British Journal of Haematology, 1992, 80, 514–518

Use of several second generation serological assays to determine the true prevalence of hepatitis C virus infection in haemophiliacs treated with non-virus inactivated factor VIII and IX concentrates

HENRY G. WATSON, CHRISTOPHER A. LUDLAM, SELMA REBUS,† LIN QI ZHANG,* JOHN F. PEUTHERER† AND PETER SIMMONDS† *Department of Haematology, Royal Infirmary, Edinburgh, *Institute of Animal, Cell and Population Biology, University of Edinburgh, and †Department of Medical Microbiology, University of Edinburgh*

Received 21 October 1991; accepted for publication 30 November 1991

Summary. To investigate the prevalence of hepatitis C virus infection in two risk groups, stored serum samples from treated haemophiliacs and intravenous drug users were tested for anti-HCV by both anti-C-100 based and second generation ELISAs (Abbott and Ortho) followed by testing in two confirmatory immunoblot assays that incorporate core as well as other non-structural antigens (Innogenetics LIA and Chiron RIBA-HCV test). Clear evidence of HCV infection was found in all but one of 78 haemophiliacs treated with non-virus inactivated clotting factor concentrates, but in none exposed only to super dry heat-treated concentrates. Only four samples gave rise to conflicting serological results between the four tests, two of these occurred in patients with advanced HIV related disease and almost certainly reflected loss of humoral immunity associated with disease progression, and the others occurred in the only two patients tested who were chronic carriers of hepatitis B infection and may reflect an interaction between the two viruses. Comparison of anti-C-100 versus second generation tests in immunocompetent drug users revealed a false negative rate of 20% using C-100 alone, indicating the advantage of using second generation assays for detection of past or current HCV infection. Of all of the antigens used in the confirmatory assay, positive sera showed strongest and most frequent reactivity with the C22 and C33c proteins (Ortho RIBA).

Assays for antibodies directed against the non-structural C-100 peptide, detect the majority of cases of HCV infection (Kuo *et al.* 1989). Such assays are, however, hindered by the frequent occurrence of false positive and negative results (Skidmore, 1990; Alter *et al.* 1989). It has previously been demonstrated that all recipients of non-virus inactivated factor VIII concentrates develop non-A non-B hepatitis (Fletcher *et al.* 1983); however despite this, testing with C-100 based assays has shown a prevalence of infection of between only 59% and 85% (Makris *et al.* 1990; Ludlam *et al.* 1989). Therefore using such assays, a significant proportion of haemophiliacs with a history of exposure to non-virus inactivated concentrates, many of whom have biochemical evidence of chronic liver disease, show no evidence of infection with HCV. Additionally, HCV RNA has been detected by polymerase chain reaction (PCR) in haemophiliacs who are seronegative for HCV by C-100 testing (Simmonds *et al.* 1990).

Correspondence: Dr P. Simmonds, Department of Medical Microbiology, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG.

Further problems have arisen in the field of blood donor screening in order to prevent post-transfusional non-A non-B hepatitis (PTNANBH). Studies in which the value of screening for HCV antibodies have been evaluated show a decrease in incidence, but not abolition of PTNANBH, when units positive for anti-C-100 are excluded (Esteban *et al.* 1990).

New antibody assays which use structural HCV antigens have recently become available and initial studies have shown high prevalence of HCV infection in patients with both PTNANBH and sporadic chronic active hepatitis (Marcellin *et al.* 1991; Craxi *et al.* 1991).

