Hepatitis C virus genotype in anti-HCV-positive hemodialysed patients

N. Colleoni¹, R. Bucci¹, M. L. Ribero², J. Zhou¹, G. D’Amico¹ and A. Tagger²

¹Department of Nephrology and Dialysis, Ospedale San Carlo Borromeo, Milan, and ²Institute of Virology, University of Milan, Italy

Abstract We investigated the influence of hepatitis C virus (HCV) genotypes on the clinical course of HCV infection in a haemodialysis population. In June 1991, a 4 year prospective follow-up programme was implemented in 184 consecutive haemodialysis patients. Alanine aminotransferase (ALT) and gamma glutamline transferase (GGT) were performed every 2 months. When HCV antibody (Ab) (by second-generation ELISA) was positive, it was confirmed by RIBA 2 and HCV RNA amplification by PCR. The pattern of nucleotide sequence variability in the 5' non-coding region was categorized according to Simmonds’ genotype classification. Risk factors including blood transfusions were evaluated. The levels of hepatic enzymes in HCV Ab-positive patients were retrospectively studied over a mean period of 11.8 years. ALT and GGT levels were assigned a score for every year of infection (0 = normal, 1 = fluctuating 2 = high levels). Fifty-two patients were HCV Ab reactive (30.4%), eight were RIBA undetermined and 44 were RIBA positive; 40 of these were HCV RNA positive (91%). Twelve patients were HCV RNA negative, suggesting that they had recovered from the infection. Four genotypes were identified: lb [26 patients (65%)], la (one patient), 2 [12 patients (30%)] and 3 (one patient). The genotype distribution was not different from that found in patients with chronic hepatitis C and normal renal function of the same geographical area. Genotype lb accounted for 75% of the cases before 1985 and an equal prevalence of the two major genotypes was observed after 1985. Patients infected with HCV subtype 1 had normal mean ALT levels, but higher levels in the follow-up period (28±15.6 IU/l) and higher ALT and GGT personal scores in the retrospective study. Genotype 1 patients had higher mean ALT levels after 6 months. HCV RNA-negative patients had lower ALT levels after 24 months. RIBA pattern could differentiate the patients. Patients with genotype 1 received a higher number of transfusions, while only 50% of HCV RNA-negative patients had been transfused. Our data suggest a worse course of HCV infection in haemodialysis patients infected with HCV subtype 1, but the severity of HCV infection can only be assessed by histology. Transaminases are only loosely correlated with severity.

Key words: ALT levels; HCV genotypes; haemodialysis

Introduction

Several studies have shown that haemodialysis patients are a high risk group for hepatitis C virus (HCV) infection [1]. Furthermore, there is clear evidence that the time on haemodialysis [2-5], the number of transfusions given [1,6,7] and anti-HCV positivity. At least five genotypes of HCV have been recognized which differ in more than 21% of about 9400 nucleotides and are designated as genotypes I, II, III, IV and V [8], corresponding to subtypes 1a, 1b, 2a, 2b and 3a [9]. These different genotypes appear to have a distinct epidemiological distribution that might be associated with differences in the severity and clinical course of HCV disease [10]. Recent evidence suggests that the genotype of HCV may even influence the response to interferon therapy [11,12].

To investigate the influence of genotype on the natural history of HCV infection in haemodialysis patients, a 4 year prospective follow-up study was implemented in our haemodialysis population. After identification of different HCV subtypes, we retrospectively evaluated the clinical and epidemiological aspects of the subgroups infected by different genotypes and of haemodialysis patients who recovered from the disease in our dialysis unit.

Subjects and methods

Patients

For the first part of the study, carried out between June 1991 and June 1995, 184 consecutive haemodialysis patients, who had been treated in our dialysis department for at least one whole year (range 1–25 years), were entered into the study.
Methods

The follow-up programme included measurements of serum alanine aminotransferase (ALT), alkaline phosphatase, aspartate aminotransferase and gamma glutamyl transferase (GGT) every 2 months (every month in patients with HCV infection). HBS antigen, anti-HBS, and anti-HBC antibodies were detected twice a year using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions.

