

# Hepatitis C viral clearance and antibody reactivity patterns in persons with haemophilia and other congenital bleeding disorders

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**Summary.** We studied hepatitis C virus (HCV) clearance and antibody reactivity patterns in a cohort of 100 haemophiliacs exposed to unsterilized blood products, of whom 25 were antiHCV negative and 75 were antiHCV positive [49 human immunodeficiency virus (HIV) negative and 26 HIV positive]. HCV RNA was measured by the 2.0 bDNA assay and an 'in-house' polymerase chain reaction assay. Antibody reactivity patterns were examined using a recombinant immunoblot assay (RIBA). Prior HCV infection was found in two (8%) of 25 antiHCV negative patients. HCV viraemia persisted in all 26 antiHCV+ patients who were coinfecting with HIV. HCV RNA clearance was found in 12 (25%) of 49 antiHCV+, HIV- patients. Viral clearance was associated with younger current age ( $P < 0.01$ ) and age at infection ( $P < 0.001$ ), but not with duration of infection or with dose or frequency of clotting factor

use. RIBA ratios reflecting an index of each patient's overall reactivity to four HCV epitopes were significantly lower in those with viral clearance ( $P < 0.0001$ ). Over a period of 15 years, those with viral clearance demonstrated significant loss of reactivity to the NS3, NS4 and NS5 epitopes, while those with viral persistence demonstrated relatively stable reactivities to all epitopes. We conclude that spontaneous HCV RNA clearance in haemophiliacs is age-related and is unlikely to occur in those coinfecting with HIV. The loss of antibody reactivity for some epitopes, especially c22 (core), may be a marker for the natural resolution of chronic HCV infection.

**Keywords:** haemophilia, HCV RNA, hepatitis C, HIV coinfection, RIBA patterns, viral clearance.

## Introduction

Nearly all haemophiliacs who were infused with clotting factor concentrates prior to the introduction of donor screening and viral inactivation techniques in the mid 1980s were infected with the hepatitis C virus (HCV) [1–3]. Greater than 80% have devel-

oped chronic hepatitis, manifested by intermittently or persistently elevated serum transaminases [4–6] and viraemia [7,8]. Although most have remained asymptomatic, it is estimated that 20–30% develop cirrhosis within 20–30 years after the acute infection [9–11]. Once cirrhosis is established, the risk of developing hepatocellular carcinoma is significantly increased [12].

It is widely accepted that approximately 15–20% of all individuals infected with HCV spontaneously resolve their infections [13]. However, the factors relating to viral clearance are poorly understood. The purpose of this study was to determine the frequency of viral clearance, to examine changes in antibody reactivity patterns over time, and to identify factors that relate to viral clearance, in a well-characterized cohort of haemophiliacs with chronic HCV.

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## Patients and methods

The study group consisted of 272 patients with haemophilia and other inherited bleeding disorders, who have been followed up regularly in our comprehensive care clinic since 1973 and enrolled in the Multicentre Hemophilia Cohort Study since 1982 [14]. Periodic evaluation consisting of an interim history, physical examination, complete blood count, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), hepatitis B surface antigen (HBsAg), and hepatitis B antibody (antiHBs) tests were performed with informed consent at intervals of 6 months to 1 year as previously described [15,16]. At each visit, serum and plasma samples were obtained and stored frozen at  $-20\text{ }^{\circ}\text{C}$  to  $-70\text{ }^{\circ}\text{C}$ .

Testing for HCV antibody was performed in 1990–92, using a first generation enzyme-linked immunosorbent assay (ELISA; Ortho Diagnostics System, Raritan, NJ, USA). Positive tests were confirmed using a second-generation recombinant immunoblot assay (RIBA-2; Chiron Corp. Emeryville, CA, USA). Tests for HIV antibody were performed using an enzyme-linked immunosorbent assay (ELISA) and confirmatory assays, as previously described [15,16].