We have re-evaluated the serological status of a large group of haemophiliacs attending one haemophilia centre and a group of immunocompetent HIV-negative individuals with a history of intravenous drug use (IVDUs), for evidence of HCV infection. Our results demonstrate the different prevalence of HCV infection in the patient groups and

Table I. Detection of HCV antibody in different risk groups

	Abbott 1st gen. ELISA		Ortho 2nd gen. ELISA		Abbott 2nd gen. ELISA		Confirmatory assay Chiron RIBA			Confirmatory assay Innogenetics LIA		
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Ind.	Neg.	Pos.	Ind.	Neg.
Haemophilia A												
HIV pos.	16	4	20	0	20	0	19	1	0	18	2	0
HIV neg.	36	4	39	1	39	1	38	1	1	40	0	0
Haemophilia B (HIV neg.)	16	2	18	0	18	0	18	0	0	18	0	0
H/T concentrate only*	0	7	0	7	0	7	0	0	7	0	0	7
IVDU (HIV neg.)	20	13	25	8	25	8	24	0	9	22	3	8

* Recipients of heat-treated factor VIII ($n=6$) or factor IX ($n=1$) inactivated at 80°C for 72 h (dry).

compares the performance of the different antigens used in the assays to detect anti-HCV antibodies.

METHODS AND SUBJECTS

Stored serum samples from 85 haemophiliacs attending the Edinburgh Regional Haemophilia Centre were randomly chosen for testing. The characteristics of the group were as follows: Haemophilia A ($n=66$) of which 20 were HIV-positive, 40 HIV-negative, and six were recipients of exclusively virus-inactivated concentrate. Haemophilia B ($n=19$), of which none were HIV-positive, and one had received only heat-treated products. IVDUs ($n=33$) of which all were HIV-negative both at the time of testing and in a subsequent sample at least 2 months later.

HCV testing was carried out according to the manufacturers' instruction. In the ELISAs (Abbott anti-C-100 EIA, Abbott HCV EIA second generation and Ortho HCV ELISA test system second generation) samples with an optical density over the calculated cut-off were scored as positive. Second generation assays for anti-HCV include core and further non-structural antigens in addition to C-100 coated on the solid phase. In the immunoblot assays antibody reactivity to structural and non-structural antigens contained in the Chiron RIBA-HCV (RIBA) (C-100, 5-1-1, C33c, and C22), and Innogenetics-LIA HCV (LIA) (NS-4, NS-5, C1, C2, C3 and C4), was measured. As instructed by the manufacturers, samples were considered positive if reactive with two antigens with scores of 1+ or greater, or with one band if 2+ or greater (LIA only). Indeterminate samples were those that were reactive with only one antigen (with a score of 1+ in the LIA or 1+ to 4+ in the RIBA). The frequency of positive bands was calculated in different patient groups by dividing the number of positive outcomes by the total number tested, and the average band intensity calculated by dividing the total score of all positive bands by the number of positive results. PCR using nested primers was performed on 200 μl of serum as previously described (Simmonds *et al.* 1990). Hepatitis B surface antigen (HBsAg) and core (anti-HBc) and surface (anti-HBs) antibodies were detected by radioimmunoassay. Patients with intermittent or persistent elevation of alanine aminotransferase (ALT) over the period of approximately 1 year around testing were designated as having biochemical evidence of chronic liver disease.

Table II. Reactivity of haemophiliac samples in Innogenetics and Chiron confirmatory assays

(A) Anti-C-100 (Abbott) positive samples

	Innogenetics LIA		
Chiron RIBA	Pos.	Ind.	Neg.
Pos.	67	1	0
Ind.	0	0	0
Neg.	0	0	0

(B) Anti-C-100 (Abbott) negative samples

	Innogenetics LIA		
Chiron RIBA	Pos.	Ind.	Neg.
Pos.	7	0	0
Ind.	1	1	0
Neg.	1	0	0

RESULTS

Of the 78 haemophilia A and B patients previously exposed to non-virus inactivated concentrates, 68 were positive by anti-C-100 testing (Table I). These results were true positives, as all were positive in the second generation Abbott ELISA (2nd-GAE) and Ortho ELISA (2nd-GOE), and all were confirmed in the Chiron RIBA. All but one were positive by the LIA (Table IIa). Ten samples were anti-C-100 negative. Of these, nine were positive by 2nd-GOE and 2nd-GAE, nine were positive and one indeterminate by the LIA, and seven positive, two indeterminate and one negative by the Chiron RIBA (Table IIb). Patients who had received only heat-treated concentrates were uniformly negative by all assays (Table I). Twenty of 33 IVDUs were positive by anti-C-100 testing; however, further testing in the second generation assays showed 25 positive results leaving eight negative sera and demonstrating a false negative incidence of five in 25 using the C-100 based assay alone (Table I).