Second generation anti-HCV enzyme immunoassays (ELISA 2, Ortho Diagnostic Sistems) were performed every 6 months. A recombinant immunoblot assay (RIBA 2, Chiron Corporation) was repeatedly used for samples positive by ELISA. The RIBA 2 assay uses four HCV antigens, comprising the c22-3 core protein and the three non-structural proteins c33e, c100-3 and 5-1-1. Samples reactive to at least two HCV antigen bands were considered RIBA positive, and samples reactive to one band were considered indeterminate. In the absence of reactivity they were considered negative. Sera from anti-HCV ELISA-reactive patients were analysed for HCV RNA on fresh samples, which were immediately separated from the clot using recommended procedures to avoid contamination. The same sera were analysed, when HCV RNA was positive, for the characterization of HCV genotypes, according to Simmonds’ classification [9-13]. HCV RNA sequences were detected by amplification of cDNA with PCR using outer (nt 1-38: GGCACACTCCACCATTAGATC, and nt 341-321: GGTGCACTGTCTACAGAGCCT) and inner (nt 45-65: CGTGTAAGGAACATCCTGTC, and nt 301-281: CCCTATTACGGCAGTCACCACCA) primers from the 5’-non-coding region of HCV, yielding products of 32 and 25 bp, respectively [13].


HCV genotypes were identified by nested PCR amplification with type-specific primers from the HCV core region [8-14]. cDNA primed with no. 186 (nt 751-732: ATGTAGCCCATGAGGTCCG) was amplified by the first PCR with universal primers no. 23 (nt 467-486: TAGTTGGGTGTGCGCGCA) and no. 186 to obtain a fragment of 285 bp. The products were then amplified by a second PCR with: (i) a subtype-specific primer 1aF (nt 507-527: TCATGCAACTCTGGAGGTAGA) and the universal primer CH 35 (nt 724-705: CACGTAA-GGTTATCGATGAC) yielding a product of 218 bp, specific for HCV subtype 1a; (ii) two subtype-specific primers (CH 74, nt 511-530: AACCTCGTGGAAGGCGACAA and no. 133, nt 632-613: GACGCTATCCTGCGCCACCC) yielding a product of 122 bp, specific for subtype 1b; (iii) the type-specific primer 2abF (nt 555-573: ACCGGCA-AGTCTCTGGGAA) and the universal primer CH 35, yielding a product of 170 bp, specific for type 2; (iv) the subtype-specific primer VF (nt 539-561: CAA-AGGCCGTCGAGCAGGGACC) and the universal primer CH 35, yielding a product of 186 bp, specific for subtype 3a; (v) two universal primers (no. 104, nt 489-508: AGGAAAGACTTCCGAGGCGTC and CH 35) to detect HCV types undetectable by the above-mentioned type-specific primers. The position of the primers are according to the numbering of the nucleotide sequence of HCV-1 [15].

The first PCR was performed for 35 cycles; each reaction cycle included denaturation at 94°C for 1 min, primer annealing at 55°C for 1.5 min and primer extension at 72°C for 2 min. A 2.5-fold amount of the products was subjected to the second PCR for 30 cycles every 2 cycles with: (i) for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1.5 min in each reaction cycle. The products of the second PCR were electrophoresed on a composite agarose gel with 2.5% NuSieve and 0.5% Seakem stained with ethidium bromide and observed under UV light.

Epidemiological studies

A retrospective study was performed with all the patients who were only dialyzed in our unit. Risk factors for the spread of HCV infection among haemodialysis patients were analyzed. In our hospital a screening test to avoid HCV contamination of blood was initiated in 1990. We excluded patients with a history of other causes of chronic hepatitis, including alcoholic liver disease, haemochromatosis, Wilson’s disease, hepatotoxic drug exposure or autoimmune hepatitis.

The number of blood transfusions at risk for the transmission of HCV (administered before 1990) were determined. We included data from 120 out of 132 HCV antibody negative patients, excluding five patients for the above-mentioned criteria and seven others because it was not possible to evaluate the risk factors. Biochemical evidence of HCV infection, indicated by ALT elevation of at least twice the normal level, was recorded and correlated to transfusion of blood products, which were administered during the period 2-26 weeks before. Events in which nosocomial transmission was possible, such as surgical procedures and particularly renal transplantation, were noted. Since 1970 ALT and GGT were determined at least every 2 months (every month for patients with hepatitis). We used the records to assign an arbitrary yearly score of ALT and GGT levels as follows: 0 = all values obtained during the year within normal range; 1 = fluctuating levels (10-50% of the total number of values above the normal range); 2 = persistently elevated levels (more than 50% of the values above the normal range). The score was calculated for every year for which biochemical evidence of HCV infection existed. The sum of the yearly scores was related to the duration of hepatitis (years), to obtain both a yearly score and a personal total score for every HCV-positive haemodialysed patient.

