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Detection of Antibodies to Hepatitis C Virus in U.S. Blood Donors

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An enzyme immunoassay (EIA) which utilizes a solid phase coated with a recombinant antigen (c100-3) derived from the hepatitis C virus (HCV) genome was evaluated for efficacy in the detection of antibodies to HCV (anti-HCV). The sensitivity of the antibody test was demonstrated by the detection of anti-HCV in a well-characterized panel of human specimens known to contain the infectious agent of non-A, non-B hepatitis. The specificity of the anti-HCV test was evaluated by testing 6,118 serum specimens from volunteer blood donors considered to be at low risk for exposure to HCV. The specificity of the anti-HCV EIA was demonstrated to be 99.56%, since 6,069 of 6,096 specimens from this low-risk group were nonreactive. A total of 49 (0.80%) of the 6,118 specimens were repeatedly reactive in the test, and 22 (46.81%) of the 47 specimens available for additional testing were confirmed as positive for antibodies to HCV c100-3. Among commercial plasma donors, 390 (10.49%) of 3,718 specimens were repeatedly reactive in the EIA. A total of 375 (97.40%) of the 385 specimens available for further testing were confirmed as positive. These limited data indicate that the prevalence of antibodies to HCV is 0.36% (22 confirmed positives among 6,118 specimens) among volunteer blood donors and 10.08% (375 confirmed positives among 3,718 specimens) among commercial plasma donors. The importance of confirmatory testing is discussed.

One major problem in assuring a safe blood supply has been the inability to identify blood donors who may transmit non-A, non-B hepatitis (NANBH). The risk of posttransfusion hepatitis was estimated to be 7 to 12% in studies prior to 1980, with approximately 90% of posttransfusion hepatitis being caused by the NANBH agent (9). Currently, it is estimated that 5 to 10% of transfused individuals will develop acute NANBH (3). In addition, 40 to 60% of those infected may become chronic NANBH carriers (3). NANBH carriers remain infectious for years (19) and perhaps for their lifetimes.

Through 1989, the only method routinely available to blood banks for the identification of potential NANBH carriers was the use of surrogate markers (detection of antibodies to hepatitis B core antigen [anti-HBc] and/or detection of elevated levels of serum alanine aminotransferase [ALT]) (1, 4, 11, 17). In 1989, Choo et al. cloned segments of an NANBH virus (designated hepatitis C virus [HCV]) from infectious chimpanzee plasma (7). A recombinant antigen (c100-3) was expressed in yeast cells as a fusion protein with human superoxide dismutase and used as the antigen in a solid-phase immunoassay to detect antibodies to HCV (anti-HCV), now believed to be the major cause of NANBH (5, 13).

In this paper, we report data on the performance of the Abbott HCV enzyme immunoassay (EIA), which makes use of the c100-3 antigen. Serological studies which document the ability of this assay to detect antibodies in both volunteer blood donors and commercial plasmapheresis donors are presented.

MATERIALS AND METHODS

HCV antigen. The c100-3 yeast recombinant antigen was purchased from Chiron Corp., Emeryville, Calif. The cloning, expression, and serological use of this fusion protein were described previously (7, 13).

Immunoassay for detection of anti-HCV. For the Abbott HCV EIA, human serum or plasma was diluted 1:41 by adding 10 μl of specimen and 400 μl of specimen diluent to the wells of a reaction tray. Two hundred microliters of this mixture was removed from the tray and added to the wells of a second tray. A polystyrene bead coated with HCV c100-3 was added to wells containing diluted specimens or controls. After 1 h at 40°C, the beads were washed three times with distilled water and reacted with a solution containing affinity-purified goat anti human immunoglobulin labeled with horseradish peroxidase. The trays were incubated for 30 min at 40°C and washed as described above. Next, 300 μl of a solution of *o*-phenylenediamine 2HCl and hydrogen peroxide was added to the beads and incubated for 30 min at room temperature; the reaction was terminated by the addition of 1 N H_2SO_4. The A_{492} was read. A cutoff value was established as a function of both the negative and the positive controls and was adjusted to provide optimal separation between specimens which contained anti-HCV and those which did not. The cutoff value was calculated as the sum of the mean negative control absorbance value plus 25% of the mean positive control absorbance value. Specimens which produced absorbance values above the cutoff were considered reactive and were tested in duplicate. Specimens which were repeatedly reactive were considered positive and were tested by alternate methods to confirm the results obtained in the primary assay.

