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Will the Real Hepatitis C Stand Up?

TO the casual observer, the claims and counter-claims with respect to the discovery of serological detection systems for non-A non-B (NANB) hepatitis must be confusing. Should the latest announcement[1,2] of yet another candidate be taken more seriously? The key to answering this question lies not only in an analysis of the specificity and reproducibility of the test results but also in an appreciation of the background to the work, since this discovery builds on a wealth of previous knowledge carefully assembled by clinical and basic research groups over many years.

The essential groundwork began in the 1970s, with the clinical recognition and description of cases of post-transfusion hepatitis which, on serological testing, were not due to hepatitis A or B infection. This long-incubation hepatitis (60 days) was notable for its mild, often subclinical, presentation but high rates of chronicity and progression to cirrhosis. A similar illness could also be transmitted by blood products such as clotting factors.[3] An important series of studies in chimpanzees clearly showed the presence of a transmissible agent in blood products and in serum from carrier blood donors.[4] The agent was sensitive to organic solvents, and less than 80 nm in diameter as assessed by filtration. Thus, even without conventional virological studies in vitro or knowledge of the genome, it was possible for Bradley[5] to make a calculated guess that the NANB agent could be a small togavirus-like envelope RNA virus.

Meanwhile, many groups, using the principles that had worked well for hepatitis A and B, were making unsuccessful attempts to design serological systems.[4] What was particularly frustrating was that the resulting "tests" often appeared to respond to something in suspect sera but were, on more rigorous investigation, either unreproducible or clearly non-specific,[6] or were detecting normal liver antigens whose production was stimulated by the infection.[7]

Houghton and colleagues,[1] working at the Chiron Corporation in California, adopted a new approach, consistent with Bradley's view of the virus. These researchers used large quantities of a well-characterised highly infectious chimpanzee plasma as a source of virus. The virus was concentrated into pellets by ultracentrifugation, the nucleic acid

extracted, and cDNA synthesised from both RNA and DNA by reverse transcriptase. Cloning into the bacteriophage λgt11 provided a cDNA library that could be screened for expression of an antigen detected by serum from a patient with chronic NANB infection. After about a million clones had been screened, one phage-infected bacterial colony was found to be producing a protein that reacted with the patient's serum. The 155 base-pair insert in this clone was then cut out and used as a hybridisation probe to extract, from the original library, a larger (353 base-pairs) overlapping clone. Neither of the strands in this double-stranded cloned DNA hybridised to human or chimpanzee DNA, but one of the strands was homologous with a single-stranded RNA, containing up to 10 000 nucleotides, in the virus-rich pellet from the original chimpanzee serum. The cDNA also hybridised to RNA from infected, but not normal, chimpanzee liver. Examination of the nucleotide sequence in this and two other overlapping clones suggested a single continuous translational open reading frame; the next step was to insert this DNA sequence into a plasmid containing the human superoxide dismutase gene in order to express the open reading frame in a system that could generate large quantities of the resultant polypeptide. The fusion protein so produced, when expressed in bacteria, reacted on immunoblotting with serum from seven of eleven patients with NANB infection, but with none of ten control sera. The demonstration of seroconversion in four chimpanzees experimentally infected with the NANB innoculum, but not in seven with hepatitis A or B infection, provided further encouragement with respect to the specificity of this antigen/antibody system.

Higher yields of the fusion protein (containing 363 viral aminoacids) were obtained from recombinant yeast cultures and used to coat microtitre wells. These could then form the basis of a radioimmunoassay for antibody in test sera.[2] Initial tests were conducted blind, with a well-characterised panel of NANB sera that have proved to be the undoing of several previous candidate test system.[8] The new test cleared this first hurdle with ease. Of seven NANB serum samples shown to be infectious in chimpanzees, six gave clearly positive results in the assay and the one negative sample came from an individual in the acute pha[illegible] post-transfusion NANB hepatitis. Seven n[illegible]

infectious control sera were all negative. In serial samples from ten well-characterised cases of post-transfusion NANB hepatitis, antibody appeared about 6 months after the transfusion in all but one, and the assay was positive in 71% of a further twenty-four patients with post-transfusion NANB hepatitis. It has always been unclear whether community-acquired NANB hepatitis without an apparent source was due to the same agent as that causing post-transfusion NANB infection, and it is of some interest that 58% of fifty-nine cases of the sporadic type were also positive at some stage after the clinical onset of acute hepatitis.