Statistical analysis

We used the Systat 5 program with ANOVA, Student’s t, K square and Fisher’s exact probability tests and non-parametric tests as appropriate. All P values are two-tailed with statistical significance at 0.05.

Results

In our haemodialysis population, 52 of 184 HBS antigen negative patients had antibodies against HCV, detectable in the second-generation ELISA assay (prevalence of 30.2%). Forty-four patients were confirmed by RIBA test, while eight had undetermined results. Among RIBA-confirmed patients, 40 (90.9%) were also HCV RNA positive; none of the RIBA-undetermined patients was HCV RNA positive. The cumulative incidence of biochemical evidence of HCV infection in our haemodialysis population was 4.2%. The yearly incidence of HCV seroconversion in 1992 was 0.86% and in 1993 was 0.82% (one case per year); we had no new cases in 1994 and in the first half of 1995.

HCV RNA after PCR amplification was positive in 40 of the 52 patients (77%), while in 12 patients (23%)
HCV RNA was negative, suggesting they had recovered from HCV infection. Four distinct genotypes were identified in HCV RNA-positive patients (Simmonds' classification). Genotype 1b accounted for 65% of all the infections (26 patients), genotype 2 accounted for another 30% (12 patients) and subtypes 1a and 3a were detected in one patient each (2.5%). All patients appeared to be infected with a single virus genotype. Patients with genotypes 1a and 1b were analysed together.

As evident in Table 1, patients with genotype 1 had mean ALT levels in the normal range, but higher than in all other patients. Mean GGT levels were also higher, but did not reach statistical significance. There was no correlation between ALT and GGT these enzyme levels were not correlated to the time on haemodialysis or to the age of the patients. Time on haemodialysis was longer in patients who acquired HCV infection, while age was not different among the patients. There was a male prevalence in genotype 1 patients (67%) and a female prevalence in genotype 2 patients (58%), but without statistical significance. We did not find gender-related differences in ALT and GGT levels.

As is evident from Table 2, most of the haemodialysis HCV ELISA 2-reactive patients had antibodies to the HCV core region c22-3, while antibodies to the non-structural regions 5-1-1 and c100-3 were more frequently found in genotype 1 patients. Antibodies to the non-structural region c33-c were less frequent in HCV RNA-negative patients.

The natural history of hepatic enzyme levels in our haemodialysis patients with HCV infection is shown in Figs 1-4. The yearly ALT and GGT scores for each patient infected with genotype 1 or 2 since the diagnosis of hepatitis C virus infection are presented.

Table 1. Mean (SD) of liver enzymes, age and time on haemodialysis for patients with different HCV genotypes, in HCV RNA-negative patients and in anti-HCV ELISA-negative patients (June 1991–June 1995)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of patients</th>
<th>ALT (IU/l)</th>
<th>GGT (IU/l)</th>
<th>Age (years)</th>
<th>Time on HD (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype 1</td>
<td>27</td>
<td>28.0 (15.6)*</td>
<td>66.8 (70.4)</td>
<td>51.4 (11)</td>
<td>14.7 (7.7)</td>
</tr>
<tr>
<td>Genotype 2</td>
<td>12</td>
<td>15.7 (6.1)</td>
<td>39.2 (43.9)</td>
<td>57.3 (11)</td>
<td>12.3 (6.8)</td>
</tr>
<tr>
<td>HCV-RNA neg</td>
<td>12</td>
<td>12.5 (5.2)</td>
<td>33.4 (22)</td>
<td>58.4 (12)</td>
<td>12.4 (6)</td>
</tr>
<tr>
<td>Anti-HCV-neg.</td>
<td>120</td>
<td>16.2 (16.8)</td>
<td>40.2 (45.4)</td>
<td>57.2 (14)</td>
<td>8.5 (7.3)*</td>
</tr>
</tbody>
</table>

*ANOVA, P<0.05.