For the current analysis, the most recent frozen serum sample on all subjects and earlier frozen sera from seronegative patients over 12 years of age who had received blood or unsterilized blood products prior to 1987 were re-tested in June 1999 for antiHCV with a second generation ELISA test (Abbott 2.0; Abbott Laboratories, Chicago, IL, USA). All who were antiHCV positive were then tested for the presence of HCV RNA by the Quantiplex 2.0 branched chain DNA-enhanced label amplification (bDNA) assay (Chiron Corp.). For samples less than the cut-off of  $0.2 \times 10^6$  genome equivalents  $\text{mL}^{-1}$ , HCV RNA levels were measured using a more sensitive 'in-house' polymerase chain reaction (PCR) assay with a lower limit of detection of 1000 copies  $\text{mL}^{-1}$ , as previously described [17,18].

Patterns of antiHCV reactivity were studied using the third generation recombinant immunoblot assay (RIBA HCV 3.0; Chiron Corp.). The intensity of antibody reactivities was measured for each epitope (c22 core, NS3, NS4, NS5) and assigned a numerical value from 0 to 4+ according to the manufacturer's instructions. A RIBA ratio, defined as the sum of reactivities for all epitopes for a given sample divided by the number of reactive epitopes, was calculated for each patient as described by Lanotte *et al.* [8].

For those with established HCV seropositivity, duration of infection was estimated by calculating the midpoint between the maximum duration of infection (years since first transfusion) and the minimum duration of infection (years since first exposure to unsterilized clotting factor concentrates) [19]. It is possible to estimate the minimum duration of infection for haemophiliacs because studies have shown an almost 100% incidence after the first infusion of unsterilized clotting factor concentrates [1,2].

Patients were assigned to one of four categories based on past clotting factor use: none, low ( $<20\ 000$  units  $\text{year}^{-1}$ ), medium ( $>20000$ ,  $<50000$  units  $\text{year}^{-1}$ ), or high ( $>50\ 000$  units  $\text{year}^{-1}$ ). Cumulative use of fresh frozen plasma (FFP) and cryoprecipitate was also calculated for each patient.

Patients were categorized based on the patterns of their three most recent serum ALT determinations [4]. Those with three consecutive values in the normal range were considered normal. All others were considered abnormal.

Differences between groups in current age, age at infection, and duration of infection were compared using Student's *t*-test on logarithmically transformed data. Fischer's exact test was used to evaluate history and intensity of clotting factor concentrate and other blood product exposure, as well as disease type and severity, and coinfection with hepatitis A and B. Wilcoxon rank sum test was used to evaluate serial RIBA results.

## Results

Of 272 persons with haemophilia or other inherited coagulation disorders, 66 were younger than 12 years of age, and would not have been exposed to contaminated blood products before the advent of donor screening programmes and viral inactivation of clotting factor concentrates in the mid-1980s. An additional 64 were 12 years of age or older, but had no documented exposure to blood products prior to 1987, leaving 142 patients at risk of having acquired HCV from blood product usage. Of these, 102 (72%) were antiHCV positive, and 40 (28%) were antiHCV negative. Forty-two had insufficient archived sera (17 antiHCV+ and 15 antiHCV-) or had been previously treated with interferon (10), leaving 75 antiHCV+ patients and 25 antiHCV- patients for further study (Table 1).

Of the 25 antiHCV negative patients, two (8%) were exposed to unsterilized clotting factor concentrate prior to 1987, while 23 (92%) were exposed to

**Table 1.** Hepatitis C and ALT test results on 100 haemophiliacs at risk for HCV exposure from unsterilized blood products\*.