In this issue we publish four series of results with the new test system. The Spanish (p 294) and Dutch (p 297) workers used the prototype radio-immunoassay originally developed by the Chiron researchers whereas the two German groups who report their data in our correspondence columns (p 324) used the second generation enzyme-linked immunosorbent assay marketed by Ortho (the antigen is the same for both assays). In general, the results support the sensitivity and specificity of the test system, and underline both the urgency of making the test system available for blood donor screening, and the importance of despositing the sequence of the viral genome in the GenBank database where it would be available to the wider scientific community.

There are many questions yet to be settled. How many other agents are involved? Is there a short-incubation agent? What is the cause of the negative cases of sporadic NANB in the community? Are any of these related to the enterically transmitted NANB virus so common in the Indian subcontinent?[3] Will the assay help to select patients for interferon treatment? Meanwhile, this new test system represents a clinically important advance in the detection of one of the causal agents of NANB hepatitis, which has the characteristics of either a togavirus or a flavivirus. It would be logical to confer the title of hepatitis C on the newcomer.

Finally, it is worth re-emphasising that the complex molecular engineering, although almost routine as far as the technology is concerned, could not have succeeded and would probably never have been conceived without the detailed clinical groundwork and chimp[illegible] studies that established the nature of the disease, broadly defined the agents involved, and

provided the essential sera. The hepatitis C story is an excellent example of the need for a broad approach to research funding. The flash of the diamond may catch the eye but the setting is often the key to the beauty of the display.

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HEPATITIS C VIRUS ANTIBODIES AMONG RISK GROUPS IN SPAIN

J. I. ESTEBAN, R. ESTEBAN, L. VILADOMIU, J. C. LÓPEZ-TALAVERA, A. GONZÁLEZ, J. M. HERNÁNDEZ, M. ROGET, V. VARGAS, J. GENESCÀ, M. BUTI, J. GUARDIA

Liver Unit and Transfusion Medicine Unit, Department of Internal Medicine, Hospital Vall d'Hebron, Universitat Autonoma, Barcelona, Spain

M. HOUGHTON, Q-L. CHOO, G. KUO

Chiron Corporation, Emeryville, California, USA

Summary The frequency of hepatitis C virus (HCV) infection in Spain was assessed by means of a recombinant-based immunoassay for serum anti-HCV antibodies. 836 serum samples were tested from 676 patients selected according to their risk of bl ome viral infections and presence of liver disease. Amo. patients at high risk of infection (with or without liver disease) anti-HCV antibodies were found in 85% of prospectively followed patients with post-transfusion non-A, non-B hepatitis, 62% of patients with chronic hepatitis or cirrhosis and a history of blood transfusion, 70% of haemophiliacs receiving replacement therapy, 70% of intravenous drug abusers, and 20% of haemodialysis patients. Only 8% of homosexual men infected with human immunodeficiency virus and 6% of female contacts of drug abusers were positive. Among patients with liver disease and no history of parenteral exposure to blood, anti-HCV antibodies were detected in 38% with cryptogenic, alcoholic, or primary biliary cirrhosis and in 44% with chronic active hepatitis. Among healthy subjects without risk factors for hepatitis the overall prevalence of anti-HCV was 1·2%.

Introduction

MORE than 90% of transfusion-associated hepatitis cases worldwide are attributed to non-A, non-B hepatitis (NANBH).[1-6] NANBH accounts for a substantial proportion of hepatitis cases among patients with frequent parenteral exposure to blood (eg, haemophiliacs,[7] intravenous drug abusers,[8] and haemodialysis patients[9]) and for more than 25% of cases of sporadic hepatitis without obvious percutaneous exposure.[10,11] Researchers at the Chiron Corporation in California have lately isolated a blood-borne NANBH agent, designated hepatitis C virus (HCV).[12] Virus isolation led to the development of a recombinant-based immunoassay for detection of specific anti-HCV antibodies. Initial evaluation of one such assay[13] in serum samples from post-transfusion NANBH cases and implicated donors in the USA and in sera from Italian and Japanese patients with acute and chronic NANBH confirmed that HCV is the major causal agent of NANBH. To estimate the prevalence of HCV infection in Spain, we have studied 836 serum samples from three categories of patients—326 at high risk of viral hepatitis (group I); 60 with biopsy-proven chronic liver disease but no apparent risk factor for viral hepatitis (group II); and 290 healthy subjects without liver disease and with no history of percutaneous exposure to blood (group III).