In HCV RNA-positive patients, the mean time of the course of the hepatitis was 11.8 years. The mean interval between the initial dialysis and the development of hepatitis was 25 months (range 1–150). Patients with genotype 1 had higher ALT and GGT personal scores, while the mean scores of these enzymes were not different between genotype 2 and HCV RNA-negative patients (Table 3).

Two-thirds of the patients (29/39) acquired the infection before 1985. There were chronological differences in the epidemiology of the two genotypes. Genotype 1 accounted for 75% (22/29) of the infections before 1985; after this date, we observed an equal prevalence between the two genotypes (five with genotype 1 and five with genotype 2).

The pattern of ALT levels in the first year of the disease was mild (ALT score = 1) in 26% of patients with genotype 1 versus 60% of patients with genotype 2 and 33% of HCV RNA-negative patients. The mean maximum ALT levels were not different among the groups of patients (Table 3).

As shown in Fig. 5, 6 months after the beginning of the infection, patients with genotype 1 had significantly higher mean ALT levels than the other two groups (Student's t test, P<0.05). After 2 years, mean ALT levels of HCV RNA-negative patients returned to normal values, significantly different from the other two groups (Student's t test, P<0.05).

Combination of the ALT and GGT personal scores resulted in the identification of three distinct patterns. Patients with low ALT and GGT personal scores (below 0.1) included nine patients with genotype 1 (33%) and nine patients with genotype 2 (75%). The second pattern included patients with normal–high ALT personal score (0.1–0.4) and high GGT personal scores (above 0.4). This group included seven patients with genotype 1 (26%) and two patients with genotype 2 (16%). The third pattern included patients with high ALT and GGT personal scores (above 0.4). In this group we found 11 patients with genotype 1 (41%) and one patient with genotype 2 (9%). All but one patient with negative HCV RNA showed normal ALT and GGT personal scores.

The mean time during which liver enzymes were elevated was not different between the patients with genotypes 1 and 2 (Table 3). HCV RNA-negative patients had a shorter course of the disease, although they took longer than usual to recover from HCV infection (Table 3). Surgical procedures were per-
HCV genotypes in haemodialysis patients

Fig. 1. Yearly ALT scores in haemodialysis patients with genotype 1 HCV infection, since the first appearance of elevated liver enzymes.

Fig. 2. Yearly ALT scores in haemodialysis patients with genotype 2 HCV infection, since the first appearance of elevated liver enzymes.

formed before the onset of hepatitis in 14% of genotype 1 patients, in 10% of genotype 2 patients and in 8% of HCV RNA-negative patients.

Most of our haemodialysed HCV ELISA-reactive patients (70%) received blood transfusions. The number of blood transfusions at risk (performed before the HCV screening) was higher in patients who acquired the infection, and among these patients it was higher in genotype 1 patients (Table 4). A significantly lower number of HCV RNA-negative patients received blood transfusions. Twelve of our patients had never been transfused; two with genotype 1 (7%), four with
Fig. 3. Yearly GGT scores in haemodialysis patients with genotype 1 HCV infection, since the first appearance of elevated liver enzymes.

Fig. 4. Yearly GGT scores in haemodialysis patients with genotype 2 HCV infection, since the first appearance of elevated liver enzymes.

genotype 2 (33%) and six negative for HCV RNA (50%) (K square, P<0.05). Two of these patients underwent surgical procedures and for the remaining 10 patients, we could not identify clustering of cross-contamination in our dialysis unit.

Discussion

The prevalence of HCV infection in our haemodialysis patients is in accordance with that reported by other dialysis units [1,15,16]. Genotype 1b is the prevalent
Table 3. Mean (SD) of clinical and serological expression of HCV infection in haemodialysis patients. Infection = years of biochemical evidence of HCV infection

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Infection (years)</th>
<th>Personal total score</th>
<th>Maximum ALT levels (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ALT score</td>
<td>GGT score</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.77 (0.6)†</td>
<td>0.92 (0.7)†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.38 (0.46)</td>
<td>0.31 (0.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.57 (0.7)</td>
<td>0.39 (0.8)</td>
</tr>
</tbody>
</table>

*P < 0.05, Student t-test; †P < 0.05, ANOVA.

