Hepatitis C virus in autoimmune liver disease in the UK: aetiological agent or artefact?

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Abstract

Hepatitis C virus antibody titres (anti-HCV) were measured in serum from 122 patients with autoimmune liver disease (96 with primary biliary cirrhosis and 26 with autoimmune chronic active hepatitis using three generations of enzyme immunoassay (EIA): first generation – Ortho, EIA1; second generation – Abbott, EIA2; and third generation – Murex, EIA3. Anti-HCV was below the positive cut-off level in all 26 autoimmune chronic active hepatitis patients for all tests, while seropositivity values in primary biliary cirrhosis were 31% (EIA1), 14% (EIA2), and 0% (EIA3). In primary biliary cirrhosis, anti-HCV values as measured by all three tests correlated positively with serum IgG concentrations, serum storage time, and a number of other indices of hepatic dysfunction. Multiple regression analysis showed that anti-HCV values were independently affected by both serum IgG and the length of storage time, although the magnitude of the effects varied between tests. When all three multiple regression models were applied to an extreme clinical example, however, EIA3 was least likely to give a false-positive result. The difference in test performance was emphasised further by examination of anti-HCV values in nine primary biliary cirrhosis patients (confirmed negative by recombinant immunoblot assay 2) in whom serial samples were tested (seven to 14 per patient, stored for one to 138 months). Apparent anti-HCV values (EIA1 and EIA2) increased with increasing serum storage time, but were unchanged when measured by EIA3. A similar pattern was evident in a limited study of five autoimmune chronic active hepatitis patients. Thus, in the largest study reported to date, there is no evidence of an important aetiological role for HCV in autoimmune chronic active hepatitis or primary biliary cirrhosis in the UK. The third generation of anti-HCV EIAs is the most reliable screening test, will avoid costly and unnecessary confirmatory testing, and may partly clarify the existing geographical heterogeneity in anti-HCV seroprevalence. An accurate diagnosis of past HCV infection is an important determinant in the choice of treatment in autoimmune chronic active hepatitis. As the second generation EIA is in widespread use and confirmatory testing is not always available, the effect of serum storage in addition to hyperglobulinaemia should be considered in the interpretation of positive results in autoimmune and in other types of chronic liver disease.

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The development of an enzyme immunoassay (EIA) in 1989 for antibodies to hepatitis C viral antigens (anti-HCV) in serum^{1,2} and the subsequent development of a recombinant immunoblot assay (RIBA)³ stimulated much investigation into the possible pathogenetic role of hepatitis C infection in chronic liver disease.

Published studies of type 1 autoimmune chronic active hepatitis (CAH) showed a wide variation in seroprevalence of anti-HCV as measured by first generation screening tests, unconfirmed by first generation RIBA (RIBA-1): 6% (USA),⁴ 14% and 40% (UK),^{5,6} 33-35% (Scandinavia),^{7,8} 44% (Spain),⁹ 50% (Japan),¹⁰ and 57% (France).¹¹ One Italian group which performed RIBA-1 tests reported that 57% of type 1 patients were positive.¹²

In type 2 autoimmune CAH, two Italian reports of RIBA-1 confirmed seropositive patients showed prevalence figures of 73-85%.^{12,13} In the study of Todros *et al*¹³ most of the patients who were given interferon experienced rapid clinical remission of their disease.

Reported seropositivity in primary biliary cirrhosis (PBC) has ranged from 0% (UK and France)^{5,6,11} to 12% (Italy),¹⁴ 13% (Japan),¹⁰ 25% (Norway),⁸ and 27% and 35% (Italy).^{15,16} One French group who reported 33% seroprevalence at initial screening was able to confirm only 1% by RIBA-1.¹⁷

As a result of these initial screening studies, the unexpectedly high prevalence in some countries suggested that HCV contributed to the pathogenesis of both PBC and autoimmune CAH; this has important therapeutic implications since a number of groups suggest some benefit from interferon treatment in chronic hepatitis C.^{13,18}

