Detection of three types of hepatitis C virus in blood donors: investigation of type-specific differences in serologic reactivity and rate of alanine aminotransferase abnormalities


The serologic reactivity and epidemiology associated with different hepatitis C virus (HCV) variants were investigated in a cohort of 113 anti-HCV-positive donors. In Scotland, HCV type 1 accounted for one-half of all infections; 40 percent of subjects were infected with HCV type 3, and the remainder were infected with type 2. Reactivity with the NS-4-encoded antigens in the first-generation anti-c100 assay was absent in 68 percent of donors infected with types 2 and 3, as compared with 10 percent for those infected with type 1. Even when combined with surrogate marker testing, first-generation tests would have failed to detect 12 percent of HCV-infected blood donors. The age distribution, incidence of past infection with hepatitis B virus, and reported risk factors were similar in donors infected with types 1 and 3 (mean ages were 31.9 and 29.9; 18 and 17.5% were positive for antibody to hepatitis B core antigen; and 47 and 48% had past intravenous drug abuse). However, the distributions of alanine aminotransferase levels were significantly different in those infected with type 3 (abnormally raised in 83%) and those infected with type 1 (55%; p<0.05) or type 2 (60%; p<0.01) and those who were nonviremic (6%; p<0.0001). These data suggest that HCV type 1 is the most common HCV infection in blood donors and that infection with HCV type 3 may be associated with more severe liver disease, because of either recent infection or because of a greater inherent pathogenicity of type 3 variants. TRANSFUSION 1993;33:7-13.
equivalent subdivision is also apparent between type 2 sequences, corresponding to the K2a (type 2a) and K2b (type 2b) groups in the NS-5 region.6

In this study, we used restriction fragment length polymorphism (RFLP) analysis of viral sequences amplified in the 5'NCR to analyze the distribution of the three main types of HCV in Scottish blood donors. The extent to which sequence variability affects the recognition of HCV antigens has been assessed by analysis of serologic reactivity between individual peptides used in commercially available confirmatory assays for anti-HCV with sera from individuals infected with different HCV types. Finally, the recall of HCV-infected individuals has permitted a comparison of risk factors for infection, age distribution, and extent of liver function abnormality associated with each type.

Materials and Methods

Samples

Approximately 147,000 volunteer blood donor samples collected in Scotland and Northern Ireland were screened between September 1, 1991, and January 15, 1992, for antibody to HCV using a second-generation HCV enzyme immunoassay (ETA, Abbott GmbH, Wiesbaden-Delkenheim, Germany) or a second-generation HCV enzyme-linked immunosorbent assay (Ortho Diagnostic Systems, Raritan, NJ). Repeatedly reactive samples were tested by second-generation recombinant immunoassay (RIBA, Chiron Corporation, Emeryville, CA) for antibody to 2-1-1, c100-3, c33c, and c22-3 antigens and by line immunoassay (LIA, Innogenetics, Antwerp, Belgium) for antibody to NS-4, NS-5, and four different core oligopeptides. We carried out all antibody tests and interpreted them strictly in accordance with the manufacturers' instructions. Donations that were positive (significant reactivity with two or more HCV antigens [1+ to 4+] or indeterminate reactivity with one antigen only) in the RIBA were tested for viral RNA by polymerase chain reaction (PCR). We tested donor samples for antibody to hepatitis B core antigen (anti-HBc) by radioimmunoassay (Abbott). Donors yielding RIBA-confirmed antibody-positive and/or PCR-positive donations were recalled for medical investigation and counseling in Glasgow and Edinburgh (Scotland). A single alanine aminotransferase (ALT) determination was made in samples from the 90 recalled donors and from 100 randomly selected HCV-negative donors. The upper limit of the normal range for the testing laboratory was 55 units per L.

HCV typing

Predicted cleavage patterns produced by all common restriction enzymes of 59 blood donor and hemophiliac sequences, obtained in this and our previous study,15 as well as a number of commercial sequences obtained elsewhere5,6,17,18,19,20 were computed with standard sequence-analysis software.21 We labeled amplified DNA by supplementing the PCR buffer with 2 μCi of [32P]-dATP (Amersham International, Amersham, UK) and reducing the concentration of unlabelled nucleotide triphosphates to 8.25 μM (8.25 μmol/L). One μL of the product was digested with 1 unit of ScraI or 1 unit each of RsaI and HaeIII (all: Boehringer-Mannheim UK, Lewes, UK) in 50 μL of supplied restriction buffer for 3 hours at 37°C.