Subjects and Methods

Group I (High-risk Patients with or without Liver Disease)

Post-transfusion hepatitis and chronic NANBH patients.—54 patients with post-transfusion NANBH who had been enrolled in a prospective study between 1978 and 1984 were studied.[6] 3–4 samples (obtained at 1–2, 6–12, and 20–32 weeks post-transfusion) we assayed in 40 patients; 5–10 serial samples (obtained shortly a sfusion, during the acute phase, and at variable intervals

during convalescence) in 10 patients; and 2 samples (1–2 and 20–25 weeks post-transfusion) in 4 patients. Samples were also tested from 8 symptom-free patients with biopsy-proven chronic active hepatitis (6 cases) or cirrhosis (2) who had a history of blood transfusion as the only known origin of their liver disease.

Haemophiliacs.—Serum samples had been obtained in 1985 from 97 patients aged 1 to 55 years (mean 20·1, SD 14) with coagulation disorders (73 haemophilia A; 7 haemophilia B; 11 von Willebrand disease; 6 other deficiencies) regularly attending the haemophilia unit at our centre. 12 patients had never been treated whereas the remaining 85 were receiving regular replacement therapy (61 factor VIII concentrate; 10 cryoprecipitate; 8 prothrombin complex; 6 fresh frozen plasma). 48 patients had antibodies against human immunodeficiency virus (HIV) but none had symptoms of immunodeficiency when the sample was obtained.

Intravenous drug abusers.—Samples were obtained from 83 drug abusers (60 M, 23 F, mean age 26·5 years, range 18–35) with a mean duration of drug use of 6·1 (SD 3·5) years. All but 1 had been referred to the hospital because of HIV-seropositivity (6 patients had AIDS, 3 AIDS-related complex, and the rest were symptom-free). All but 3 admitted frequent needle sharing. A history of hepatitis of unknown type was reported by 34 (41%).

Homosexual men and female contacts of intravenous drug abusers.—There were 26 homosexual men (96% anti-HIV positive) who had engaged in active and passive rectal intercourse and had a mean of 10·8 sexual partners/year. 10 patients had a history of hepatitis; 4 were chronic HBsAg carriers; and 15 were positive for anti-HBc. 10 of the 18 female partners of drug abusers were anti-HIV positive and 2 were positive for anti-HBc. All had had unprotected regular sexual intercourse with at least 1 drug abuser for a mean of 3·2 years. None had a history of hepatitis or intravenous drug abuse. In 5 cases the partner who abused drugs was later found to be anti-HCV positive.

Haemodialysis patients.—There were 42 patients on chronic haemodialysis who were seronegative for all HBV markers; 30 had received a mean of 2·6 (SD 2·4) blood units whereas the remainder had never been transfused.

Group II (Low-risk Patients with Liver Disease)

There were 34 patients (3 M, 31 F; aged 13–76 years, mean 54) with biopsy-proven chronic active hepatitis (20) or active cirrhosis (14). 14 patients were symptom-free and had been studied because of persistently increased alanine aminotransferase (ALT) values, 4 had arthritis, 4 presented with symptoms of acute hepatitis, and 11 presented with jaundice, ascites, or variceal bleeding. 32 patients had antinuclear antibodies, in most cases together with other autoantibodies (anti-liver-membrane antigen, anti-smooth-muscle, or anti-gastric-parietal-cell). None had been transfused or recalled percutaneous exposure to blood. Mean ALT level was 98 U/l (range 11–599, normal range 8–25).

3 patients with primary biliary cirrhosis, 8 with cryptogenic cirrhosis, and 15 men (mean age 61·5) with alcoholic cirrhosis were also included in this group. Most patients had been admitted to hospital because of ascites or variceal bleeding. None had a history of transfusion before the test sample was obtained.

Group III (Low-risk Healthy Subjects)

There were 49 unselected blood donors and 241 healthy pregnant women from whom blood samples had been taken at delivery as part of a vaccination programme of babies born to HBsAg carrier mothers. Of the pregnant women, 98 were healthy HBsAg carriers with repeatedly normal ALT values on several occasions for more than a year who had never received blood or had any known risk factor for hepatitis.

Methods

Anti-HCV testing.—All samples were shipped in dry ice to the assay laboratory and tested under code in duplicate. Anti-HCV was assayed with a microtitre radioimmunoassay in which a recombinant HCV polypeptide obtained in yeast was used to capture specific viral antibodies, as previously described.[13] Samples were considered positive when the counts per minute (cpm) were above the mean plus 3 SD cpm of 138 blood donor control sera (> 3549 cpm).

Statistical analysis.—Statistical methods included Fisher's exact test to compare relative frequencies within groups and Student's *t*-test to evaluate the significance of differences among groups. All p values were two-sided.

