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An Assay for Circulating Antibodies to a Major Etiologic Virus of Human Non-A, Non-B Hepatitis

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A specific assay has been developed for a blood-borne non-A, non-B hepatitis (NANBH) virus in which a polypeptide synthesized in recombinant yeast clones of the hepatitis C virus (HCV) is used to capture circulating viral antibodies. HCV antibodies were detected in six of seven human sera that were shown previously to transmit NANBH to chimpanzees. Assays of ten blood transfusions in the United States that resulted in chronic NANBH revealed that there was at least one positive blood donor in nine of these cases and that all ten recipients seroconverted during their illnesses. About 80 percent of chronic, post-transfusion NANBH (PT-NANBH) patients from Italy and Japan had circulating HCV antibody; a much lower frequency (15 percent) was observed in acute, resolving infections. In addition, 58 percent of NANBH patients from the United States with no identifiable source of parenteral exposure to the virus were also positive for HCV antibody. These data indicate that HCV is a major cause of NANBH throughout the world.

VIRAL HEPATITIS COMMONLY OCCURS in the absence of serologic markers for such known hepatotropic agents as hepatitis A virus (HAV), hepatitis B virus (HBV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV) (*1–4*). Termed non-A, non-B hepatitis (NANBH), this entity represents greater than 90% of transfusion-associated hepatitis cases in the United States, and up to 10% of transfusions have been estimated to result in NANBH (*5, 6*). More recently, the frequent occurrence of NANBH in the absence of any obvious parenteral exposure has been well documented (*7–9*). Whereas acute disease is often subclinical, at least half of NANBH infections result in chronic hepatitis, which may result in cirrhosis in approximately 20% of cases (*10*). A potential association with hepatocellular carcinoma has also been proposed (*11*). Because of the frequency and severity of NANBH, there is an urgent need to develop a direct diagnostic test for the causative agent or agents. We have recently cloned the genome of a NANBH agent (*12*), designated the hepatitis C virus (HCV), and now report the development and use of a recombinant-based assay for HCV antibodies.

Three overlapping clones were isolated by means of the cDNA in HCV clone 5-1-1, which was used as a hybridization probe to the original cDNA library (*12*). These clones have one common open reading frame (ORF) extending throughout them that encodes part of a viral antigen associated with NANBH (*12*). This continuous ORF was reconstructed from these clones and then expressed in yeast (*13*) as a fusion polypeptide with human superoxide dismutase (SOD), which facilitates the efficient expression of foreign proteins in yeast and bacteria (*13–15*). In this way, a SOD/HCV polypeptide (C100-3) containing 363 viral amino acids was synthesized at high levels (~4% total protein) in recombinant yeast. After solubilization and purification, C100-3 was used to coat the wells of microtiter plates so that circulating HCV antibodies in blood samples could be captured and measured. Detection of bound antibody was achieved with a radioactive second antibody.

Initially, to test the specificity and sensitivity of this assay, sera of known NANBH infectivity was assayed in a blind fashion (Table 1). This panel of well-pedigreed and well-characterized samples has been accepted widely as a crucial test of the validity of putative specific assays for NANBH (*16*). Of seven NANBH serum samples shown to be infectious in chimpanzees, all but one gave very high signals in the assay as compared to the results obtained with sera from two control patients with alcoholic hepatitis or primary biliary cirrhosis and five non-infectious normal blood donors. These results were reproducible in quadruplicate analysis (Table 1). The only proven infectious sample that was negative in the assay was obtained from an individual in the acute phase of post-transfusion NANBH (PT-NANBH), although another acute-phase serum of unproven infectivity was similarly negative. A blood donor implicated in transmission of NANBH but whose serum was of equivocal infectivity in chimpanzees was also found negative in this assay. Thus, the data from this panel of sera indicates a high sensitivity and specificity of the antibody assay for blood-borne NANBH. No other assay evaluated by this panel has achieved this degree of specificity and sensitivity (*16*).