The cleaved product was heated to 65°C for 5 minutes and electrophoresed on a 12-percent polyacrylamide gel in 1× TBE (134 mM Tris, 68 mM boric acid, 2.5 mM EDTA, pH 8.0) at 50 V for 18 hours or 160 V for 6 hours. After fixation in changes of 5-percent acetic acid and 5-percent methanol (5 min each), the gel was dried and exposed to x-ray film for 3 days prior to development. We sized DNA fragments by comparison with migration distances of standard-size DNA markers (pBR322/HaeIII digest; Boehringer).
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![Image of a table with restriction patterns](image)

**Fig. 2.** A) Predicted combinations of RFLP patterns obtained from sequences with HaeIII/Rfl and ScrFI associated with sequences of HCV types 1 to 3. Patterns A and B for ScrFI (Fig. 1) are not differentiable. B) Observed RFLP patterns of HCV variants amplified from blood donor samples, showing inferred HCV type.

Type I sequences, produced restriction patterns a and b; type 2 sequences produced patterns c, d, and e, and all type 3 sequences produced patterns f and g (Fig. 2A).

ScrFI independently differentiates all known type 1 sequences (pattern A, B) from type 2 (C, D, E, and F) and type 3 (G, H, I). The two adjacent ScrFI sites present in type 3 and type 2 sequences are not resolved when the restriction digest is electrophoresed, so patterns A and B are, for practical purposes, equivalent and will subsequently be referred to as type 1.

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**Clinical correlations with HCV type**

Anti-HBc was detected in 19 percent of samples from HCV-infected subjects (100 PCR-positive subjects; 13 samples from individuals who were anti-HCV positive but PCR negative; Table 1). We found similar frequencies among subjects infected with different HCV types.

A total of 90 HCV-infected blood donors (38 infected with HCV type 1, 10 with type 2, 29 with type 3, and 13 who were confirmed antibody positive but were PCR negative and therefore were not typed) attended a donor follow-up clinic. As the ages of the donors were not distributed normally, they were compared by the nonparametric Wilcoxon rank-sum test. This showed a significant difference between those infected with HCV types 1 and 3 (combined mean age, 30.8) and those infected with HCV type 2 (mean age, 37.9; p < 0.05; Table 2; Fig. 4A). No significant difference was observed in the age distributions of those infected with type 1 or type 3 or those who were PCR negative (Fig. 4A).

ALT values from each of the four categories of HCV-infected individuals and a control group of 100 HCV antibody-negative donors are shown in Fig. 4B. The Wilcoxon rank-sum test was used to compare the distribution of values, and the x² test was used for contingency analysis of numbers of samples over the upper limit of the normal range (55 units/L; Table 2). Both statistical methods indicated significant differences in ALT values in PCR-positive donors (i.e., types 1-3) and PCR-negative and HCV-uninfected donors (all p values < 0.02). We observed no difference between the PCR-negative group and the controls. In both statistical tests, ALT levels were significantly higher (p < 0.05, < 0.025) in donors infected with HCV type 3 than in those infected with other types (type 3, 24/29 had abnormal ALT; type 1, 10/38; type 2, 6/13; Fig. 4B).

A total of 27 of the 90 donations had normal ALT values (< 55 units/L) and were anti-HBc negative (totals of 12, 3, 4,
Table 1. Comparison of anti-c100, anti-HBc*, and history of intravenous drug abuse in subjects infected with different HCV type.

