Hepatitis C Virus in Blood Samples from Volunteer Donors

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The specificities of four assays for hepatitis C virus (HCV) were compared by using units from volunteer blood donors. Upon Food and Drug Administration licensure of the first immunoassay for anti-HCV, EIA-1, units previously deemed acceptable for transfusion and all subsequent blood donations were screened. EIA-1 repeat-reactive (RR) units were tested for HCV by a second-generation enzyme-linked immunoassay (EIA-2) and by a four-antigen recombinant immunoblot assay (RIBA II) and for HCV RNA by reverse transcriptase polymerase chain reaction. All HCV RNA-positive samples were reactive by both RIBA II and EIA-2. All RIBA II-reactive sera were EIA-2 RR. In EIA-1, 0.45% of the prescreened units and 0.59% of the prospectively screened donors were RR. Of these, 52.5 and 54%, respectively, were EIA-2 RR, 71.4 and 69% of the EIA-2 RR units were reactive on RIBA II, and 93 and 88% of the RIBA II-reactive samples were HCV RNA positive. When the sample/cutoff ratio for EIA-2 was greater than 5, 91% of the samples were RIBA II reactive and 82% of the samples were HCV RNA positive. None of EIA-2 RR units with a sample/cutoff ratio of <5 was RIBA II reactive or HCV RNA positive. In conclusion, RIBA II and RT PCR results are highly concordant. A sample/cutoff ratio of >5 in EIA-2 is a good discriminator for the likelihood of a true HCV infection on the basis of RT PCR and RIBA II assays.

The flavivirus-like entity hepatitis C virus (HCV) is the principal etiological agent of parenterally transmitted non-A, non-B hepatitis (6). The first anti-HCV enzyme immunoassay (EIA-1) established HCV as the major cause of transfusion-associated and community-acquired non-A, non-B hepatitis (10). EIA-1 measures antibodies that bind to a superoxide dismutase fusion protein containing c100-3, a polypeptide that spans the distal nonstructural domain 3 to the proximal nonstructural domain 4 of the putative viral polyprotein.

On 2 May 1990, the anti-HCV EIA-1 was licensed by the U.S. Food and Drug Administration for screening samples from blood and plasma donors in an effort to reduce the incidence of transfusion-associated hepatitis. Subsequently, a second-generation enzyme immunoassay (EIA-2) and a recombinant immunoblot assay (RIBA II) that detected antibodies to a putative core protein (c22) and another part of the NS3 region (c33c) were developed (11). Since the 5' untranslated region of the HCV genome is conserved, nested reverse transcriptase polymerase chain reaction (RT PCR) amplification of this region allows confirmation of viremia.

EIA-1 and EIA-2 detect antibodies to both superoxide dismutase and HCV-related epitopes. Hence, supplemental assays such as RIBA II and RT PCR may be needed to confirm HCV infection (1, 7, 11). In this investigation, we compared the diagnostic efficacy of repeat reactive (RR) results in EIA-1 with those obtained with EIA-2, RIBA II, and RT PCR. The sample/cutoff (S/C) ratios of both EIA-1 and EIA-2 were good predictors of the results of the supplemental assays.

**MATERIALS AND METHODS**

**Blood donors.** When the Food and Drug Administration approved the Ortho/Chiron anti-HCV EIA-1, the Sacramento Medical Foundation implemented testing of all inven-

tory and donated blood and components, a total of 8,921 units. These units had already been found suitable for transfusion. Before blood donation, each donor passed a medical history that included as exclusion criteria demographic data and practices that correlated with acquisition of HCV infections. Units with an elevated alanine aminotransferase level (>53 IU/liter) or that were RR in immunoassays for hepatitis B core antibody, hepatitis B surface antigen, human T cell lymphotropic virus I antibody, and human immunodeficiency virus I antibody and a serological test for syphilis were excluded. Forty RR anti-HCV EIA-1 units were identified. Because these units were obtained from previously screened volunteer blood donors, an additional 148 prospective donors were subsequently identified as EIA-1 RR on the basis of routine screening with the Food and Drug Administration-licensed EIA-1 as part of the normal testing procedures for volunteer blood donations. This constituted 0.6% of our donors. Blood donor serum samples were kept frozen at −20°C and not thawed more than twice before testing.