	<i>n</i>	%
AntiHCV negative, HIV negative	25†	
Previously antiHCV negative	23	92
Previously antiHCV positive	2	8
AntiHCV positive, HIV positive	26	
HCV RNA detectable	26	100
ALT abnormal	23	88
AntiHCV positive, HIV negative	49‡	
HCV RNA detectable	37	75
ALT abnormal	26	70
ALT normal	11	30
HCV RNA not detectable	12	25
ALT normal	12	100

\**n* = 75 antiHCV positive and 25 antiHCV negative exposed individuals. ALT, serum alanine aminotransferase. †Factor VIII deficiency, *n* = 16; factor IX deficiency, *n* = 5; von Willebrand disease, *n* = 4. ‡Factor VIII deficiency, *n* = 37; factor IX deficiency, *n* = 8; von Willebrand disease, *n* = 2; factor V deficiency, *n* = 1; factor VII deficiency, *n* = 1.

only whole blood, fresh frozen plasma, or cryoprecipitate prior to 1987. None were infected with HIV. Twenty-three (92%) of the earlier samples were antiHCV- by ELISA; two (8%) were antiHCV+. Both patients had been infected in early childhood. One, who was coinfecting with HCV and HBV in 1977, remained a carrier for HBsAg for 10 years before developing antiHBs and clearing HBsAg. By 1988 he had cleared his HCV viraemia and lost antibody reactivity by ELISA. He had received only small amounts of cryoprecipitate and only one vial of clotting factor concentrate. The other was antiHCV+ by ELISA and RIBA in 1986; he was antiHCV- by ELISA but repeatedly RIBA indeterminate over the course of the last 12 years. He had received only 56 bags of cryoprecipitate and no clotting factor concentrate prior to seroconversion.

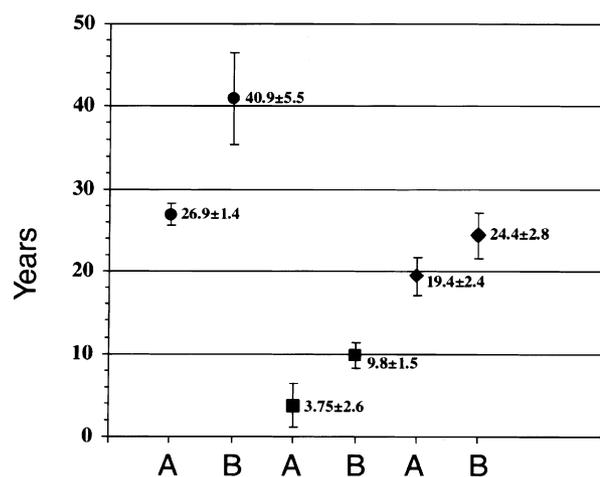
Of the 75 antiHCV+ patients, 26 were coinfecting with HIV. All 26 had detectable HCV RNA and 23 (88%) had abnormal ALT values (Table 1). Of the 49 HIV-, antiHCV+ patients, 37 (75%) had detectable HCV RNA. Thirty-five (71%) were positive by the bDNA assay. Two (4%) were positive only by the PCR assay. Twenty-six (70%) had at least one abnormal serum ALT level within the span of their previous three clinic visits. Twelve (25%) had no detectable HCV RNA. All 12 had repeatedly normal serum ALT levels.

The 37 HIV- patients with HCV RNA persistence and the 12 HIV- patients with HCV RNA clearance were compared with respect to current age, age at infection, duration of infection, type and severity of haemophilia, frequency and intensity of previous

exposure to clotting factor concentrate or other blood products, history of infection with hepatitis A and hepatitis B, and alcohol, acetaminophen, and illegal parenteral drug use. Statistically significant differences between those with and without viral clearance were found for current age (*P* < 0.01) and age at infection (*P* < 0.001) (Fig 1). Duration of infection (*P* > 0.05) was not found to be significant. Among the 37 viraemic patients, the 11 with normal ALT values did not differ from the 26 with abnormal ALT values with respect to current age, duration of infection, age at infection, or viral load.