Results

Group I

Post-transfusion hepatitis and chronic NANBH patients.—Unequivocal seroconversion was documented in 42 patients (78%). In 4 additional patients low-level anti-HCV antibodies were present from the first early post-transfusion sample, presumably owing to passive transfer of antibody from the donor. Thus, 85% of our post-transfusion NANBH cases became anti-HCV positive (table 1). Of the 34 seroconverters from whom enough serial samples were available, anti-HCV was first detected during the acute phase of infection (6–8 weeks post-transfusion) in 13; between 20 and 26 weeks post-transfusion in 19; and at 38 and 52 weeks, respectively, in 2. In 6 of the 8 patients who remained seronegative the last tested sample had been

TABLE 1—ANTI-HCV ANTIBODIES IN SPANISH PATIENTS ACCORDING TO RISK OF HEPATITIS AND PRESENCE OF LIVER DISEASE

Group	Tested	Anti-HCV positive (%)
Group I		
Post-transfusion NANBH	54	46 (85)
Chronic NANBH	8	5 (62)
Intravenous drug abusers	83	59 (70)
Haemophiliacs	97	62 (64)
Haemodialysis patients	42	8 (20)
Homosexual men	26	2 (8)
Female contacts of drug abusers	18	1 (6)
Group II		
Autoimmune chronic active hepatitis	34	15 (44)
Primary biliary cirrhosis, alcoholic and cryptogenic cirrhosis	26	10 (38)
Group III		
Healthy pregnant women	241	3 (1·2)
Random blood donors	49	0 ..

obtained a mean of 26 weeks after transfusion (range 20–32). 5 of the 8 patients with chronic active hepatitis or cirrhosis and a history of blood transfusion were anti-HCV positive.

Haemophiliacs.—64% of patients in this group were anti-HCV positive (table I). 60 of the 85 treated patients (70%) were anti-HCV positive. By contrast, of the 12 who had never been treated, only 2 had anti-HCV antibodies ($p < 0.05$). 1 of the latter was a year-old infant whose mother's status was unknown. Among the treated patients, anti-HCV positivity was unrelated to age, type of haemophilia (71% haemophilia B, 68% haemophilia A, 58% von Willebrand disease, and 50% of patients with other deficiencies), type of replacement therapy (factor VIII 70%, cryoprecipitate 60%, prothrombin complex 87%, and fresh frozen plasma 50%) or serological status for HIV (anti-HIV positive 71%, anti-HIV negative 58%).

Intravenous drug abusers.—70% of patients were positive (table I). Overall, there was no difference between anti-HCV positive and negative addicts with respect to age (26·6 [SD 4·5] *vs* 25·8 [4·1], respectively), mean duration of drug abuse (6·4 [3·6] *vs* 5·5 [3·3]) and mean ALT levels (61·7 [53] *vs* 47·5 [38·4]).

Haemodialysis patients.—8 of the 42 tested patients were positive. Of the 30 patients who had received blood transfusions in the past, 7 were positive *vs* 1 of the 12 without a history of transfusion (difference not statistically significant).

Homosexual men and heterosexual contacts of intravenous drug abusers.—8% of homosexual men and 6% of female contacts of drug abusers had anti-HCV antibodies (table I). None of the 5 contacts of abusers known to be HCV infected had anti-HCV. Comparison of serum markers for HIV, hepatitis B virus (anti-HBc), and HCV in both groups is shown in table II.

Group II

Autoimmune chronic active hepatitis.—15 patients (44%) had circulating antibodies to HCV. Antibody-positive patients were significantly older and had lower mean ALT levels than those who were anti-HCV negative (table III).

Primary biliary cirrhosis, cryptogenic cirrhosis, and alcoholic cirrhosis.—Anti-HCV antibodies were detected in 1 of 3 patients with primary biliary cirrhosis, in 2 of 8 with cryptogenic cirrhosis, and in 7 of 15 with alcoholic cirrhosis. Overall frequency of anti-HCV in this group was 38%.

TABLE II—SEROLOGICAL STATUS FOR HIV (ANTI-HIV), HEPATITIS B VIRUS (ANTI-HBC), AND HCV (ANTI-HCV) AMONG HOMOSEXUAL MEN AND FEMALE CONTACTS OF INTRAVENOUS DRUG ABUSERS

Subjects	Anti-HIV positive (%)	Anti-HBc positive (%)	Anti-HCV positive (%)
Homosexuals	25/26 (96)	15/	2/26 (8)
Heterosexual contacts of drug abusers	10/18 (56)	2/18 (11)	1/18 (6)

