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The Clinical Relevance of Persistent RIBA Indeterminate Reactions: Insights into the Natural History of HCV infection and Implications for Donor Counseling

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Abstract

BACKGROUND—A solid phase recombinant-immunoblot-assay(RIBA) is often used to determine the specificity of antibody to hepatitis-C-virus(anti-HCV). The RIBA result is recorded as positive, negative or indeterminate. The interpretation of RIBA indeterminate reactivity and its significance to patients and blood donors are unclear. We attempted to address the clinical relevance of RIBA-indeterminate reactions in the context of the natural history of HCV infection in a prospectively followed cohort of anti-HCV positive blood donors.

STUDY-DESIGN AND METHODS—Donor demographics, HCV exposure history, humoral and cell-mediated immunity(CMI) to HCV were compared in 15 RIBA-indeterminates, 9 chronic-HCV-carriers and 8 spontaneously-recovered subjects. Serum samples were tested for the presence of anti-HCV by a liquid phase Luciferase-Immunoprecipitation-System(LIPS) assay. CMI was assessed by IFN- γ -ELISpot assay.

RESULTS—In the quantitative LIPS assay, the sum of antibody responses to 6 HCV-antigens showed significant ($p < 0.001$) step-wise diminution progressing downward from chronic-carriers to spontaneously-recovered to RIBA-indeterminates. CMI responses in RIBA-indeterminates were similar to spontaneously-recovered subjects, and greater than chronic-carriers and negative controls ($p < 0.008$). A parenteral risk factor was identified in 13% of RIBA-indeterminates as compared with 89% of chronic-carriers and 87% of spontaneously-recovered subjects. On average, donors in the RIBA-indeterminate group were older than the other groups.

CONCLUSION—The combined CMI and LIPS results suggest that persistent RIBA-indeterminate reactions generally represent waning anti-HCV responses in persons who have recovered from a remote HCV infection. In such cases, detectable antibody may ultimately

disappear leaving no residual serologic evidence of prior HCV infection, as previously reported in a minority of long-term HCV-recovered subjects.

Keywords

HCV; RIBA indeterminate; HCV infection spontaneously recovered; Chronic HCV infection; RIBA 3.0; Cell-mediated immunity; IFN γ ; Luciferase immunoprecipitation system (LIPS) assay

INTRODUCTION

Among the approximately 200 million people estimated to harbor hepatitis C virus (HCV) worldwide¹ are asymptomatic blood donors who may have transiently engaged in high risk behavior in the distant past. In the US, the estimated prevalence of HCV infection is 3.9 million with 2.7 million people found to have chronic infection (detectable HCV RNA).^{2, 3} The incidence of HCV infection associated with blood transfusion was reduced from 3.84% to 0.57% per-recipient (0.03% per-unit blood) after HCV screening was introduced in 1990.^{2, 4} Nonetheless, new HCV infections continue to occur, primarily among intravenous drug users.

Currently, screening tests for detecting exposure to HCV include a third generation enzyme immunoassay (EIA) for antibodies to HCV (anti-HCV) and molecular amplification of HCV RNA.⁵ The presence of antibody, particularly in those who are HCV RNA negative, is confirmed by solid phase qualitative recombinant immunoblot assay (RIBA HCV 3.0). A positive RIBA shows reactivity to at least 2 of the 4 antigens displayed while a negative RIBA shows no reactive bands. Not infrequently, only one band is present and designated as an indeterminate RIBA pattern.

The vast majority of blood donors with indeterminate RIBA result are HCV RNA negative by polymerase chain reaction (PCR), though RIBA indeterminate reactions have been observed occasionally in HCV RNA positive subjects who are immunocompromised.⁶ A single RIBA indeterminate result in a HCV RNA negative donor may represent a false positive reaction, but a prospective study has shown that RIBA indeterminate reactivity can be persistent over time, suggesting that these results may be clinically relevant.⁷

Currently available solid phase assays, such as EIA and RIBA, are unable to identify antibodies directed against conformational pathogen-specific antigens or epitopes. Since the detection of antibodies against specific components of the HCV viral particle may provide additional specificity and sensitivity, we supplemented RIBA with a recently described liquid-phase immunoprecipitation assay, the Luciferase Immunoprecipitation System (LIPS), that can quantitatively detect antibody response against multiple pathogen-specific antigens.⁸

Cell mediated immune (CMI) responses to HCV can be measured in individuals with reduced antibody response and strong CMI responses have been observed in persons who spontaneously recover from HCV infection.⁹ CMI response has been shown to be critical to recovery from HCV infection and is strongest in individuals who have the serologic and molecular pattern of recovery (anti-HCV+, RIBA+, HCV RNA-) and weakest in those who are chronic carriers (anti-HCV+, RIBA+, HCV RNA+).^{10, 11} Thus, measurement of CMI in persistent RIBA indeterminates can be used to ascertain whether such individuals are similar to or distinct from HCV recovered subjects.