Table III. Features of patients with indeterminate HCV results

ID	ALT[1]	CDC[2] status	CD4 [3] ×10⁶/l	HBV serology Anti HB-C	Anti HB-S	HB_sAg	HCV ELISA OD 1st GE[4]	Abbott 2nd GE[5]	Ortho 2nd GE[6]	HCV confirmation LIA NS-4	NS-5	RIBA C_1	C_2	C_3	C_4	5-1-1	C-100	$C33_c$	C22	HCV PCR
1	P	IV	90	+	weak +	–	0·698	> 2	> 2	–	–	1+	±	–	–	–	–	2+	4+	+
2	I	IV	140	weak +	weak +	–	0·377	1·733	> 2	1+	–	±	±	–	–	±	±	–	4+	+
3	P	NA[3]	400	+	–	+	0·299	0·806	1·458	–	–	–	–	–	2+	±	–	–	2+	–
4	P	NA[3]	310	+	–	+	0·242	0·05	0·180	±	–	–	1+	–	1+	–	–	–	–	–

[1] ALT level over 1 year around testing P (persistent), I (intermittent) elevation.
[2] Centre for Disease Control classification of HIV related illness.
[3] Not affected.
[4] EIA OD control cut off 0·522.
[5] EIA OD control cut off 0·668.
[6] EIA OD control cut off 0·459.

Amongst the haemophiliacs, the indeterminate results were confined to four patients. The details of antibody reactivity and PCR results are given in Table III. Two of these individuals had late stage infection with HIV ($CD4+ < 200 \times 10^6/l$), but were positive for HCV RNA and therefore certainly infected, the weak antibody reactivity reflecting their state of immunosuppression. The other two indeterminate samples were PCR negative, but both were antibody positive in at least one assay (Table III). Interestingly these two samples were from the only two persistent carriers of hepatitis B virus infection and may reflect an interaction between the two viruses resulting in altered expression as has been previously shown (Brotman *et al*, 1983; Tanaka *et al*, 1991).

Comparison of antibody reactivity to the individual peptides showed that in all groups C22 and C33c were the most sensitive indicators of infection with detection rates ranging 96–100% and 95·2–100% respectively (Fig 1). The intensity of antibody reactivity was in general slightly greater in HIV-negative than in HIV-positive samples (Fig 2).

DISCUSSION

Overall our results provide convincing serological evidence for the expected universal prevalence of HCV infection in haemophiliacs treated with non-virus inactivated factor VIII (Fletcher *et al*, 1983) and IX concentrates. In contrast, all seven patients exposed only to concentrates, heat treated dry for 72 h at 80°C, were uniformly negative in all serological assays (Table I). Although the numbers of patients studied was small, the absence of serological reactivity in this group provides reassurance about the viral safety of currently available Scottish National Blood Transfusion Service factor VIII and IX concentrates. Other studies have demonstrated absence of reactivity to C-100 in patients receiving only concentrate treated by this method (Evans *et al*, 1990), in contrast to others which demonstrated that the early forms of heat treatment although decreasing the incidence, did not abolish PTNANBH (Columbo *et al*, 1985; Kernoff *et al*, 1987).