Differences in frequency and intensity of blood product use between the viral clearance and viral persistence groups were not found to be statistically significant. Patients with viral clearance had a mean FFP use of 800 mL and mean cryoprecipitate use of 13 354 units; 50% (6/12) had no FVIII or FIX exposure while 50% (6/12) had low factor exposure. Patients with viral persistence, regardless of ALT values, had a mean FFP use of 4700 mL and cryoprecipitate use of 13 686 units; 39% (14/36) had no FVIII or FIX exposure, 56% (22/36) had low factor exposure, and 5% (2/36) had medium levels of factor exposure.

Mean RIBA reactivities for each epitope were lower for the 12 patients with viral clearance than for the 37 patients with viral persistence (Table 2). The NS3, NS4, and NS5 epitopes showed the most marked differences, with NS4 showing the greatest change. No patient lost c22 (core) reactivity. Only two (8%) of the 12 patients with viral clearance had



**Fig. 1.** Current age, age at HCV infection and duration of infection in 12 patients with viral clearance and 37 patients with viral persistence. A, viral clearance; B, viral persistence. (●), Mean current age; (■), mean age at infection; (◆) mean duration of infection ± 95% C.I.

a RIBA ratio greater than or equal to 3.5 (mean 2.08), compared to 27 (73%) of the 37 patients with viral persistence (mean 3.6,  $P < 0.0001$ ).

Changes in mean antibody reactivities over a mean of 15 years for the 12 patients with viral clearance and 18 representative patients with viral persistence were compared, using the most recent frozen serum sample, and an earlier frozen serum sample taken within 6 months to 1 year after the first noted rise in serum ALT levels for each patient (Table 3). There were no significant differences between the two groups for any epitope in the early samples. However there were significant differences between the two groups for all epitopes in the most recent samples. In addition, all viraemic patients showed stable reactivities over time for all epitopes, while those with viral clearance showed a highly significant loss of reactivity to the NS3, NS4, and NS5 epitopes (Table 3). In spite of the substantial losses in reactivities over time for these three epitopes, patients with viral clearance demonstrated stable reactivities for the core epitope, with only two showing at least two-fold decreases in core epitope reactivity (Fig. 2).

## Discussion

We found that 12 of 49 (25%) of our cohort of HIV-, antiHCV+ haemophiliacs who had not been exposed to contaminated blood products for over 12 years had cleared HCV RNA from their sera a mean of 24 years after infection. All had persistently normal ALT values. The remaining 75% had measurable viraemia, with or without elevated serum ALT values. These findings are consistent with the 23% rate of spontaneous viral clearance described by Seeff *et al.* in transfusion recipients [20]. None of our 26 HIV-infected antiHCV+ patients cleared HCV RNA from their sera.

Viral clearance in HIV- patients was associated with younger current age, and younger age at infection, but not with duration of infection. In a population of 155 antiHCV+ patients, Schulman *et al.* found that those younger than 40 years of age were statistically more likely to have no detectable HCV RNA than those 40 years of age or older [21]. In a study of 67 children who acquired HCV by blood transfusion following cardiac surgery, Vogt *et al.* reported a 45% rate of viral clearance a mean of 20 years after initial infection [22]. All of these children were infected at a single point in time, with a definite but limited viral inoculum from blood transfusion. It remains to be seen whether haemophilic children, who were transfused multiple times with HCV-contaminated plasma products, will fare as well. Larger, long-term studies are needed to fully evaluate the roles of age and route of infection on HCV persistence.

RIBA ratios reflecting an index of each patient's overall reactivity to the four HCV epitopes presented were significantly lower in those with viral clearance. In addition, those with viral clearance demonstrated a clear loss of antibody reactivity to all HCV epitopes over time. Early antiHCV reactivity profiles were similar in both groups, but over a period of 15 years, those in the viral clearance group demonstrated significant loss of reactivity to the NS3, NS4, and NS5 epitopes. In contrast, during the same period of time, patients with viral persistence demonstrated relatively stable reactivities to all epitopes.