The specificity of the first generation assay that uses recombinant antigen c100-3 derived from non-structural regions 3 and 4 of the HCV genome was questioned, however, by McFarlane *et al*, who reported falsely high anti-HCV values in autoimmune CAH patients with hyperglobulinaemia.⁶ Other groups reported interference in the assay from superoxide dismutase antibodies,¹⁹ rheumatoid factors,²⁰ and paraproteinaemia.²¹ The possibility that sample storage might cause false-positive results has been raised^{8,17,22,23} but has not been systematically investigated in either CAH or PBC, and very few authors make any comment about duration of serum storage. Since most published studies have relied on stored sera, information on the importance of prolonged storage on anti-HCV is vital. For most study centres, confirmatory immunoblot tests are expensive and routine measurement of HCV-RNA is unavailable.

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Thus, it is of utmost importance to have a reliable screening test available.

Second generation EIAs are now routinely in use, although results from first generation tests are still being reported.²⁴ It has been claimed that the addition of new recombinant antigens derived from the core (c22-3) and non-structural regions 3 and 4 (c33c) allows detection of anti-HCV in an additional 10–20% of patients from high risk groups²⁵ and has decreased the length of the window period between HCV infection and seroconversion.²⁶ It was also hoped that the introduction of second generation tests would result in increased specificity, but the few studies of autoimmune liver disease which have been reported suggest that the problem of false-positive results persists, though the mechanism has not previously been investigated. Discrepancies in seroprevalence figures using only RIBA-2 confirmed cases show that geographical heterogeneity is still apparent (Table I).^{14 25 27 28}

A third generation test, which includes for the first time an antigen derived from the NS5 region in addition to antigens from the core and NS3 region, has recently become available from Murex.

This paper reports results from the largest group of patients with autoimmune liver disease examined to date. The prevalence of anti-HCV has been determined in 122 UK patients with PBC and autoimmune CAH using the Ortho first generation (EIA1), Abbott second generation (EIA2), and Murex third generation (EIA3) EIAs. Seropositive samples were retested in selected cases using the second generation Chiron four-antigen RIBA test (RIBA-2).²⁹ We have investigated the possible mechanism of false-positive anti-HCV tests by examining the relationship between anti-HCV values and parameters of inflammatory activity and hepatic dysfunction. In addition, we have investigated in detail the effect of prolonged serum storage on anti-HCV values in both disease groups.

Patients and methods

Ninety six patients with PBC were studied: 77 were investigated using EIA1 and all 96 were tested with EIA2 and EIA3. All had raised serum alkaline phosphatase activities, positive anti-mitochondrial antibody titres (>1:100), and histological features diagnostic of, or compatible with, primary biliary cirrhosis. Serum samples were stored at –20°C, with storage time varying from less than one month to 161 months.

Sera from 26 patients with CAH were tested for anti-HCV. Testing with EIA1 was performed on the day of collection (n=22) or after storage for less than two weeks at –20°C (n=4). Testing with EIA2 and EIA3 was carried out 33 months later after storage at –20°C. At presentation, all patients had histological features of CAH. None gave a history of drug or alcohol abuse. Serum from all patients showed normal copper oxidase activity and was negative for hepatitis B markers. In 22 patients, titres of antinuclear antibody or smooth muscle antibody, or both, exceeded 1:100 (autoimmune group).

In nine PBC and five autoimmune CAH patients, sequentially obtained serum samples

were tested for anti-HCV (PBC: stored 1–138 months (EIA1); 3–135 months (EIA2 and EIA3); CAH: stored 7–112 months (EIA1); 8–136 months (EIA2 and EIA3)).