**Anti-HCV testing.** The 8,921 inventory serum samples were tested for anti-HCV by EIA-1 (Ortho Diagnostic Systems, Inc., Raritan, N.J.). Repeat assays were performed twice on samples exceeding the cutoff on the initial assay. If at least two of the three results were over the cutoff, the sample was considered anti-HCV RR. All RR samples were subsequently tested by EIA-2 (Ortho), which includes c200 (c100-3 and c33c constructs fused to yeast superoxide dismutase) and the core epitope, c22. The samples were also tested by RIBA II (Chiron, Emeryville, Calif.), which identifies antibodies to c22 (core), c33c (NS3), c100-3 (NS3-4), and 5-1-1 (NS4) in a Western immunoblot-like format. RIBA II results were scored as reactive if more than one HCV-related antigen was labeled with antibody and the superoxide dismutase control was not labeled, indeterminant if one HCV-related antigen was labeled or the superoxide dismutase control was labeled in the presence of labeled HCV antigens, and negative if no bands were present. EIA-1,

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RT PCR. The following sequences were used as external primers: 5' GGCAGACTCCACCATAGATC 3' (sense orientation, nucleotides 9 through 29) and 5' CATGGTGCACT GTCTACGAGA 3' (antisense orientation, nucleotides 315 through 335). The internal primers were 5' GGAACACTCT GTCTTACGCGAGA 3' (sense orientation, nucleotides 42 through 63) and 5' TCGAACACCTACGAGA 3' (antisense orientation, nucleotides 280 through 301) (4).

RNA was extracted from plasma or serum by the method of Chomczynski and Sacchi (5), with several modifications. Serum (100 μl) was mixed with 600 μl of extraction buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 2% sarcosyl, 0.5 M mercaptoethanol, 5 μg of carrier RNA). Then 50 μl of 2 M sodium acetate (pH 4.0), 600 μl of water-saturated phenol, and 100 μl of chloroform-isoamyl alcohol mixture (49:1) were added. The suspension was placed on ice for 5 min and then centrifuged at 10,000 × g for 30 min. Proteins were removed by extraction with phenol-chloroform (1:1) and then extraction with chloroform alone. After isopropanol precipitation, the RNA pellet was resuspended in 70% ethanol, sedimented, and vacuum dried.

Reverse transcription was performed in a 20-μl reaction volume containing 20 U of RNase inhibitor (Promega Biotech, Madison, Wis.), reaction buffer (0.05 M Tris-HCl [pH 8.3], 0.75 M KCl, 3 mM MgCl₂, 10 mM dithiothreitol), 100 pmol of random primers (Pharmacia, Piscataway, N.J.), 200 μM (each) deoxynucleoside triphosphates (Pharmacia), the template, and 200 U of Moloney murine leukemia virus reverse transcriptase ( Gibco-BRL, Grand Island, N.Y.). The mixture was incubated at room temperature for 15 min and then at 42°C for 30 min.

The PCR was carried out under mineral oil in a 50-μl volume containing 2.5 U of Taq polymerase (Promega Biotech), PCR buffer (5 mM Tris-HCl [pH 8.0], 10 mM NaCl, 0.01 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, 0.1% Triton X-100, 1.5 mM MgCl₂), 2 μl of cDNA, each deoxynucleoside triphosphate at 200 μM, and 50 pmol of each external primer. Samples were incubated at 95°C for 5 min, subjected to 35 rounds of thermal cycling for 30 s at 95°C, 1 min at 55°C, and 1 min at 72°C, and then incubated for 7 min at 72°C. One microliter of PCR product was amplified for another 35 cycles with the internal primers.