The product of the core region of the HCV genome is regarded to be the most highly immunogenic of the HCV proteins. In the study by Lanotte *et al.* stable antiHCV reactivities over time were found in 95% of viraemic haemophiliacs, while 7/8 (87.5%) patients with viral clearance showed a decrease in the level of antibody reactivity [8]. Reactivities against core

**Table 2.** Hepatitis C RIBA reactivity patterns for 37 haemophiliacs with viral persistence and 12 with viral clearance.

Epitope	Number of subjects who are reactive for each viral epitope						Mean reactivity score
	Band intensity						
	-(0)	+/- (0.5)	+1	+2	+3	+4	
<b>Viral persistence</b>							
NS3	0	0	0	0	2	35	3.89
NS4	0	2	2	2	5	26	3.41
NS5	7	1	1	0	2	26	3.01
c-22	0	0	0	0	0	37	4.00
<b>Viral clearance</b>							
NS3	0	1	3	3	1	4	2.38
NS4	0	3	4	4	1	0	1.38
NS5	3	3	0	4	1	1	1.38
c-22	0	1	0	1	4	6	3.20

**Table 3.** Comparisons of early vs. recent and change over time in serum samples from 30 haemophiliacs with chronic hepatitis C.

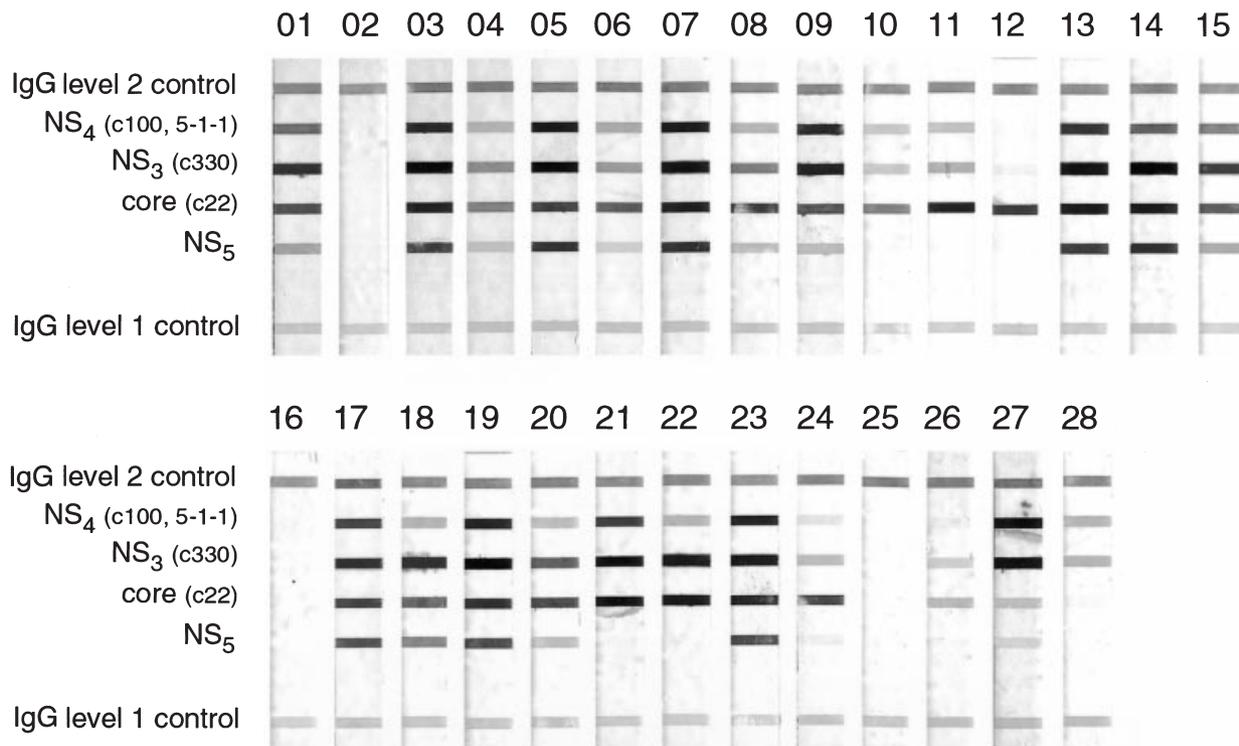
Epitope		Mean RIBA reactivity scores		P-value*
		Viral persistence (n = 18)	Viral clearance (n = 12)	
c22	early	3.31	3.83	0.30
	recent	4.00	3.29	0.003
	change	+0.69	-0.54	0.002
NS3	early	3.83	3.92	0.84
	recent	4.00	2.25	0.0001
	change	+0.17	-1.67	0.0001
NS4	early	3.33	3.83	0.31
	recent	3.08	1.42	0.004
	change	-0.25	-2.42	0.0002
NS5	early	2.53	2.67	0.77
	recent	2.64	1.04	0.03
	change	+0.11	-1.63	0.004

