino acids of HCV genotypes 1 to 3 in core and NS3. Identical residues are indicated by -. The proteins used in the RIBA™ assay are indicated by the box. The immunogenic epitopes and regions of published sequences are indicated.
Figure 3c1: Comparison of amino acids of HCV genotypes 1 to 3 in NS5. Identical residues are indicated by -. The proteins used in the RIBA™ assay are indicated by the box. The immunogenic epitopes and regions of published sequences are indicated.
Figure 3c-2. Comparison of amino acids of HCV genotypes 1 to 3 in NS5. Identical residues are indicated by -. The proteins used in the RIBA™ assay are indicated by the box. The immunogenic epitopes and regions of published sequences are indicated.
Statistical analysis of HCV levels and antibody profiles in individuals with persistent viremia versus individuals with apparent resolution of HCV RNA in blood

Samples with known HCV load, known HCV genotype, and known antibody responses to core, NS3, NS4, and NS5 were analyzed and used to compare individuals with persistent viremia (n=5) and individuals with apparent resolution of HCV RNA in blood (n=5). To avoid the genotype-specificity bias of the Chiron RIBA™ HCV titering Strip Immunoblot Assay, we compared groups of individuals infected with the same HCV genotype. In the group infected with genotype 1, the subgroup with persistent viremia had significantly higher median HCV loads (2.7 x 10^6 HCV RNA/ml vs <0.2 x 10^5 HCV RNA/ml; P<0.0001). In the group with apparent resolution of HCV RNA in blood and infected with HCV genotype 1, median antibody titers of NS3 were significantly decreased as compared to the group with persistent viremia (0.38 RI vs 2.61 RI; P=0.003), whereas median antibody titers to core, NS4, and NS5 were not statistically different between the 2 groups (Fig.4a).

In the group with apparent HCV RNA resolution infected with genotypes other than HCV genotype 1, median antibody titers of NS3 (0.03 RI vs 0.89 RI; P=0.0004) and median anti-

Figure 4a. Scattergram of antibody responses to core, NS3, NS4, and NS5 in individuals infected with HCV genotype 1 with persistent viremia in comparison to individuals infected with HCV genotype 1 with apparent viral clearance from blood. Median values are indicated by short horizontal bars. The dotted line represents the detection limit of the bDNA assay. Statistical analysis was done using the Mann-Whitney test for comparison of groups. A value of P<0.05 was considered to be significant.
body titers to NS5 (0.01 RI vs 1.86 RI; P=0.006) were significantly lower as compared to the group with persistent viraemia, whereas median antibody titers to core and NS4 were not statistically different between the 2 groups (Fig. 4b).

**Other genotypes**

![Figure 4b](image)

Figure 4b: Scattergram of antibody responses to core, NS3, NS4, and NS5 in individuals infected with HCV genotypes other than 1 with persistent viraemia in comparison to individuals infected with HCV genotype 1 with apparent viral clearance from blood. Median values are indicated by short horizontal bars. The dotted line represents the detection limit of the bDNA assay. Statistical analysis was done using the Mann-Whitney test for comparison of groups. A value of P<0.05 was considered to be significant.
Although less stronger, these differences remained significant when individuals with only 1 HCV RNA negative time-point (1102, 1213, and 3006) were included in the subgroup with apparent HCV resolution. Median HCV loads, were regardless of HCV genotype, significantly higher in the groups with persistent viraemia as compared to the group with apparent viral clearance from blood ($P<0.0001$), and significantly higher median NS3 antibody titers ($2.61\ RI$ vs $0.55\ RI; P=0.02$) than the subgroup with apparent resolution of HCV RNA in blood. Median antibody titers to core, NS4, and NS5 were not statistically different between the 2 subgroups (Fig. 5a).