Serological studies. A panel (NANBH panel II) of coded human serum or plasma samples which has been used to evaluate putative assays for antibodies or antigens associated with exposure to NANBH was provided by H. Alter, Department of Health and Human Services (Bethesda, Md.). This 44-member panel consists of 22 human specimens supplied in duplicate under code and specimens from NANBH carriers whose serum was proven to be infectious in chimpanzees, donors implicated in the transmission of NANBH to recipients, and acute NANBH patients. Speci-

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mens from highly pedigreed negative controls and other disease controls were also included in this panel.

A total of 6,118 serum specimens from volunteer blood donors were tested at three geographically distinct clinical sites in the United States (west coast, midwest, and southeast).

In addition, 3,718 plasma specimens from commercial plasmapheresis donors were tested at two geographically distinct clinical sites in the United States (southeast and southwest).

Confirmatory procedures. To distinguish true-positive from false-positive results, we developed two confirmatory assay procedures. In the first procedure, an EIA was developed with polystyrene beads coated with synthetic peptides representing sequences of known epitopes within the c100-3 antigen. Four synthetic peptides comprised of amino acid sequences contained within c100-3 were synthesized at Abbott Laboratories and used for serological studies (14, 15). These peptides were selected after reviewing previous studies conducted by scientists at Chiron Corp. and reported in the European Patent Application (6) filed by Chiron Corp. These studies indicated that some individuals produce antibodies which react with one, but not both, of the initial HCV recombinant antigens located within the c100 region of the HCV genome. Synthetic peptides corresponding to these antigens were generated at Abbott Laboratories. One of these recombinant antigens was designated clone 5-1-1 and served as the model for the generation of a synthetic peptide comprised of 42 contiguous amino acids (designated sp42). Another recombinant antigen, designated clone 81, served as the model for sp117. sp67 was generated as an intermediate-size synthetic peptide which could be used along with sp42 and sp117 for studies comparing the efficacy of each of these peptides as antigenic targets. In addition to these overlapping synthetic peptides, a fourth peptide (sp65) which contained the carboxyl region of c100-3 was synthesized; the hydrophilicity profile suggested the presence of an additional epitope in this region. The amino acid sequences of these synthetic peptides were as follows: sp42, I-I-P-D-R-E-V-L-Y-R-E-F-D-E-M-E-E-C-S-Q-H-L-P-Y-I-E-Q-G-M-M-L-A-E-Q-F-K-Q-K-A-L-G-L; sp117, S-G-K-P-A-I-I-P-D-R-E-V-L-Y-R-E-F-D-E-M-E-E-C-S-Q-H-L-P-Y-I-E-Q-G-M-M-L-A-E-Q-F-K-Q-K-A-L-G-L-L-Q-T-A-S-R-Q-A-E-V-I-A-P-A-V-Q-T-N-W-Q-K-L-E-T-F-W-A-K-H-M-W-N-F-I-S-G-I-Q-Y-L-A-G-L-S-T-L-P-G-N-P-A-I-A-S-L-M-A-F-T-A-A-V-T-S-P-L-T-T-S-Q; sp67, G-R-V-V-L-S-G-K-P-A-I-I-P-D-R-E-V-L-Y-R-E-F-D-E-M-E-E-C-S-Q-H-L-P-Y-I-E-Q-G-M-M-L-A-E-Q-F-K-Q-K-A-L-G-L-L-Q-T-A-S-R-Q-A-E-V-I-A-P-A-V; and sp65, F-K-I-M-S-G-E-V-P-S-T-E-D-L-V-N-L-L-P-A-I-L-S-P-G-A-L-V-V-G-V-V-C-A-A-I-L-R-R-H-V-G-P-G-E-G-A-V-Q-W-M-N-R-L-I-A-F-A-S-R-G-N-H-V-S (underlined amino acids indicate amino acid sequence homology with sp42).