<table>
<thead>
<tr>
<th>HCV type</th>
<th>Frequency (%)</th>
<th>Anti-c100 (%)</th>
<th>Anti-HBc (%)</th>
<th>IVDA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50/100 (50)</td>
<td>45/50 (90)</td>
<td>9/50 (18)</td>
<td>18/38 (47)</td>
</tr>
<tr>
<td>2</td>
<td>10/100 (10)</td>
<td>3/10 (30)</td>
<td>3/10 (30)</td>
<td>2/10 (20)</td>
</tr>
<tr>
<td>3</td>
<td>40/100 (40)</td>
<td>13/40 (33)</td>
<td>7/40 (18)</td>
<td>14/39 (43)</td>
</tr>
<tr>
<td>Total</td>
<td>60/100 (60)</td>
<td>61/100 (61)</td>
<td>20/100 (20)</td>
<td>34/77 (44)</td>
</tr>
</tbody>
</table>

PCA*: positive
PCR-negative
Total HCV-positive

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Discussion

Distribution of HCV types

The data presented in this article confirm our previous conclusion that all three major types of HCV are present in the British blood donor population. In Japan, type 1 appears more commonly than type 2 in blood donors and patients with non-A, non-B hepatitis, cirrhosis, an hepatocellular carcinoma.6,8,24,25 When it has been possible to differentiate type 1a sequences from type 1b, it has been reported that the majority of Japanese and Taiwanese patients are infected with type 1b, with the exceptions being hemophiliacs treated with imported (from the USA) factor VIII.6,8 However, while almost all blood donors in this and our previous study6 appear to be infected with type 1a, it has recently been reported that type 1b is more common in Holland26 and Belgium (Maertens G, verbal communication, December 1991). Type 1b also accounts for a proportion of HCV infections in France.27

HCV type 2 was originally found in Japanese and Taiwanese patients;6,8,24,25 its presence in Italy6,15 and the UK hints at a much more widespread distribution than anticipated by the original reports. Sequences of type 3 group have been found in the UK14,15 and in blood donors from India, Finland (McOmish F. and Simmons P., unpublished observations, 1992), and Brazil (Maertens G, verbal communication, February 1992). Two of the donors in this study appear to have been infected with HCV type 3 outside of the UK (Belgium, Italy). We have also detected type 3 sequences in patients with non-A, non-B posttransfusion hepatitis from numerious countries (Sweden, Italy, USA; unpublished data). Together, these data indicate that all three types have worldwide distribution, with varied frequencies in particular countries.

Serologic crossreactivity

The region of the HCV genome encoding to c100- and 5-1-1 is known to be extremely variable between HCV types 1 and 2,7 and within types 9,10,12,13 Thus, it is not surprising that serologic crossreactivity between type 1a antigens and antibody elicited by infection with type 2 and 3 is limited. In this study, sera from only 2 of the 51 patients infected with type 2 and 3 (4/11 and 17/40, respectively) reacted with NS-4-encoded antigen.
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Comparison of the distributions of ALT* values in donors infected with HCV types 1, 2, and 3 and in PCR-negative and HCV-uninfected donors using $x^2$ (upper right quadrant) and Wilcoxon Rank-Sum (lower left quadrant).

<table>
<thead>
<tr>
<th>HCV type</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>PCR-negative</th>
<th>HCV-uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.002 (NS)</td>
<td>5.63 (p &lt; 0.025)</td>
<td>8.04 (p &lt; 0.005)</td>
<td>42.46 (p &lt; 0.005)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1136 (p &lt; 0.05)</td>
<td>135 (p &lt; 0.02)</td>
<td>24.22 (p &lt; 0.005)</td>
<td>74.9 (p &lt; 0.005) $x^2$</td>
<td></td>
</tr>
<tr>
<td>PCR-negative</td>
<td>4010 (p &lt; 0.001)</td>
<td>865 (p &lt; 0.02)</td>
<td>3168 (p &lt; 0.001)</td>
<td>610 (NS)</td>
<td></td>
</tr>
</tbody>
</table>

ALT: alanine aminotransferase.

Polymerase chain reaction.

*Values for each pair-wise comparison of numbers of ALT values greater than 55 unit/mL (probability of obtaining the observed numbers by chance in parentheses).

Wilcoxon Rank-Sums of ALT values, W, for each pair-wise comparison.

*Values > 0.05 considered nonsignificant (NS).