\*Wilcoxon rank-sum test.

epitope were stable for longer periods than other epitopes, and anticore antibodies remained present

on the last sample of all patients. Similarly, in five of 30 HCV infected thalassaemic patients with partial or complete seroreversion, Lefrere *et al.* found that reactivity to core was the most persistent. They proposed a sequence of seroreversion, with successive loss of antiNS5, then antiNS3, followed by loss of antiNS4, and finally by loss of anticore [23]. These findings support the immunogenic strength of the HCV core antigen, strongly linking the presence of HCV viraemia with anticore antibodies.

Whether or not patients with viral clearance and partial seroreversion have true resolution of their HCV infection is uncertain. Serum HCV RNA levels fluctuate during chronic infection and may be transiently undetectable in some individuals by current methods [24,25]. In such cases it is possible that the viral load may be insufficient to elicit the full host response, but sufficient to provoke an anticore response. Another potentially confounding factor is the possibility that HCV RNA may exist intrahepatically in the absence of measurable circulating serum HCV RNA [26]. In these cases, virus may be replicating in a hepatic reservoir, thus providing



**Fig. 2.** Serial recombinant immunoblot assays of 12 subjects with viral clearance demonstrating loss of reactivity to NS3, NS4, and NS5 epitopes with stable reactivity to core epitope over time. Lanes 1, 15 = positive controls; lanes 2, 16 = negative controls. Odd numbers = early samples; even numbers = recent samples. Samples less than the level 1 control are scored as +/- . Samples greater than the level 1 control and less than the level 2 control are scored as 2+. The sample in lane 25 was obtained in 1984 prior to seroconversion. A 1987 sample (not shown) showed 2+ NS4, 3+ NS3, 4+ core and absent NS5. A 1997 sample (lane 26) showed +/-NS4, 1+ NS3, 2+ core, and +/-NS5.

continued antigenic stimulus for the production of core antibodies without the presence of viraemia.

Complete seroreversion following acute HCV infection appears to be rare. We were able to document complete loss of antibody reactivity as measured by a second-generation ELISA test in only two (8%) of 25 haemophiliacs who were exposed to unsterilized blood products prior to 1987 and were antiHCV- at study entry in 1999. Larger retrospective-prospective studies are needed to determine the frequency of complete seroreversion in haemophiliacs who have cleared HCV RNA and are now antiHCV antibody negative 12 or more years after infection.

The shortcomings of our study include small sample size, lack of a standardized qualitative PCR assay to detect very low levels of viraemia, and lack of liver biopsy to assess the extent of inflammation and fibrosis. Future studies should include a comparison of viral clearance and antibody reactivities with liver histology and measurement of intrahepatic HCV RNA to determine whether partial or complete seroreversion by RIBA is a reliable indicator of viral clearance.

We conclude that spontaneous HCV RNA clearance in haemophiliacs is age-related and is unlikely to occur in those coinfecting with HIV. Although complete seroreversion is rare, loss of antibody reactivity, especially to the c22 (core) epitope, may be a marker for the natural resolution of chronic HCV infection.

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