The synthetic peptide-based assays were performed on specimens diluted 1:41 by use of the procedure used for the HCV EIA. Specimens with absorbance values equal to or greater than four times the mean absorbance value of the negative control specimen were considered reactive and confirmed as having antibodies to c100-3.

In the second procedure, an EIA was developed with a blocking reagent to neutralize the reactivity of true-positive specimens. The blocking reagent is a recombinant antigen which contains 256 of the 363 amino acids of c100-3 and contains all known epitopes from within the c100-3 region. It is expressed in *Escherichia coli* rather than in yeasts and is a chimeric protein with CTP:CMP 3-deoxy-D-manno-octulosonate cytidyltransferase (CMP-KDO synthetase) rather than superoxide dismutase. This blocking reagent does not prevent antibodies directed against yeast proteins or superoxide dismutase from binding to the solid phase but does prevent antibodies specifically directed against c100-3 from binding to the antigen-coated beads. Each specimen which was repeatedly reactive in the HCV EIA was retested in duplicate with the standard reagents and with the blocking reagent added to the specimen diluent. Specimens which had sample/cutoff (S/CO) ratios of greater than 0.8 and which demonstrated greater than 50% neutralization in the presence of the blocking reagent were considered reactive and confirmed for antibodies to HCV c100-3.

Prior to conducting the studies described here, we found that 55 specimens from individuals diagnosed with NANBH were repeatedly reactive in the HCV EIA; all 55 were confirmed as positive with epitopes contained within the blocking reagent. These data provided the rationale for the confirmatory strategy presented in this paper. Although some individuals may respond to potential c100-3 epitopes not contained within the recombinant antigen produced in *E. coli*, this phenomenon has not yet been observed.

The percent neutralization for this blocking assay can be determined as follows: percent neutralization = (A_{492} of standard assay − A_{492} of blocking reagent))/(A_{492} of standard assay − A_{492} of negative control) × 100. Specimens which were repeatedly reactive in one or both confirmatory procedures were considered confirmed as having antibodies to HCV c100-3.

TABLE 1. Anti-HCV results for NANBH panel II

Panel identification	Abbott HCV EIA	
	Specimen	Result
Proven infectious in chimps		
Chronic posttransfusion NANBH	1	+
	2	+
	3	+
Implicated donors with elevated ALT levels	4	+
	5	+
	6	+
Acute posttransfusion NANBH	7	−
Equivocally infectious in chimps: implicated donors with ALT levels within normal limits	8	−
Probably infectious: patients with acute posttransfusion NANBH		
Bleed 1 (? wk posttransfusion)	9	−
Bleed 2 (? wk posttransfusion)	10	−
Bleed 3 (13 wk posttransfusion)	11	+
Disease controls		
Primary biliary cirrhosis	12	−
Alcoholic hepatitis in recovery	13	−
Pedigreed negative controls	14	−
	15	−
	16	−
	17	−
	18	−
Potential NANBH antigens		
JS-80-01T-O	19	−
Asterix	20	−
Zurtz	21	+
Becassdine	22	−

TABLE 2. Abbott HCV EIA study of volunteer blood donors

Site	No. of specimens tested	No. of specimens repeatedly reactive (% of total tested)	No. of confirmed specimens (% of available repeatedly reactive specimens)
1	1,999	14 (0.70)	7 (50.0)
2	2,000	17 (0.85)	8[a] (47.1)
3	2,119	18[b] (0.85)	7 (43.75)
Total	6,118	49 (0.80)	22 (46.81)

[a] One specimen was confirmed by peptides only, and one specimen was confirmed by neutralization only.
[b] Two specimens were not available for confirmatory testing.