Four of the 10 haemophiliacs negative for anti-C-100 were HIV-positive and therefore these false negative results may be attributable to immunosuppression as has been demonstrated with other antibody responses in HIV infected individuals (Mannucci *et al*, 1989). However false negative results in the anti-C-100 assay were also found in six haemophiliacs and five IVDUs all of whom were HIV-negative (Table I), giving an overall prevalence of false negatives of 13% in the HIV negative group. HIV negative haemophiliacs have been shown to have minor alterations in their immune systems which may reflect a degree of immunosuppression (Madhok *et al*, 1986; Carr *et al*, 1984); however, even if these are excluded, five of 25 presumed immunocompetent IVDUs still had false negative results by C-100 testing alone, and therefore it can be presumed that screening of similarly immunocompetent donors by the Blood Transfusion Service by anti-C-100 testing would fail to detect HCV infection in a considerable number of cases. Esteban *et al* (1990) have previously demonstrated that exclusion of units positive by C-100 assays does not completely abolish the risk of PTNANBH

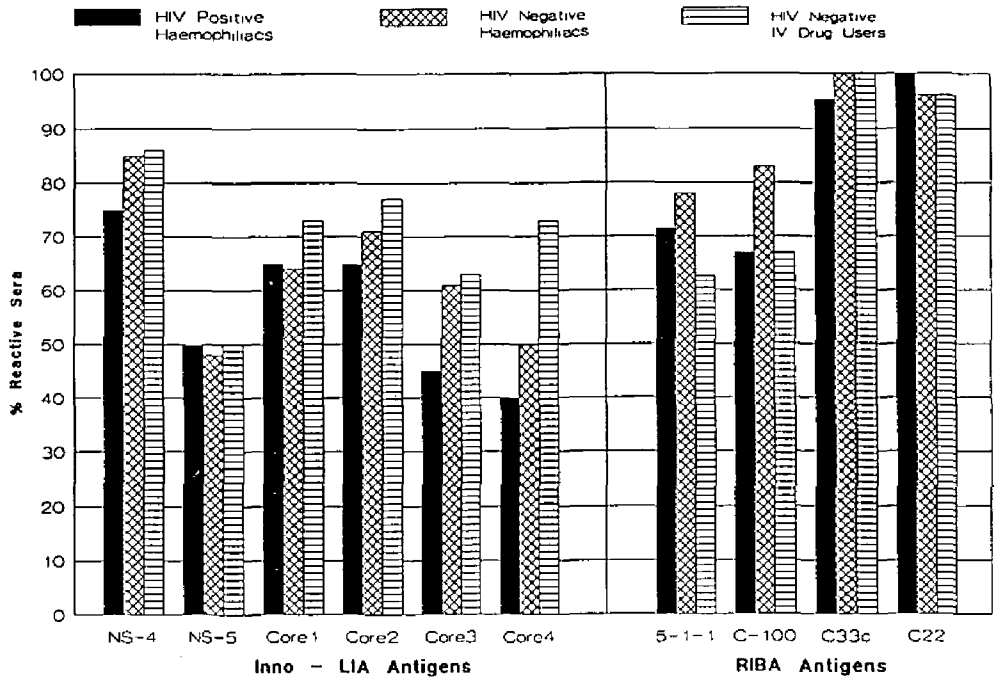

Fig 1. Frequency of positive results for individual antigens.