All samples analyzed for HCV RNA by the RT PCR were performed in duplicate on different days. Each run contained water and a known HCV-negative human serum as negative controls that were subjected to the RNA extraction procedure. An anti-HCV positive serum that was known to contain HCV RNA was also employed as a positive control. A 10-μl sample from the second amplification was analyzed by electrophoresis in a 2% agarose gel and visualized by UV (300-nm) fluorescence after staining with ethidium bromide. In the few instances in which the results of the two assays differed with each other, a third assay was performed. Samples that were positive for HCV RNA twice were then scored as HCV RNA RT PCR positive.

The sensitivity of the RT PCR assay was determined to be five 50% chimpanzee-infective doses by analyzing serial dilutions of a plasma whose infectivity titer had been previously determined (gift of Dan Bradley, Centers for Disease Control, Atlanta, Ga.). The specificity of the amplified HCV RNA products were confirmed by hybridization to probes complementary to sequences internal to the oligonucleotide primers and by direct DNA sequencing of the PCR products.

RESULTS

The prevalence in EIA-1 of anti-HCV among 8,921 volunteer donors whose blood was acceptable for transfusion was 0.45%. Of the 40 samples that were RR in EIA-1, 21 and 15 were also reactive in EIA-2 and RIBA II, respectively (Fig. 1). All but one of the RIBA II-reactive units had detectable HCV RNA.

After routine implementation of EIA-1 for detection of anti-HCV, we found that 0.6% of our volunteer blood donors were EIA-1 RR. We therefore decided to analyze an additional 148 anti-HCV EIA-1 RR units for their reactivity in the EIA-2, RIBA II, and RT PCR assays (Fig. 2). Eighty-eight (59%) of the 148 units were anti-HCV EIA-2 RR; of these, 61 (69%) were RIBA II reactive. None of the EIA-2-negative samples was RIBA II reactive. Fifty-five (37%) of the 148 units were HCV RNA positive by RT PCR. Eighty-nine...
percent of the RIBA II-reactive units were also HCV RNA positive, but only 1 of 12 RIBA-indeterminate samples contained HCV RNA. None of the RIBA II-negative units had detectable HCV RNA.

The concentration of antibodies to HCV epitopes as expressed by the S/C ratio of the enzyme immunoassay sample was very predictive of the results of the RIBA II and HCV RNA confirmation assays. When the S/C ratios of the RR EIA-1 samples were greater than 5, then 86% of the units were also EIA-2 RR, 79% were RIBA II reactive, and 75% were HCV RNA positive. Conversely, when the S/C ratios of the units in EIA-1 were 5 or less, 31% were EIA-2 RR, 13% were RIBA II reactive, and 9% were HCV RNA positive. Better specificity was obtained with EIA-2. For an S/C ratio of greater than 5 in the EIA-2, 91% were confirmed by RIBA II and 82% were HCV RNA positive. When the S/C ratio was less than 5 in the EIA-2 (n = 21), no sample was RIBA II or HCV RNA reactive.

In the two groups of blood donors evaluated above, the EIA-1-nonreactive units were not tested by the EIA-2, RIBA II, and HCV PCR assays. Thus, we may not have identified all units that were potentially infectious for HCV. Using the EIA-2, we prospectively screened an additional 28,805 units from volunteer blood donors who were EIA-1 nonreactive. Only 171 (0.59%) were EIA-2 RR. Of these, only 24 (14%) were confirmed with RIBA II; however, in each instance, the S/C ratio was greater than 5. Thus, even when sera are EIA-1 nonreactive, the S/C ratio in EIA-2 is a good predictor of reactivity in supplemental assays.