Anti-HCV was measured using three EIAs: EIA1 – Ortho Diagnostics employing c100-3 (NS3/NS4); EIA2 – Abbott employing c200 (NS3/NS4) and c22 (core); EIA3 – Murex employing BHC28 (a fusion protein of three antigens from core, NS3 and NS5). Typical anti-HCV test cut-off levels measured in our laboratory were 0.57 (Ortho), 0.33 (Abbott), and 0.65 (Murex) optical density (OD) units. Samples which were initially positive were confirmed by retesting in duplicate. Eight seropositive PBC samples (by EIA1 and 2) were retested using the Chiron second generation RIBA (RIBA-2) employing four recombinant HCV antigens.¹²

Serum immunoglobulins, aspartate and alanine aminotransferases (AST, ALT), bilirubin, and albumin had been measured by routine techniques when samples were initially obtained.

Correlations between pairs of variables were assessed using Pearson correlation coefficients employing two-tailed tests of significance. A logarithmic transformation was used to obtain a Gaussian distribution of data points for all parameters with the exception of serum storage time (EIA-1) and albumin. The independent relationships between these variables and anti-HCV levels were further explored using multiple regression analysis.

Results

In the group of 77 patients with PBC tested by EIA1, anti-HCV values were positive in 24 (31%), and correlated positively with serum storage time (r=0.55, p<0.001). Anti-HCV values were positive in only two of 46 patients in whom serum had been stored for less than 18 months but were positive in 22 of 31 in whom serum had been stored for longer than 18 months (p<0.001: χ^2 with Yates's correction). Of the 24 patients with raised values, 38% had OD values more than twice the cut-off value.

Of the 96 PBC patients tested by the second generation assay, anti-HCV seroprevalence was 14% and values again correlated positively with the serum storage time (r=0.71, p<0.001). Of the 13 patients with raised anti-HCV levels, all had OD values less than twice the cut-off value.

No patients tested positive by the third generation assay. In the whole group of 96, however, there was a positive correlation

TABLE I Hepatitis C virus antibody seroprevalence in autoimmune liver disease: second generation screening test results

Country (disease type)	Positivity rate (%)	Confirmed (RIBA-2) (%)
Autoimmune chronic active hepatitis:		
Italy (type 1)	46	39
USA (type 1)	45	3
France (type 2)	76	49 (all HCV-RNA +ve)
Primary biliary cirrhosis:		
Italy	17	7
USA	44	6

RIBA = recombinant immunoblot assay; HCV = hepatitis C virus.

TABLE II Three models derived by multiple regression analysis showing parameters which have independent effects on hepatitis C virus antibody (anti-HCV), as measured by three generations of screening test. Units are in months (storage time) and g/l (IgG, IgA, albumin).

Ortho-1:
$\log_{10} \text{ anti-HCV} = -2.56 + 0.004 \text{ storage time}^{***} + 1.58 \log_{10} \text{ IgG}^{***}$
Abbott-2:
$\log_{10} \text{ anti-HCV} = -1.62 + 0.303 \log_{10} \text{ IgG}^{**} + 0.263 \log_{10} \text{ IgA}^{***} + 0.258 \log_{10} \text{ storage time}^{***}$
Murex-3:
$\log_{10} \text{ anti-HCV} = -2.29 + 0.00979 \text{ albumin}^* + 0.737 \log_{10} \text{ IgG}^{***} + 0.101 \log_{10} \text{ storage time}^*$

*p<0.05, **p<0.01, ***p<0.001.

TABLE III Hepatitis C virus (anti-HCV) antibody values as predicted by each of the three models followed by 95% prediction intervals (in brackets), assuming the clinical conditions of a 'worst case scenario' for potential false-positive anti-HCV results: serum IgG=30 g/l, serum IgA=7 g/l, serum albumin=25 g/l, and serum storage time=60 months or 120 months. Typical anti-HCV test cut-off levels measured in our laboratory are 0.57 (Ortho), 0.33 (Abbott), and 0.65 (Murex). Results printed in bold type represent positive anti-HCV titres

Storage time	Enzyme immunoassay		
	Ortho-1 (OD units)	Abbott-2 (OD units)	Murex-3 (OD units)
60 Months	1.006 (0.433-2.342)	0.324 (0.190-0.550)	0.165 (0.081-0.336)
120 Months	1.685 (0.703-4.035)	0.387 (0.227-0.659)	0.177 (0.087-0.360)

between the anti-HCV titre and the serum storage time ($r=0.27$, $p<0.01$).