Next, we assayed matched blood donor and prospectively obtained recipient sera from ten well-characterized cases of chronic PT-NANBH in the United States. The results of the HCV antibody assays of sequential samples taken at 3-month intervals from each recipient during the development of NANBH and in stored samples from the corresponding donors are shown (Table 2). Each of the ten recipients seroconverted against HCV during the course of disease, although seroconversion in case 4 was marginal and not apparent until 12 months after transfusion. In contrast, seroconversion against HCV was not observed in prospectively studied individuals infected with other viral hepatitis agents. Antibody seroconversion was generally detectable within 6 months of transfusion. The prolonged interval to antibody development may explain the observed absence of HCV antibodies in the acute-phase samples assayed in Table 1.

With one exception, significant levels of HCV antibody were detected in at least one

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Table 1. Detection of HCV antibodies in proven infectious blood samples. Assays were performed (*22*) under code and in quadruplicate on a panel (*16*) containing sera from three patients with biopsy-proven chronic PT-NANBH, three implicated blood donors, and one patient with acute PT-NANBH, all of which had been proven to transmit NANBH to chimpanzees. Also included were sera from a patient with acute NANBH and a donor thrice implicated in NANBH, each of which were equivocally infectious in the chimpanzee. Control sera were assayed from five normal blood donors who had each donated blood on at least ten occasions without the development of NANBH in the recipients, from a patient with alcoholic hepatitis, and from an individual with primary biliary cirrhosis. Sera scoring positive in these assays were negative when purified SOD was used to coat wells instead of C100-3. Such samples were also positive in immunoblot analyses containing recombinant HCV polypeptides, but not SOD alone (*12*).

Serum	Counts per minute			
Proven infectious in chimp				
Chronic NANBH patients				
1 (PT-NANBH)	31,962	32,107	32,121	28,584
2 (PT-NANBH)	22,871	17,483	21,623	19,863
3 (PT-NANBH)	25,381	20,983	21,039	20,047
Acute PT-NANBH patient	909	726	767	580
Implicated blood donors				
1	40,883	33,521	35,870	34,526
2	25,812	23,512	26,476	23,723
3	31,495	30,907	33,723	33,043
Unproven infectivity in chimp				
Acute PT-NANBH patient	1,207	740	1,786	1,489
Implicated blood donor	590	469	477	461
Pedigreed normal controls				
Blood donors				
1	998	775	647	584
2	887	632	561	469
3	591	446	459	327
4	634	533	758	649
5	584	531	553	429
Disease controls				
Alcoholic hepatitis	842	571	586	566
Primary biliary cirrhosis	915	1,118	741	750

Table 2. Detection of HCV antibodies in the blood donors and recipients of ten cases of chronic PT-NANBH from the United States. There were 138 blood donations of apparent negativity that closely followed a normal distribution with a mean of 1536 cpm (range, 187 to 3097 cpm) and a standard deviation (SD) of 671 cpm. Samples >3549 cpm (mean + 3 SD) are considered positive. All prospectively studied blood recipients developed chronic NANBH as diagnosed by the persistent elevation of serum ALT levels (>6 months) in the absence of immunoglobulin M antibody to HAV, HBV surface antigen (HBsAg), antibody to HBsAg and HBcAg, and serologic markers for CMV and EBV infection. Biopsies from all ten patients confirmed the diagnosis of chronicity. Recipient sera were assayed at 3-month intervals (0 represents a sample obtained immediately before transfusion). Control samples consisted of sera from a prospective study of male homosexuals (*23*) that were assayed for up to 1 year after the onset of hepatitis as a result of infection with either HAV (18 cases), HBV (20 cases), or CMV (5 cases). None of these disease controls showed positive seroconversion to anti-HCV. The results of every positive donor unit are shown.