Reactivity with cl00-3 was lower in recipients with type 2 in one study but similar to that in type 1-infected patients in another. It is possible that such “crossreactivity” is the result of multiple infection, as has been described for hemophilia patients.25 It could be hypothesized that antibody to type 1 antigens persists longer than the type 1 viremia upon reinfection with type 2 or 3. Multiple exposure is possible in intravenous drug abusers, as that practice is the predominant risk factor for infection identified in blood donors in this study (45%) and others.29 Reactivity to c33c in the RIBA (encoded by NS-3) and the NS-5 peptides (LIA) with sera from type 2- and 3-infected donors was more frequent than reactivity with the NS-4 antigens (Fig. 3A), and almost all sera from donors infected with any of the three types reacted with c22-3, the most highly conserved protein. Similarly, the frequency and strength of reactivity with each of the core peptides in the LIA differed little in regard to HCV type (Fig. 3B). In interpreting these results, it must be borne in mind that the sera were preselected by the original second-generation screening test. Thus, there is no information on the proportion of type 2 and 3 infections that elicit such a restricted serologic response as to be undetectable by current blood donor screening.

The lack of serologic reactivity on the part of the eight donors infected with HCV type 1 to some or all of the nonstructural antigens could be explained by proximity to seroconversion, although only three of the six individuals recalled showed abnormal ALT values. Sequential studies have shown that antibody to c22-3 or c33c may appear before antibody to cl00-330,32 and that this restricted profile may persist for more than a year in noninfected or immunocompromised individuals. We therefore postulate that some individuals infected with types 2 or 3, who have a delayed or absent response to the core protein of HCV, may not be identified by present serologic screening assays. To detect such “seronegative,” HCV-infected donors, the sensitivity of current second-generation assays could be improved by incorporation of the homologues of the cl00-3 and other proteins from HCV types 2 and 3 as antigens in screening and confirmatory assays.

Approximately one-third of HCV-infected donors were negative for anti-HBc and had ALT values in the normal range, which is consistent with previous studies.33-35 Even if surrogate marker testing were combined with anti-
c100 (first-generation) screening, 11 of the 90 HCV-infected donors would have been missed. Whereas most donors infected with HCV type 1 would be excluded on the basis of anti-c100 reactivity, exclusion of those infected with type 3 would have been, in almost all cases, due to raised ALT values.

Course of disease associated with different HCV types

All of the recalled donors were asymptomatic, although approximately 60 percent had ALT values above the upper limit of the normal range. A significant finding was a clear difference in the distribution of ALT values in those who were PCR positive and those who were PCR negative (p < 0.0001). Indeed, the ALT values of the latter group were comparable to those of the HCV-negative control donors.

It has been suggested that HCV type 2 may cause more severe disease and be less susceptible to interferon therapy than type 1.\(^\text{28}\) In our study, the distribution of ALT values of type 2-infected donors was similar to that of type 1-infected donors. However, their age distribution was not typical of blood donors and tended to be higher than that of donors infected with type 1 and type 3 variants, which suggests that infection had occurred longer ago and had had more time to resolve.

In contrast, donors infected with HCV types 1 and 3 were of similar age and had a similar incidence of past hepatitis B virus infection and reported risk factors for infection (in both cases, approximately 50% admitted past intravenous drug abuse). There was no association between HCV type and the geographical region within which the donor lived in Scotland or Northern Ireland, nor was there any association with specific areas, such as public housing, within a city. All but three of the donors were born in Scotland or Northern Ireland, and the majority are likely to have acquired infection from there. Thus, it is unlikely that simple epidemiologic differences can account for the marked difference in the distribution of ALT values in the two groups (type 3: 83% abnormal ALT, compared with type 1: 55% abnormal; p < 0.05). These differences raise the possibility that infection with type 3 is associated with greater liver damage and possibly a more severe course of disease than infection with type 1 and indicate a potential clinical role for HCV typing. However, despite the epidemiologic investigations, it remains possible that the higher ALT values in type 3-infected donors are the result of more recent infection and are perhaps associated with the infection of current drug abusers and their contacts in certain areas of Scotland. In addition, the higher ALT values do not necessarily reflect greater long-term damage to the liver or the likelihood of complications such as cirrhosis and hepatocellular carcinoma. The full significance of the differences in ALT values can therefore be determined only by further observation of the donors, to study the course of infection in more detail and to carry out liver biopsies to investigate directly the extent of liver disease.

Acknowledgments

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References

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