The identification of HCV genotypes may have important implications because an association between the type of genotype and the severity of the disease has been found. Simmonds et al. recently observed that 72% of patients infected with types 1a and 1b HCV had severe liver disease, while only 28% had mild to moderate disease without cirrhosis. In contrast, patients infected with other HCV genotypes showed approximately equal numbers of severe and mild disease [18]. Patients with type 1 infection seem to be less sensitive to interferon treatment than patients with type 2 or 3 [11,12,19]. Our data suggest that anti-HCV-reactive haemodialysis patients have mild ALT changes, although baseline levels are depressed in anti-HCV-negative patients, as already observed in dialysis patients [20]. After more than 10 years of biochemical evidence of hepatitis, patients with genotype 1 had higher ALT levels than patients with genotype 2 and HCV RNA-negative patients. ALT levels in genotype 1 patients were significantly higher after 6 months, although the mean maximum ALT levels were not different.

After prolonged observation, most of the patients with genotype 2 had normal ALT and GGT personal scores, while patients with genotype 1 had high GGT activity, with either normal or elevated ALT levels. As already observed, GGT activity is a reliable marker of long-term HCV infection [16]. RIBA 2 pattern is different, as antibodies to 5-1-1 and c-100-3 regions are more frequently positive in genotype 1 patients and to c 33-c more frequently negative in HCV RNA-negative patients. Our data show a changing pattern of HCV genotype prevalence over time, with genotype 1b accounting for 75% of cases before 1985 and an equal prevalence of the two genotypes after 1985.

In accordance with other reports, most of our patients derived their infection from transfusion with contaminated blood [15]. Patients with type 1 infection received a higher number of blood transfusions. Only 50% of our HCV RNA-negative patients had received blood transfusions, and had received a low number of them. This factor may influence the outcome of the disease, as these patients apparently cleared HCV infection, although in a longer than usual time. We have not performed liver biopsy in these patients and, therefore, we can only presume that they cleared HCV infection. However, these patients had a long follow-up after the acute phase with normal ALT and GGT levels, a particular RIBA 2 pattern and were repeatedly negative for HCV RNA by PCR. Some of these patients may have been infected by dialysis-related cross-contamination with low amounts of infected blood. The source of infection was not identified in 10 of our HCV Ab-reactive patients. In our dialysis unit we have never used dialysis machines with closed circulation of dialysate. Prophylactic measures, such as the use of disposable materials and efficacious disinfectants, have been always observed. Despite these basic measures, it is possible that dialysis-related cross-contamination accounts for sporadic cases, although we did not observe a cluster of HCV infection among our patients using the same dialysis machines. The distribution of genotypes in our haemodialysis population is similar to that observed in patients with type C chronic hepatitis without renal insufficiency in the same

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Table 4. Mean (SD) of the number of blood transfusions administered before 1990 to haemodialysis patients

<table>
<thead>
<tr>
<th>Category</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV Ab-positive patients (n = 51)</td>
<td>14.5 (30)†</td>
</tr>
<tr>
<td>HCV Ab-negative patients (n = 120)</td>
<td>3.3 (7.4)</td>
</tr>
<tr>
<td>HCV genotype 1 (n = 27)</td>
<td>23.4 (70.3)†</td>
</tr>
<tr>
<td>HCV genotype 2 (n = 12)</td>
<td>4.3 (8.5)</td>
</tr>
<tr>
<td>HCV RNA-neg. patients (n = 12)</td>
<td>3.2 (4.8)</td>
</tr>
</tbody>
</table>

*(N par test) Mann-Whitney U, P = 0.012; †(N par test) Kruskall–Wallis, P = 0.002.
geographical area [17], and this observation suggests that the risk factor related to dialysis therapy is not the prevailing one.

In conclusion, ALT and GGT levels together with RIBA pattern can help to identify patients with HCV genotypes 1 and 2 and those negative for HCV RNA. Genotype 1 was responsible for 70% of hepatitis C among our haemodialysed patients, most of them infected before 1985. These patients have higher ALT levels both in the acute phase and after more than 10 years of HCV infection. During the whole course of the disease the scored ALT and GGT levels were higher in genotype 1 patients, but the severity of HCV infection can only be assessed by histology. Transaminases are only loosely correlated with severity.

References


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