RESULTS

NANBH panel II. NANBH panel II was assayed in duplicate under code for anti-HCV. Six serum specimens (specimens 1 to 6) which were proven infectious in chimpanzees were positive (Table 1). Three of these were obtained from individuals with chronic hepatitis, and three were obtained from individual blood donors (with elevated ALT levels) implicated in posttransfusion NANBH. Specimens from two additional individuals (specimens 7 and 8) with a history of hepatitis were nonreactive. The specimen from one of these individuals (specimen 7) was obtained during acute hepatitis and was proven infectious in the animal model. The other nonreactive specimen was obtained from an individual (specimen 8) who had normal ALT levels and whose serum was found equivocally infectious when tested in chimpanzees.

In the group labeled probably infectious were three samples (specimens 9 to 11) taken from the same posttransfusion hepatitis patient. The first two acute-phase samples were negative, but the third sample was reactive. This sample was taken 13 weeks posttransfusion and 5 weeks after the onset of posttransfusion hepatitis. The disease control samples (specimens 12 and 13) and pedigreed negative control samples (specimens 14 to 18) were uniformly negative.

The last group (specimens 19 to 22), referred to as potential NANBH antigens, contained specimens proposed to be positive in earlier putative NANBH assays by others but never confirmed. From this group, the sample Zurtz was found reactive. All repeatedly reactive specimens were confirmed positive by additional testing.

Volunteer blood donors. A total of 49 (0.80%) of 6,118 serum specimens obtained from three clinical sites were repeatedly reactive. Twenty-two (46.81%) of the 47 specimens available for additional testing were confirmed positive for antibodies to HCV c100-3 (Table 2). The distribution of absorbance values obtained with the 6,118 specimens is presented in Fig. 1. The mean absorbance value of confirmed positive specimens was 1.760 (corresponding to a mean S/CO ratio of 4.48), while that of nonconfirmed specimens was 0.784 (mean S/CO ratio, 1.81).

Plasmapheresis donors. A total of 390 (10.48%) of 3,718 plasma specimens were repeatedly reactive. Five of these 390 specimens were not available for confirmatory studies. A total of 375 (97.40%) of the remaining 385 specimens were confirmed positive for antibodies to HCV c100-3 (Table 3). Nine specimens were found positive by peptide assays only;