TABLE III—CHARACTERISTICS OF PATIENTS WITH AUTOIMMUNE LIVER DISEASE ACCORDING TO ANTI-HCV STATUS

Characteristic	Anti-HCV positive (n = 15)	Anti-HCV negative (n = 19)	p
Mean age (range)	60·9 (41–76)	47·3 (13–76)	p < 0·01
Symptoms at diagnosis			
None	7	7	NS
Liver-related*	5	8	NS
Mean ALT (U/l) (2 SD)†	53·8 (40·1)	128 (68)	p < 0·05
Mean total immunoglobulin (2 SD; range)	2·05 (1·1; 1·3–4·4)	2·98 (1·8; 1·0–6·6)	NS
Autoantibodies (% positive)			
Antinuclear antibodies	13	19	NS
Other‡	17	17	NS
Liver histology			
Chronic active hepatitis	9	12	NS
Active cirrhosis	6	7	NS

*Ascites, variceal bleeding, or symptoms of acute hepatitis.
†Mean of three consecutive values per patient taken to obtain group mean ALT.
‡Anti-liver-membrane antigen, anti-smooth-muscle, anti-gastric-parietal-cell, or anti-reticulin.
NS = not significant.

Group III

Antibodies to HCV were detected in 2 of 143 non-carrier pregnant women, 1 of 98 HBsAg carrier women, and none of 49 random blood donors. Overall frequency of anti-HCV in this group was 1·2%.

Discussion

Our results show that HCV accounts for most cases of post-transfusion hepatitis in Spain. Although seroconversion may occur during the acute phase of the infection (in about a third of cases), in more than half of our patients anti-HCV antibodies were first detected 4–6 months after transfusion, and in some the antibody response was considerably later. This delayed response could explain why anti-HCV was not detected in all our post-transfusion cases. In 6 of the 8 seronegative cases the last tested sample had been obtained between 20 and 32 weeks after transfusion, so we might have underestimated seroconversion by almost 10%. In the remaining 2 patients there was a very long interval between the acute and convalescent samples tested (more than a year). Since HCV antibodies may disappear with time (H. Alter, personal communication), as observed in 1 of our patients, transient seroconversion might have been missed because of

infrequent sampling. The possibility that these seronegative patients did not have viral hepatitis or that their hepatitis was caused by a different agent, although unlikely, cannot be excluded.

The high frequency of anti-HCV antibodies among haemophiliacs and drug abusers was not unexpected. Among treated haemophiliacs seropositivity for anti-HCV was independent of age, type of haemophilia, type of replacement therapy, or serological status for HIV. Similarly, among intravenous drug abusers, presence of anti-HCV was independent of age, duration of drug abuse, absolute number of T-helper lymphocytes (data not shown), or clinical stage of HIV infection. However, within this group, mean ALT values of anti-HCV positive and negative patients (after excluding those with chronic HBV infection) did not differ significantly. 48% of patients in the anti-HCV negative group had abnormal ALT values in the absence of active HBV infection; liver biopsy was done in 5 patients in this group and histological examination showed chronic active hepatitis. This finding rises the possibility of chronic seronegative anti-HCV infection or chronic hepatitis by a different viral agent, although cryptic HBV infection or toxic hepatitis cannot be excluded.

The 20% seropositivity among haemodialysis patients without a history of HBV exposure seems to be almost exclusively related to blood transfusion (7 of the 8 who were anti-HCV positive had received blood). Although homosexual men have a high risk of HBV infection, the frequency of anti-HCV among HIV-infected homosexuals in our study was low—58% of the homosexuals tested were anti-HBc positive whereas only 2 (8%) had anti-HCV antibodies. The similarly low frequency (6%) of anti-HCV among female contacts of intravenous drug abusers (5 of whom were known to be anti-HCV positive) seems to indicate that HCV is not readily transmitted by sexual contact.

Perhaps the most important finding of our study is the high prevalence of anti-HCV among patients in group II. 30% of patients with chronic active hepatitis or cirrhosis of unknown or alcoholic origin and 44% of those with autoimmune chronic active hepatitis had anti-HCV in the absence of exposure to blood. Since the finding of anti-HCV seems to be associated with chronic infection, HCV may be important in the pathogenesis of almost half of the patients whose liver disease is currently attributed to non-viral causes. For patients with chronic active hepatitis and markers of autoimmunity who are infected with HCV, it remains to be established whether autoimmune manifestations are induced by viral infection or whether the association is coincidental. In any event, the high frequency of infection among these patients would influence management: HCV testing should now become mandatory in the routine evaluation of these patients. The seroprevalence of anti-HCV among healthy subjects in our study does not differ significantly from that reported in preliminary estimates from the USA.[13]

Correspondence should be addressed to J. I. E., Liver Unit, Department of Internal Medicine, Hospital Vall d'Hebron, Paseo Vall d'Hebron s/n, 08035-Barcelona, Spain.

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