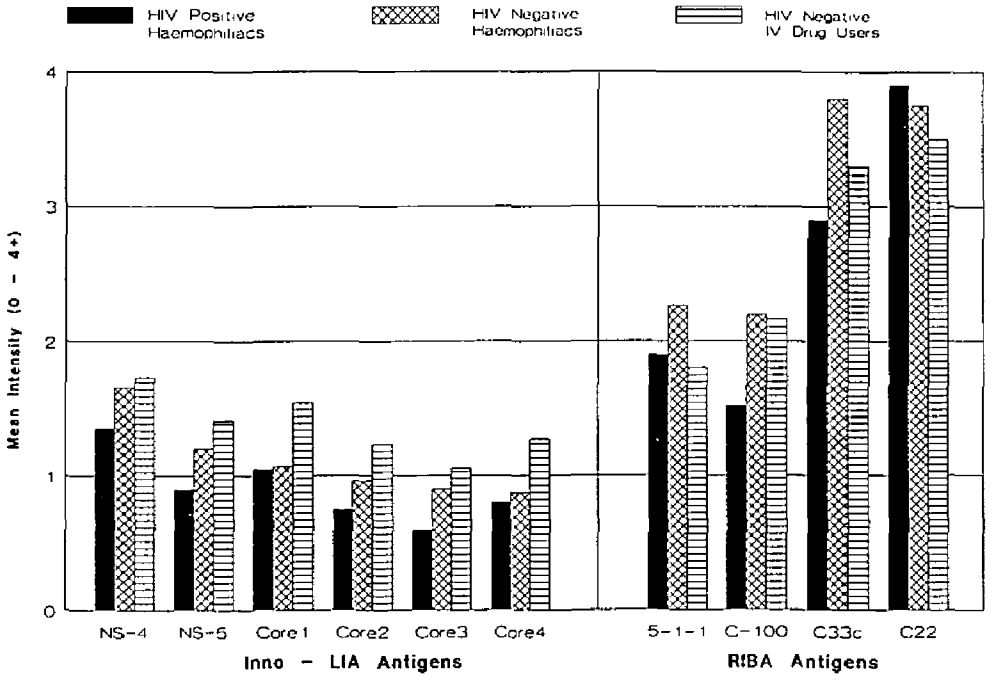

Fig 2. Mean intensity of reaction for individual antigens.

however, our data would suggest that these alternative serological assays would detect most HCV infected samples. It is, however, still possible that despite screening of donors by more sensitive second generation assays, cases of PTNANBH will occur as virus with a different serotype, not detectable by present assays may exist. In addition the long period to seroconversion may still provide a window by which infection in donors may be missed.

The finding of indeterminate, or in one case possibly negative, results of HCV serology in the only HBV surface antigen carriers is consistent with a previous study that showed a decreased prevalence of anti-HCV in patients with hepatocellular carcinoma and liver cirrhosis and who are HBsAg-positive (Tanaka *et al*, 1991), suggesting that concurrent infection interferes with viral replication (Brotman *et al* (1983).

The high frequency of positive results obtained using the antigens C22 and C33c, and the intensity of the bands seen in the immunoblot assays, suggests that these antigens are both conserved and strongly immunogenic and are the most useful of the antigens evaluated for the detection of HCV infection.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance of Mrs G. H. Leadbetter and Mr P. McCulloch in carrying out some of the serological assays. We are also indebted to n.v. Innogenetics s.a. and Ortho Diagnostics for supplying LIA strips and second generation EIA kits for evaluation. We thank Mr E. Thomson for help in producing the figures.

REFERENCES

Alter, H.J., Purcell, R.H., Shih, J.W., Melpolder, J.L., Houghton, M., Choo, Q.L. & Kuo, G. (1989) Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *New England Journal of Medicine*, 321, 1494–1500.

Brotman, B., Prince, A.M., Huima, T., Richardson, L., Van den Ende, M.C. & Pfeifer, V. (1983) Interference between non-A, non-B and hepatitis B virus infection in chimpanzees. *Journal of Medical Virology*, ii, 191–205.

Carr, R., Edmund, E., Prescott, R.J., Veitch, S.E., Peutherer, J.P., Steel, C.M. & Ludlam, C.A. (1984) Abnormalities of circulating lymphocytes subsets in haemophiliacs in an AIDS free population. *Lancet*, i, 1431–1434.

Columbo, M., Mannucci, P.M., Carnelli, V., Savidge, G.F., Gazengel, C. & Schimpf, K. (1985) Transmission of non-A, non-B hepatitis by heat-treated factor VIII concentrate. *Lancet*, ii, 1–4.

Craxi, A., Fiorentino, G., DiMarco, V., Marino, L., Magrin, S., Fabriano, C. & Pagliaro, L. (1991) Second generation tests in the diagnosis of chronic hepatitis. C. *Lancet*, 337, 1354.