This retrospective-prospective study examines the relevance and clinical interpretation of a reproducible RIBA indeterminate result and provides insight into the natural history of HCV

infection, showing quantitatively that RIBA indeterminacy represents a progression in the spontaneous clearance of HCV infection.

MATERIALS AND METHODS

Study population

Donors to the Department of Transfusion Medicine, Clinical Center, NIH and the Greater Chesapeake Region of the American Red Cross found to be anti-HCV positive were offered the opportunity to enroll in a prospective study of the natural history of HCV infection. In this study, the risk factors that resulted in HCV infection, the extent of liver disease over time and the long term consequences of HCV infection were assessed. Although the primary thrust of the study was to assess chronic HCV infection, donors were also followed if they had evidence of recovery from prior HCV infection and if they were RIBA indeterminate.

Within this study population, we identified 15 repeatedly (6 determinations during an average follow-up duration of 13 years) reactive RIBA indeterminates who were available for recall blood sampling to collect fresh peripheral blood mononuclear cells (PBMCs). Laboratory results were compared to 9 chronic HCV carriers and 8 spontaneously recovered patients. Thirteen healthy volunteer blood donors who were anti-HCV and HCV RNA negative were included as negative controls in the IFN- γ ELISpot assays and 16 healthy donors were used as controls for the LIPS assay. There are no clinical data available for the anonymized healthy volunteer control donors.

Relevant demographic and clinical data, particularly a history of parenteral exposure, were obtained from study subjects. The frequency of parenteral exposure was compared among RIBA 3.0 indeterminate donors and chronically infected or spontaneously recovered subjects. In those with an identified parenteral exposure, the date of transfusion or the first year of intravenous drug use (IDU) was used to estimate the duration of infection.

All subjects, except anonymized negative controls, gave written informed consent for research testing under a protocol approved by the Institutional Review Board of the National Institute of Diabetes, Digestive and Kidney Disease (NIDDK), NIH.

Humoral Immune Responses

Recombinant Immunoblot assay 3.0, a solid phase assay, and Luciferase Immunoprecipitation System (LIPS), a liquid phase assay, were used to assess humoral immune responses.

Recombinant Immunoblot Assay 3.0 (RIBA 3.0)—Blood units collected from healthy volunteer donors at NIH or the American Red Cross Greater Chesapeake Region were routinely screened for the presence of anti-HCV antibody using a commercial enzyme linked immunosorbent assay (EIA 2.0; ORTHO-Clinical Diagnostics, Raritan, NJ). Repeat EIA reactive samples were confirmed with a qualitative strip immunoblot assay, RIBA HCV 3.0 SIA (Chiron Corp., Emeryville, CA).

According to the information provided by the manufacturer, the antigens used in RIBA 3.0 SIA are two recombinant antigens c33c (NS3) and NS5 and two synthetic peptides c100p (NS4) and 5-1-1p (NS4) derived from putative nonstructural regions of the virus, while the third peptide c22p corresponds to the nucleocapsid (core) viral protein. Band reactivity is graded by visual calibration against IgG control bands present on each strip. A sample is considered positive when at least two HCV bands have 1+ or greater reactivity, considered indeterminate when only a single HCV band has 1+ or greater reactivity and considered negative when no HCV bands having 1+ or greater reactivity are present.