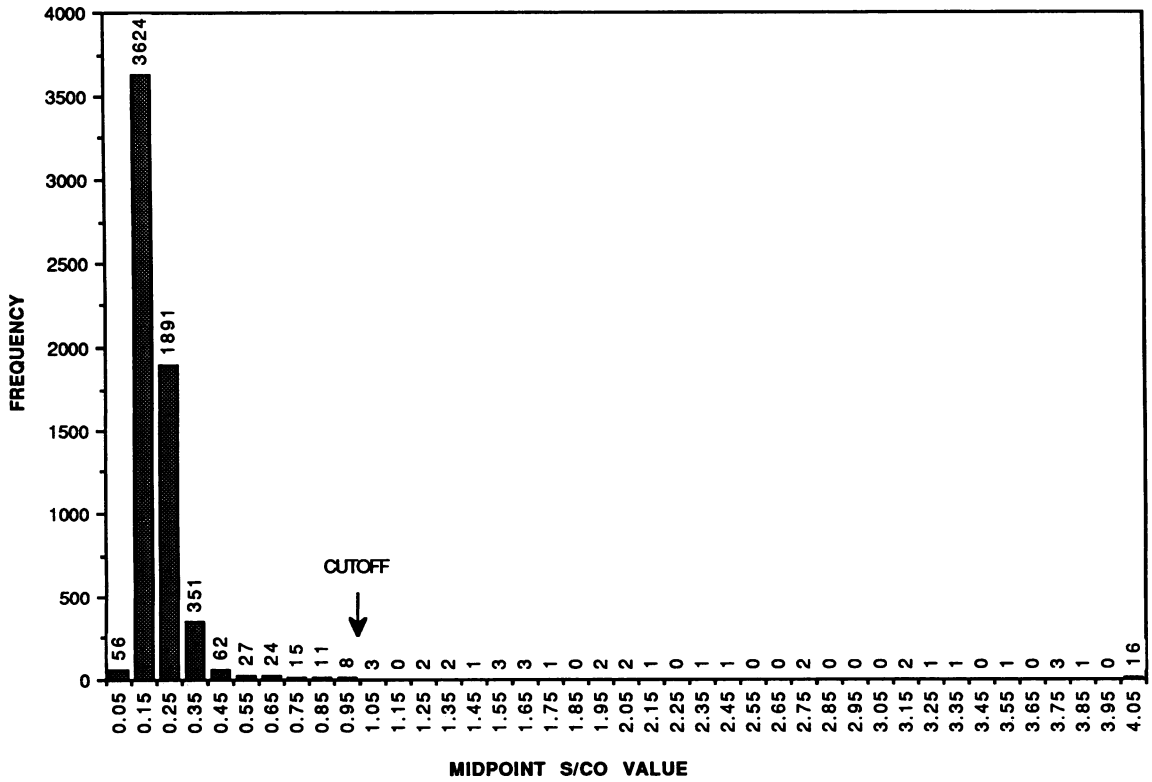

FIG. 1. Distribution of S/CO ratios among volunteer blood donors tested with the Abbott HCV EIA. Specimens with S/CO ratios above 1.0 were considered reactive.

TABLE 3. Abbott HCV EIA study of commercial plasmapheresis donors

Site	No. of specimens tested	No. of specimens repeatedly reactive (% of total tested)	No. of confirmed specimens (% of available repeatedly reactive specimens)
8	1,718	174[a] (10.13)	165[b] (95.93)
10	2,000	216[c] (10.80)	210[d] (98.59)
Total	3,718	390 (10.49)	375 (97.40)

[a] Two specimens were not available for confirmatory testing.
[b] Three specimens were confirmed by peptides only; 171 specimens were confirmed by both peptides and neutralization.
[c] Three specimens were not available for confirmatory testing.
[d] Six specimens were confirmed by peptides only; 204 specimens were confirmed by both peptides and neutralization.

the remaining 366 were found positive by both procedures. Ten specimens were found negative by both procedures. The distribution of absorbance values obtained when these 3,718 donors were tested is presented in Fig. 2. The mean absorbance value of confirmed positive specimens was 1.725 (mean S/CO, 4.70), while that of nonconfirmed specimens was 0.744 (mean S/CO, 1.51).

Study of potentially interfering specimens. Serum or plasma obtained from selected populations of individuals known to have antibodies to viruses (other than HCV) or substances which could potentially interfere with the assay were evaluated. A total of 12 (3.39%) of 354 specimens from the eight population categories tested were initially and repeatedly reactive (Table 4). Eleven of the 12 repeatedly reactive specimens were confirmed positive for antibodies to HCV c100-3; 9 of the 12 specimens were confirmed by both the synthetic peptide and the neutralization methods, and 2 of the 12 specimens were confirmed by the synthetic peptide method only. The remaining repeatedly reactive specimen was not confirmed by either method.

Anti-HCV-positive individuals positive for surrogate markers of NANBH. Specimens obtained from individuals with positive results for surrogate markers associated with NANBH were tested for anti-HCV (Table 5). Seven (7.00%) of 100 specimens from individuals having elevated ALT levels were repeatedly reactive for anti-HCV; all 7 specimens were confirmed positive. Among individuals whose serum or plasma was anti-HBc positive, 6 (3.47%) of 173 specimens were repeatedly reactive for anti-HCV; 4 of these 6 specimens were confirmed positive. However, 35 (44.87%) of 78 specimens from individuals who had both elevated ALT levels and positive results for anti-HBc were repeatedly reactive for anti-HCV. All 35 specimens were confirmed positive for antibodies to HCV c100-3.