**DISCUSSION**

The isolation and cloning of the HCV genome led to the development of first-generation and then second-generation assays for antibodies to various putative virus-related epitopes. EIA-1 identified 0.3 to 3% of volunteer blood donors as RR for antibodies either to the c100-3 epitope of HCV or to yeast superoxide dismutase. In the United States, the overall prevalence of EIA-1 RR blood donors is approximately 0.6% (2). In the first part of our investigation, 0.45% of volunteer blood donor units that had been made available for blood transfusion were RR for anti-HCV in EIA-1. Because the anti-HCV immunoassay also detects antibodies to superoxide dismutase, not all anti-HCV EIA-1 RR units are potentially infectious (8, 13). In the volunteer blood donor setting, less than 60% of EIA-1 RR samples have been confirmed with supplemental testing (1, 7). These findings highlight the need for improved screening test systems with better specificity and the need for a confirmatory test. We herein report that for otherwise acceptable blood units only 53% of 40 EIA-1 RR samples from volunteer blood donors are also EIA-2 RR. Only among the EIA-2 RR samples were RIBA II reactivity and HCV RNA detected.

Our results indicate that the addition of structural (c22) and nonstructural (c200) HCV antigens to the EIA-2 assay increases the specificity of detection of anti-HCV antibodies in asymptomatic blood donors who deny a history of viral hepatitis. The detection of 171 EIA-2 RR donors from 28,805 EIA-1-nonreactive units implies that EIA-2 is more sensitive than EIA-1. The RIBA II is highly predictive for the presence of infectious HCV as measured by RT PCR (14). In this study, the high concordance rates in positive (89 to 93%) and negative (100%) results between the RIBA II and RT PCR are not surprising. Some HCV RNA RT PCR-negative samples that are RIBA II positive may contain virus concentrations below the limit of sensitivity of the RT PCR assay (less than five 50% chimpanzee-infective doses per sample) or may have had borderline-low concentrations of virus that deteriorated due to RNA degradation from repeated freezing and thawing of specimens. No sample was frozen and thawed more than twice in this investigation. However, we and others have observed declines in plasma HCV RNA concentrations after a single freeze and thaw (3; unpublished data). The sensitivity of the HCV RT PCR assay in this study was confirmed by hybridization to oligonucleotide probes and by DNA sequencing. Another alternative is that some donors may have cleared their infection but still retained anti-HCV antibodies.

Of note, the concentration of antibody to HCV peptides as expressed by the S/C ratio of the enzyme immunoassays, especially EIA-2, was very predictive of the results of the supplemental assays. A S/C ratio of greater than 5 in EIA-2 was more than 90% predictive of a reactive RIBA II result, and a S/C ratio of less than 5 was predictive of a negative RIBA II result. Our results extend those of Giulivi and colleagues, who screened 20,186 volunteer blood donors with EIA-1 (9). A S/C ratio of 2 discriminated somewhat between RIBA II-reactive and -nonreactive samples. For an S/C ratio >2, 27 (82%) of 33 of the EIA-1 RR results were confirmed in RIBA II; however, only 4 (15%) of 26 EIA-1 RR samples with an S/C ratio of <2 were confirmed. More recently, Roth et al. determined that 6 of 10 patients whose S/C ratio was less than 4 had normal liver histology, whereas all 14 patients with S/C ratios of >4 had either chronic persistent hepatitis or chronic active hepatitis (12).

The S/C ratio on EIA-2 is a good predictor of reactivity on supplemental assays, even when the EIA-1 result is nonreactive. In situations such as organ donation, in which time to perform supplemental assays is limited before a decision is made on whether to use an organ, the S/C ratio may be a useful discriminator of a true- or false-positive anti-HCV EIA-2 result.

In summary, EIA-2 increases the sensitivity and the specificity of anti-HCV detection among volunteer blood donors. Furthermore, a high S/C ratio in EIA-2 is a useful indicator of potential HCV infectivity, on the basis of the correlation with reactive results of the RIBA II and RT PCR assays.

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**REFERENCES**