A series of univariate analyses showed that anti-HCV as measured by all three tests correlated with serum IgG concentrations and a number of other indices of hepatic dysfunction. With multiple regression analysis, however, most of these correlations became non-significant. Variables which affect anti-HCV values independently are shown in Table II. Both serum IgG and storage time were independently associated with anti-HCV values as measured by all three assays, although the magnitude of the effects varied between assays. Additionally, anti-HCV, as measured by EIA2, was affected by serum IgA concentrations, and anti-HCV, as measured by EIA3, was affected by serum albumin concentrations.

Although these correlations are of statistical significance, their effect on anti-HCV values may be of little clinical relevance. To assess their

practical importance a theoretical 'worst clinical case' was examined in which predictions of the anti-HCV value were made on the basis of the three multiple regression models using an IgG concentration of 30 g/l, IgA 7 g/l, albumin 25 g/l, and serum storage times of 60 or 120 months (Table III). This example shows clearly that use of the Murex screening test was highly unlikely to give a false-positive result even under these extreme conditions.

In order to study further the effects of the serum storage time, nine PBC patients were studied sequentially. Mean IgG concentrations did not change significantly over the time that they were followed (median follow up of 96 months). However, anti-HCV titres, as measured by EIA1 and EIA2, rose progressively with increasing serum storage time, with r values for the nine individual patients ranging from 0.55-0.93. Thus, anti-HCV values were consistently higher in the earliest obtained, longest stored sera. An EIA positive sample from eight of these nine patients was retested using RIBA-2 and all eight were found to be negative. When the same nine patients were studied sequentially with the third generation Murex test, results showed that anti-HCV values remained below the cut-off point and were virtually unchanged regardless of increasing storage time (Fig 1).

Fresh serum samples were tested by EIA1 in all 26 patients with CAH. Further testing by EIA2 and EIA3 was performed after storage for 33 months. In every case, for all three tests, anti-HCV values were below the positive cut-off value. Even within this normal range, however, significant positive correlations were found between anti-HCV values (EIA1 and EIA3) and indices of inflammatory disease activity: serum IgG and AST (Table IV). Patient numbers were too small to perform multiple regression analysis and therefore these significant univariate associations may *not* be independent. The effect of storage time could not be examined in this group as the samples had all been stored for the same length of time but in five patients in whom sequential serum samples were tested, anti-HCV values (EIA1 and EIA2) rose with increasing storage time in four, but remained unchanged and were always well below the cut-off point when tested by EIA3 (Fig 2).

Discussion

There now seems to be general agreement that the hepatitis C virus plays some part in autoimmune CAH in Italy, and may even act as the antigenic trigger in autoimmune liver disease. Previous studies of HCV based on the use of first generation assays have reported an unexpectedly variable frequency of anti-HCV seropositivity in autoimmune liver disease, and in some results re-examined by RIBA, a high level of apparent false-positives. McFarlane *et al* suggested hyperglobulinaemia as the source of the artefact with non-specific binding of protein to the assay solid phase.⁶

It was hoped that the introduction of second generation screening tests would reduce the incidence of false-positive results, clarifying the apparent geographical heterogeneity. Second

Figure 1: Hepatitis C virus antibody (anti-HCV) levels in three primary biliary cirrhosis patients followed over 12 years. The dashed line represents the cut-off level: 0.57 (Ortho), 0.33 (Abbott), and 0.65 (Murex). OD=optical density.