DISCUSSION

The performance of a newly developed EIA designed to detect anti-HCV was examined. To evaluate assay performance, we developed two types of confirmatory assays to allow discrimination between repeatedly reactive specimens which were false-positives and those which were true-positives. In the first type of confirmatory assay, the EIA was developed with an assay format similar to that of the HCV EIA, except that synthetic peptides from within the c100-3 region of the HCV genome were used as the antigenic targets. In the second type of confirmatory assay, the c100-3

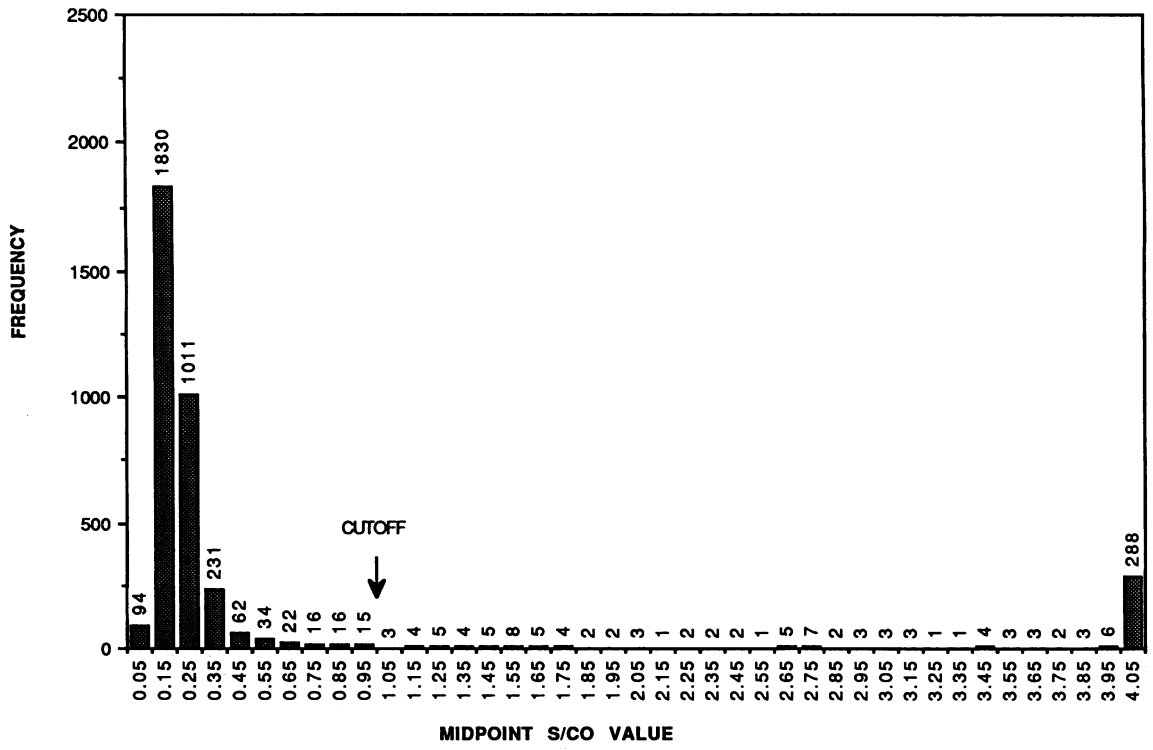

FIG. 2. Distribution of S/CO ratios among commercial plasmapheresis donors tested with the Abbott HCV EIA. Specimens with S/CO ratios above 1.0 were considered reactive.