**Figure 5a.** Scattergram of antibody responses to core, NS3, NS4, and NS5 in individuals infected with HCV genotype 1 with persistent viraemia in comparison to individuals infected with HCV genotype 1 with transient or apparent viral clearance from blood (including 1102, 1213, 3006). Median values are indicated by short horizontal bars. The dotted line represents the detection limit of the bDNA assay. Statistical analysis was done using the Mann-Whitney test for comparison of groups. A value of $P<0.05$ was considered to be significant.
In groups infected with genotypes other than genotype 1, significantly higher median HCV loads (6.4 x 10^6 HCV RNA/ml vs <0.2 x 10^5 HCV RNA/ml; P<0.0001), significantly higher median NS3 antibody titers (0.89 RI vs 0.03 RI; P=0.0015), and significantly higher median NS5 antibody titers (1.86 RI vs 0.01 RI; P=0.01) were found in the individuals with persistent viraemia than in those with apparent resolution of HCV RNA in blood. Median antibody titers to core and NS4 were not statistically different between the 2 subgroups (Fig. 5b).

**Other genotypes**

(including 1102, 1213, 3006)

![Figure 5b. Scattergram of antibody responses to core, NS3, NS4, and NS5 in individuals infected with HCV genotypes other than 1 with persistent viraemia in comparison to individuals infected with HCV genotypes other than 1 with transient or apparent viral clearance from blood (including 1102, 1213, 3006). Median values are indicated by short horizontal bars. The dotted line represents the detection limit of the bDNA assay. Statistical analysis was done using the Mann-Whitney test for comparison of groups. A value of P<0.05 was considered to be significant.]

**Discussion**

Acute HCV is heralded by detectable HCV RNA in serum, usually followed by an antibody response to HCV after a certain incubation period. Occasionally acute HCV is followed by the disappearance of HCV RNA leading to a resolved infection. Despite detectable antibody responses to various proteins, apparently the majority of HCV infected individuals develop chronic HCV infection. The factors that lead to chronic HCV infection with lacking protective properties of antibody responses are not well defined, but the quasispecies nature of HCV
probably plays an important role in escaping the immune system (22,35-39). Most studies have attempted to correlate antibody responses with the presence of HCV RNA and a particular HCV genotype, using antibody assays at single time-points without any quantitative data (9-16). Quantitative data of HCV-specific antibody responses were determined on serial samples of 13 HCV seroconverters. To better understand the various clinical profiles that emerged, the quantitative antibody responses were related to HCV genotypes and viraemia.

In all 13 individuals, there was an antibody response to at least two proteins. The highest antibody responses were raised to NS4, whereas responses to NS5 were generally the lowest and the most likely to be missing. This may confirm that NS4 is the most immunogenic protein (40). The analysis of antibody responses with respect to HCV genotypes revealed a difference between genotype 1 and other genotypes. The median antibody responses to all four proteins showed a higher trend in individuals infected with HCV genotype 1 than in individuals infected with other genotypes. Moreover, median antibody responses to core and NS4 were significantly higher in individuals infected with HCV genotype 1. Since it is well known that RIBA™ assays are based on HCV genotype 1, these differences in antibody responses could be an artifact of the assay. Several studies show, that serotyping is possible using immunodominant epitopes of core and NS4. Core serotyping was based on the following regions: amino acid (aa) 1-140 (30), both aa 39-74 and aa 65-81 (28), and spanning aa 67-81 (41). For NS4, serotyping was based on 2 major antigenic regions that were highly variable between HCV genotypes: aa 1691-1728 and 1710-1728 (27). Our alignments of amino acids in these regions, as used in the RIBA™ assay, of both core and NS4 revealed genotype-specific sequences, especially in the 5-1-1p peptide. Although the core region of HCV is the most conserved region used in the RIBA™ assay, we observed that even antibody titers to core were influenced by the HCV genotype-specificity of the assay. Conversely, NS3 and NS5, which show high sequence diversity among HCV subtypes and HCV genotypes, displayed a higher degree of cross-reactivity than both core and NS4 between heterologous genotypes. This finding may reflect the presence of recombinant proteins of NS3 and NS5 in the RIBA™ assay, which cover approximately the whole region of both NS3 and NS5 containing multiple epitopes. Hwang et al. (42) found different linear immunoreactive regions within the middle portion of the NS3 region, spanning aa 1251-1498, whereas Khudyakov (31) found 22 immunoreactive peptides (70% of all immunoreactive NS5 peptides) at the carboxyl terminus of NS5, thus recognition of shared epitopes in NS3 and NS5 may be evident. We and others have proposed that the RIBA™ assays probably are suboptimal for screening of populations infected with HCV genotypes other than genotype 1 (41,43-45).