Case	Number of donors per transfusion	Anti-HCV assay (cpm)				
		Positive donors	Recipients (months)			
			0	3	6	12
1	18	3,910	1,870	3,220	13,120	26,780
2	18	4,590 3,800	2,530	1,170	11,400	20,750
3	13	6,140	1,800	1,850	14,990	4,720
4	18	None	1,430	1,370	750	4,260
5	16	24,420	2,230	790	13,960	22,020
6	11	6,080 25,600	2,100	10,160*	21,490	24,900
7	15	15,970	2,120	2,090	10,470	16,140
8	20	13,240	1,920	2,860	8,160	22,510
9	8†	32,790	3,370	5,800*	4,700	11,380
10	15	20,430 19,760	1,530	5,830*	19,960	20,580

*These moderately high counts per minute were shown in additional studies to be due to passive transfer of antibody from donors who had high antibody titers. †Only six of the eight donors were assayed.

donor to each of the ten recipients with NANBH (Table 2). Case number 4 had no positive donors and represented the recipient with the weakest seroconversion observed. Some of the positive donors had no surrogate markers for NANBH [elevated serum alanine aminotransferase (ALT) concentrations or the presence of antibody to the hepatitis B core antigen (HBcAg), or both (*6, 17–19*)]. The prevalence of HCV antibody in voluntary blood donors from New York with normal ALT levels (<45 international units per liter) and no antibody to HBcAg was about 0.5% (2 of 412). This frequency increased to 44% (16 of 36) in donors with both elevated ALT levels and antibody to HBcAg (*20*).

These data from characterized NANBH panels combined with previous data (*12*) indicate a specific association between HCV antibody and blood-borne NANBH. This conclusion was also supported from assays of other chronic PT-NANBH patients (Table 3). These cases differ from the NANBH cases cited in Tables 1 and 2 in that they were not prospectively monitored from the time of transfusion and, in many cases, only one serum sample was assayed. This may account for the observed lower prevalence of HCV antibody.

Table 3. HCV antibody in NANBH patients from the United States.

Transmission	Total patients	Percent positive
Blood transfusion	24	71*
No identifiable source (community-acquired)	59	58†

*Between one and three serum samples assayed from patients who had received transfusions and who were diagnosed with chronic NANBH on the basis of clinical symptoms, elevations of serum ALT for >6 months, serologic exclusion of infection with other agents (Table 2), and the exclusion of other apparent causes of liver injury. †Sequential serum samples obtained prospectively up to 3 years after the onset of clinical hepatitis associated with elevated serum ALT in the absence of serologic markers for other agents (Table 2) and other identifiable causes of liver injury.

Table 4. HCV antibody in PT-NANBH cases from Italy and Japan.

Country	Number of patients	Disease	Percent positive
Italy	32	Chronic	84*
Japan	23	Chronic	78†
Japan	13	Acute, resolving	15†

*Serum samples (about three) assayed from each patient with transfusion-related chronic NANBH (diagnosed as in Tables 2 and 3). †A prospective study in which sequential serum samples were assayed for at least 6 months after the onset of acute NANBH (diagnosed as in Tables 2 and 3). The serum ALT of acute, resolving patients returned to normal and stable levels, whereas chronic patients displayed abnormal levels for at least 6 months.

Assays were also performed on a group of patients with well-defined clinical NANBH who were prospectively monitored for up to 3 years after onset of illness but who had no identifiable source of infection (*9*). More than 50% of these individuals were either positive for HCV antibody at the time of the initial consultation with the physician or seroconverted subsequently (Table 3). Thus, it appears that HCV is a major cause of community-acquired NANBH as well as PT-NANBH.

To initiate investigations into the contribution of HCV to global NANBH, a collection of sera from NANBH patients from Italy and Japan was assayed for HCV antibody. The results indicate that 84% of Italian patients diagnosed with chronic PT-NANBH contained HCV antibody (Table 4). A similar frequency was observed in prospectively studied chronic PT-NANBH cases from Japan, but a much lower prevalence was seen in Japanese patients with NANBH that had resolved their acute infection without progression to chronic hepatitis (Table 4). The lower incidence of antibody to HCV in acute, resolving NANBH has also been observed in other human studies (*21*) and may reflect a lower stimulation of the immune system in these cases as compared with chronic, persistent infections.

These data suggest that HCV is a major cause of chronic NANBH throughout the world. The advent of the specific, sensitive test for HCV antibody described here should improve the safety of the world's blood supply as well as provide an important clinical diagnostic tool. With this assay and the availability of HCV hybridization probes (*12*), it should also be possible to address the issue of whether other parenteral NANBH agents exist.

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