TABLE 4. Anti-HCV results for potentially interfering specimens

Category[a]	No.		
	Tested	Positive	Confirmed
Anti-EBV VCA			
Positive	23	1	1
Negative	6	0	NA[b]
Anti-CMV			
Positive	40	2	2
Negative	20	0	NA
Anti-HSV positive	18	1	1
Anti-RV positive	69	1	1
Anti-flavivirus positive			
Yellow fever vaccinees	7	1	1
Dengue fever patients	6	0	NA
St. Louis encephalitis patients	7	1	0
Japanese encephalitis patients	1	0	NA
Anti-fungus positive			
Aspergillosis	5	0	NA
Candidiasis	4	0	NA
Coccidioidomycosis	16	0	NA
Cryptococcosis	8	2	2[c]
Histoplasmosis	7	0	NA
Others[d]	6	0	NA
ANA			
Positive	60	0	NA
Negative	20	1	1
Rheumatoid factor positive	31	2	2
Total (% of total)	354	12 (3.39)	11 (3.11)

[a] Anti-EBV VCA, Antibody to Epstein-Barr virus viral capsid antigen; anti-CMV, antibody to cytomegalovirus; anti-HSV, antibody to herpes simplex virus; anti-RV, antibody to rubella virus; ANA, antibody to nuclear antigen.

[b] NA, Not applicable.

[c] Two specimens were confirmed by peptides and were not available for the neutralization test.

[d] Includes other antifungal sera from patients infected with *Pseudallescheria boydii*, *Bipolaris hawaiiensis*, *Torulopsis glabrata*, and three unidentified mycoses.

anti-HCV EIA was run in the presence and absence of a blocking reagent which inhibits antibodies directed against c100-3 from binding to the solid phase. Unlike the c100-3 antibody assay with yeast-derived recombinant antigens, these confirmatory assays were not subject to false-positive results because of the presence of antibodies directed against superoxide dismutase or against yeast proteins which may copurify with the c100-3 protein.

TABLE 5. Correlation between anti-HCV and surrogate markers for NANBH (anti-HBc and elevated ALT levels)

Category	No. positive/total (%)	
	HCV EIA	Confirmed/repeatedly reactive specimens
Elevated ALT levels	7/100 (7.00)	7/7 (100.00)
Anti-HBc positive	6/173 (3.47)	4/6 (66.67)
Elevated ALT levels and anti-HBc positive	35/78 (44.87)	35/35 (100.00)

The sensitivity of the antibody test for HCV can be defined as the ability to detect as reactive specimens from individuals known to be associated with an HCV infection. Using NANBH panel II and assuming that the appropriate specimens had anti-HCV, we determined the sensitivity of the HCV EIA for detecting antibodies in human serum proven infectious for NANBH to be 85.71% (six of seven); the only specimen not detected was obtained during the acute phase of the disease, in which antibodies may not yet be at detectable levels. All of the repeatedly reactive specimens were confirmed positive. The same set of specimens was reactive when tested with the antibody assay developed by scientists at Chiron Corp. (13).

Since the number of specimens in NANBH panel II which were used to evaluate sensitivity was limited, additional studies to evaluate sensitivity were performed by testing specimens from individuals diagnosed as having chronic NANBH. The Abbott HCV EIA detects as reactive 80 to 85% of specimens from individuals with NANBH (14). The detection rate reported here is similar to that obtained with the research anti-HCV assay described by Kuo et al. (13). Individuals who are negative for anti-HCV c100-3 may have antibodies to HCV antigens not included in the current EIAs or, alternatively, these individuals may not be infected with HCV but may have chronic NANBH caused by an agent other than HCV. This method of evaluating sensitivity is dependent on clinicians who have the difficult task of categorizing hepatitis infections without tests which exclusively identify the etiologic agent(s).