Viral persistence was found independent of HCV genotype and particular humoral immune responses, suggesting that antibodies to core, NS3, NS4, and NS5 have no protective properties. The algorithm for virological remission from blood was defined if 3 or more consecutive samples were HCV RNA-negative as measured by RT-PCR, our finding in an unexpectedly high number of 5 individuals (38.5%). However, levels below the sensitivity of the RT-PCR may exist, and the virus may persist in these individuals at low levels and definitive proof of viral clearance from blood, still has to be addressed by more sensitive methods. Moreover, if viral clearance from blood actually exists, does this also reflects the situation in the liver which is considered to be the main HCV reservoir?

However, transient HCV RNA levels or apparent virological remission observed in these individuals, was correlated with a significant reduction of NS3 antibody titers, independent of HCV genotype, as compared to individuals with persistent viraemia. Moreover, in these individuals infected with HCV genotypes other than genotype 1, the reduction of NS3 antibody titers was even more significant and accompanied with a significant reduction of NS5 antibody titers. With one exception (0073), all of our subjects with resolving HCV infection had unde-
detectable antibodies to NS5 throughout follow-up, whereas antibodies to NS3 were undetectable in 1 individual (1085) and decreased significantly in the other 3 individuals (0146, 3009, 7095). These findings suggest that NS3 and NS5 antibody titers may be a marker for chronicity, as recently was found in a study with experimentally infected chimpanzees (46). The complete disappearance of antibodies to HCV, or seroreversion observed in individual 3009, was earlier described in immunocompetent patients without antiviral-therapy and was accompanied by virological and clinical resolution (20,47-49).

In 6 individuals (0073, 1102, 1213, 3006, 3009, 7095), HCV infection was transient. These subjects were either reinfected with HCV of the same or another genotype, or possibly the initial HCV was reactivated. Recurrence of HCV infection in chimpanzees (21) and in polytransfused thalassaemic children (20) has been shown to result from reinfection and not to reactivation of the initial HCV infection. However, among our subjects in whom the same HCV genotype was found after apparent clearance of the initial HCV infection, a reinfection can not be ruled out because genotyping was based on the highly conserved 5'-UTR, using Innolipa or direct sequencing without cloning. Injecting drug users (17) and also haemophiliacs (18), haemodialysed patients (19), and polytransfused thalassaemics (20) are known to be at high risk for exposure to HCV through reinfections, with multiple distinct episodes of acute HCV. Chimpanzees have been super infected with homologous and heterologous HCV strains having intermittent and low replication rates (50), and HCV is known to circulate as a quasispecies both in vitro and in vivo (51,52). Therefore, an individual infected with HCV may not harbour a single virus strain but a mixture of related strains. Such a mixture could lead to the rapid emergence of viruses that can escape the immune system. The finding of transient HCV RNA of different genotypes may not indicate reinfection but the outgrowth of a strain may have occurred.

In summary, this study shows that antibody responses to core and NS4 are influenced by the antigenic variation of HCV. In our subjects, the antibody responses to core, NS3, NS4, and NS5 were erratic and not protective. In general, individuals with viral persistence had higher HCV RNA loads with higher overall antibody responses then individuals with apparent viral clearance. Apparent viral clearance was observed in an unexpected high percentage, associated with a significant decrease of NS3 and NS5 antibody titers as compared to individuals with persistent viraemia. Of this group, apparent viral clearance with gradual loss of antibodies to the various HCV proteins occurred in 4 individuals within approximately 1 year after HCV seroconversion, whereas 1 of these individuals apparently cleared the virus from blood with complete seroreversion. These findings suggest that NS3 and NS5 antibody titers may be a marker for chronicity and an alternative for monitoring efficacy of HCV therapy.

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