Esteban, J.I., Gonzalez, A., Hernandez, J.M., Viladomiu, L., Sanchez, C., Lopez-Talevera, J.C., Lucea, D., Martin-Vega, C., Vidal, X., Esteban, R. & Guardia, J. (1990) Evaluation of antibodies to hepatitis C virus in a study of transfusion-associated hepatitis. *New England Journal of Medicine*, 323, 1107–1112.

Evans, J.A., Pasi, K.J. & Hill, F.G.H. (1990) Consistently normal CD4, CD8 and immunoglobulin levels in hemophilic boys only treated with a new high purity factor VIII concentrate. (Abstract). *Blood*, 76, 420a.

Fletcher, M.L., Trowell, J.M., Craske, J., Pavier, K. & Rizza, C.R. (1983) Non-A, non-B hepatitis after transfusion of factor VIII in infrequently treated patients. *British Medical Journal*, 287, 1754–1757.

Kernoff, P.B.A., Miller, E.J., Savidge, G.F., Machin, S.J., Dewar, M.S. & Preston, F.E. (1987) Reduced risk of non-A, non-B hepatitis after a first exposure to wet-heated factor VIII concentrate. *British Journal of Haematology*, 67, 207–211.

Kuo, G., Choo, Q.L., Alter, H.J., Gitnick, G.L., Redeker, A.G., Purcell, R.H., Miyamura, T., Dienstag, J.L., Alter, M.J., Stevens, C.E., Tegtmeier, G.E., Bonino, F., Colombo, M., Lee, W.S., Kuo, C., Berger, K., Shuster, J.R., Overby, L.R., Bradley, D.W. & Houghton, M. (1989) An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science*, 224, 362–364.

Ludlam, C.A., Chapman, D., Cohen, H. & Litton, P.A. (1989) Antibodies to hepatitis C virus in haemophilia. *Lancet*, ii, 560.

Madhok, R., Gracie, A., Lowe, G.D.O., Burnett, A., Froebel, K., Follet, E. & Forbes, C.D. (1986) Impaired cell mediated immunity in haemophilia in the absence of infection with human immunodeficiency virus. *British Medical Journal*, 293, 978–980.

Makris, M., Preston, F.E., Trigger, D.R., Underwood, J.C.E., Choo, Q.L., Kuo, G. & Houghton, M. (1990) Hepatitis C antibody and chronic liver disease in haemophilia. *Lancet*, ii, 1117–1119.

Mannucci, P.M., Zanetti, A.R., Gringeri, A., Tanzi, E., Morfini, M., Messori, A., Tirindelli, M.C., de Biasi, R., Ciavarella, N. & Colombo, M. (1989) Long-term immunogenicity of a plasma-derived hepatitis B vaccine in HIV seropositive and HIV seronegative hemophiliacs. *Archives of Internal Medicine*, 149, 1333–1337.

Marcellin, P., Martinot-Peignoux, M., Boyer, N., Pouteau, M., Aumont, P., Erlinger, S. & Benhamou, J.P. (1991) Second generation (RIBA) test in diagnosis of chronic hepatitis C. *Lancet*, 337, 551–552.

Simmonds, P., Zhang, L.Q., Watson, H.G., Rebus, S., Ferguson, E.D., Balfe, P., Leadbetter, G.H., Yap, P.L., Peutherer, J.F. & Ludlam, C.A. (1990) Hepatitis C quantification and sequencing in blood products, haemophiliacs and drug users. *Lancet*, 336, 1469–1472.

Skidmore, S. (1990) Recombinant immunoblot assays for hepatitis C antibody. *Lancet*, i, 1346.

Tanaka, K., Hirohata, T., Koga, S., Sugimachi, K., Kanematsu, T., Ohryohji, F., Nawata, H., Ishibashi, H., Maeda, Y., Kiyokawa, H., Tokunaga, K., Irita, Y., Takeshita, S., Arase, Y. & Nishino, N. (1991) Hepatitis C and hepatitis B in the aetiology of hepatocellular carcinoma in the Japanese population. *Cancer Research*, 51, 2842–2847.