TABLE IV Univariate regression analysis in autoimmune chronic active hepatitis; correlations with hepatitis C virus values

	Ortho-1			Abbott-2			Murex-3		
	No	r	p Value	No	r	p Value	No	r	p Value
IgG	26	0.52	p<0.01	24	0.17	NS	24	0.85	p<0.001
AST	26	0.54	p<0.01	25	0.06	NS	25	0.78	p<0.001
ALT	26	0.34	NS	25	0.04	NS	25	0.69	p<0.001

AST=aspartate aminotransferase; ALT=alamine aminotransferase.

generation testing has resulted in improved sensitivity in high risk groups but there are still discrepancies in figures reported for lower risk groups and two studies have questioned the specificity of the test particularly in autoimmune disease.^{14 25} No clear explanation currently exists for the divergent findings of both first and second generation tests. The clinician's dilemma hinges on whether the anti-HCV assays will help in the selection of patients for anti-viral treatment. The serious consequences of inadvertently treating patients with true autoimmune disease with interferon instead of steroids have been well documented.^{30 31} Since confirmatory tests may not always be available there is an urgent need for screening assays which minimise the possibility of false-positive results.

In our study of autoimmune CAH patients, the prevalence of anti-HCV in sera, whether freshly obtained or stored frozen for 33 months, using all three generations of test, was zero. Most of our patients with CAH had clinically inactive disease with normal or only slightly raised serum IgG values; this probably accounts for our finding of zero positivity compared with the 40% observed by McFarlane *et al*, also in patients from the UK.⁶ Sequential anti-HCV analysis of serial samples from a small autoimmune CAH group, which had been stored over a longer period (up to 136 months) showed a pattern of

rising anti-HCV values in four out of five cases with EIA1 and EIA2; no such rise was evident, however, with EIA3.

The overall incidence of anti-HCV in our group of primary biliary cirrhosis patients decreased with each generation of screening test from 31% (EIA1), to 14% (EIA2), to 0% (EIA3). Full analysis suggests that the positive results found with EIA1 and EIA2 are 'false-positives' and arise for two reasons. In all assay systems multiple regression analysis showed positive, independent links between anti-HCV and serum IgG and also, for the first time, with serum storage time. By applying multiple regression models to a theoretical 'worst clinical case' for all three tests, we have shown that from a practical viewpoint, use of the third generation test is highly unlikely to give a false-positive result. In addition, sequential analysis of nine primary biliary cirrhosis patients, confirmed as anti-HCV negative by RIBA-2, showed that apparent anti-HCV values (EIA1 and EIA2) rose with increasing storage time and eight became positive, independent of changes in inflammatory activity or hepatic function. Sequential anti-HCV analysis of the same patients by EIA3 resulted in no change in anti-HCV values despite storage for up to 136 months.

We have witnessed an explosion of interest in HCV and the application of assays to establish HCV prevalence in chronic liver disease. Most groups have moved on to second generation screening tests, but high levels of false-positives are still apparent and geographical heterogeneity is yet to be explained. We propose that the use of stored samples in retrospective studies in addition to differences in serum IgG values may account for some of the variability in the incidence of HCV seropositivity in previous reports. We have shown that use of the Murex screening EIA eliminated the problem of false-positive results in the two forms of liver disease in the UK that are classically thought to have an autoimmune basis.

The introduction of interferon treatment for HCV positive CAH now makes the accurate assessment of HCV status in every patient with CAH of great importance. In the current situation where routine measurement of HCV-RNA in serum is not a likely prospect for most study centres and confirmatory immunoblot tests are expensive and may not be readily available, it is highly desirable to have available a frontline screening test that is both sensitive and specific. We propose that the third generation of EIA is the most reliable screening test to date. From the present study, the largest so far reported, we find no evidence for a significant aetiological role for HCV in primary biliary cirrhosis or autoimmune type 1 CAH in the UK.

Figure 2: Hepatitis C virus antibody (anti-HCV) levels in five patients with autoimmune chronic active hepatitis followed over 12 years. OD=optical density.

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