The specificity of the HCV EIA can be defined as the ability to detect as nonreactive specimens from a population of individuals considered to be at low risk for infection with HCV. Specimens which are reactive and confirmed as positive for anti-HCV are not included in the determination of specificity. Using volunteer blood donors from our clinical studies as the low-risk population and assuming that 100% of this population should test negative, we determined the specificity of this assay to be 99.20% (6,069 of 6,118 specimens were negative). When the repeatedly reactive confirmed specimens were removed from this population, the specificity was determined to be 99.56% (6,069 of 6,096). A total of 375 (10.08%) of 3,718 specimens from plasma donors were repeatedly reactive in the HCV EIA and were confirmed positive. Therefore, the specificity of the assay was determined to be 99.55%, since 3,328 of the remaining 3,343 specimens were nonreactive and 15 repeatedly reactive specimens could not be confirmed positive. Thus, the specificities of the HCV EIA for volunteer blood donors and commercial plasmapheresis donors are very similar, at 99.56 and 99.55%, respectively. Conversely, the false-positive specimen rates (or nonconfirmable repeatedly reactive specimen rates) are similar, at 0.44 and 0.45%, respectively, for volunteer blood donors and commercial plasmapheresis donors. Previous studies indicated that the incidence of hepatitis caused by either hepatitis B virus or NANBH viral agents is higher in commercial plasmapheresis donors than in volunteer blood donors (2). Our limited studies indicated that the prevalence of anti-HCV is relatively high among commercial plasma donors (at approximately 10%) when compared with volunteer blood donors (at approximately 0.36%). Without confirmatory testing, it would have been difficult to determine whether the increased reactivity rates noted among plasmapheresis donors were related to techni-

cal problems (instrumentation, operator, reagent lot) or to differences in antibody prevalence.

In studies of volunteer donors with the anti-HCV assay developed by Ortho Diagnostics Systems, the repeatedly reactive specimen rates were reported to be 0.68% (10), 0.79% (12), 0.87% (16), and 0.50 to 1.00% (8). Although these repeatedly reactive specimen rates are very similar to those found with the Abbott HCV EIA (Table 2), no confirmatory testing was reported in these previous studies. Thus, it is difficult to assess the true prevalence of anti-HCV (via superoxide dismutase–c100-3 antigen) in these studies. Our limited data indicate that antibody prevalence among volunteer donors is approximately 0.36%, since 22 of 6,118 blood donors were confirmed positive for anti-HCV.

Specificity can also be evaluated by testing specimens which could potentially produce false-positive results. While 12 of 354 such specimens were repeatedly reactive in the assay, 11 of these 12 specimens were confirmed positive, indicating that only 1 of 354 specimens was false-positive and thereby demonstrating a specificity of 99.72%.

The correlation between NANBH surrogate markers and anti-HCV has been discussed in several reports (5, 10, 12, 16, 18, 20). Our data indicate that while the incidence of anti-HCV was relatively low in individuals positive for anti-HBc (3.47%) or with elevated ALT levels (7.00%), the incidence was much higher than that in volunteer blood donors (0.36%). The incidence of anti-HCV in individuals having both anti-HBc and elevated ALT levels was much higher (44.87%) than that in individuals positive for only one of these markers. The continued use of surrogate markers may be important in detecting HCV-infected individuals who may be seronegative in current assays; alternatively, surrogate markers may also detect individuals who harbor agents which may cause NANBH but which are not related to HCV.

Two separately developed anti-HCV EIAs are currently licensed for use in the United States (Ortho Diagnostics Systems and Abbott Laboratories); both of these assays make use of the c100-3 antigen produced by Chiron Corp. There is a clear indication that the use of the HCV c100-3 antigen in antibody tests will reduce the transmission of NANBH (5, 13, 21). However, it is unlikely that antibodies to c100-3 are sufficient to detect all individuals exposed to HCV, since this recombinant antigen is derived from a domain of the genome which is tentatively believed to encode nonstructural viral proteins. Additional tests will be necessary to detect antibodies to proteins encoded by other regions of the HCV genome, including viral structural proteins such as core and envelope proteins.

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