Luciferase Immunoprecipitation System (LIPS)—The plasmid, pREN2, was used to transfect mammalian cells that then expressed encoded antigens as fusion products with Renilla luciferase.(Ruc).^{8, 12} Genomic DNA and cDNA templates for the HCV antigens were obtained from HCV-1a genotype and all Ruc-HCV antigens have been previously described.^{8, 13}

LIPS with Ruc fusion proteins: Cos1 cell lysates were prepared as described.^{8, 13} Total luciferase activity in 1 µL of each crude extract was determined by adding it to 9 µL of phosphate-buffered saline (PBS) in a 1.5 ml microfuge tube, followed by the addition of 100 µL of substrate (Renilla Luciferase Reagent Kit, Promega), vortexing, and measuring light-forming units (LU) with a luminometer (20/20n Turner Scientific).

An immunoprecipitation assay modified from the originally reported format was then utilized.¹² Serum samples were first diluted 1:10 in assay buffer A (50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1% Triton X-100) using a 96-well polypropylene microtiter plate. Antibody titers were measured by adding 40 µl of buffer A, 10 µl of diluted sera (1 µl equivalent of serum), and each of the Ruc-HCV antigen fragments containing crude Cos1 cell extract to wells of a polypropylene plate and incubated for 60 minutes at room temperature on a rotary shaker. For several of the Ruc-HCV antigen including Env1 and Env2, less than 1×10^7 light units (LU) of input was used. Next, 5 µl of a 30% suspension of Ultralink protein A/G beads (Pierce Biotechnology Inc, Rockford, IL) in PBS was added for an additional 1 hr at 4°C with tumbling. The protein A/G beads and captured proteins were washed on a vacuum manifold. Each well was washed 8 times with 100 µl buffer A and twice with 1.0 ml of PBS. Following the last wash, the filter plate was removed and blot dried to remove moisture on the top and bottom of the plate and LU measured using an LB 960 Centro microplate luminometer to determine luminescence in each well using a single injector. The LU data presented are corrected for background by subtracting the LU obtained from beads plus extract without the addition of sera.

Cell Mediated Immune Responses

Interferon-γ (IFN-γ) ELISpot Assay

Synthetic HCV peptides: Six hundred 15-mer peptides (Mimotopes, Clayton, Australia), overlapping by 10 amino acids and covering the complete HCV genotype 1 polyprotein sequence were resuspended at 20 mg/mL in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO), pooled, and further diluted with PBS solution to obtain 18 mixes covering HCV core (1 mix), E1 (1 mix), E2 (2 mixes), p7 (1 mix), NS2 (1 mix), NS3 (3 mixes), NS4A (1 mix), NS4B (2 mixes), NS5A (3 mixes), NS5B (3 mixes). The concentration of each single peptide was 24 µg/mL.

Isolation of peripheral blood mononuclear cells (PBMC): PBMC were isolated from leukopheresis packs or from acid citrate dextrose (ACD)-anticoagulated blood tubes that were drawn from 15 persistent RIBA-indeterminate subjects, 8 spontaneously HCV-recovered patients, 9 patients with chronic HCV infection and from 13 healthy blood donors lacking any serological markers of HCV infection. PBMC were separated from plasma, thrombocytes and erythrocytes by density gradient centrifugation exactly as previously described and washed three times with PBS (Mediatech, Manassas, VA).¹⁰ Cells were used immediately or cryopreserved for later use.

IFN-γ ELISpot assays: IFN-γ ELISpot assays were performed as previously described with minor modification.⁹ Specifically, 96-well plates (Millipore, Bedford, MA) were coated with 0.5 µg/mL of an antibody against human IFN-γ (Endogen, Woburn, MA) in PBS, held overnight at 4°C, washed four times with PBS, blocked with 1% bovine serum

albumin (Sigma, St. Louis, MO) in PBS for 1 h at 25°C, washed twice more with PBS and blocked for 0.5 h with complete cell culture medium (Roswell Park Memorial Institute 1640; RPMI1640) containing 5% fetal bovine serum (Serum Source International Inc., Charlotte, NC), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine (all from Mediatech, Manassas, VA).

Cryopreserved/fresh PBMCs (2×10^5 or 3×10^5 depending on available cell number) were thawed and stimulated in duplicate cultures with or without HCV peptide pools at a final concentration of 1 µg/mL per individual peptide. Stimulation with 1 µg/mL phytohemagglutinin (PHA, Sigma, St. Louis, MO) served as the positive control. After a 30 hour incubation the plates were washed three times with PBS and four times with PBS/Tween (1:2000) and incubated overnight with 100 µL of 0.25 µg/mL biotin-conjugated secondary antibody against IFN- γ (Endogen, Woburn, MA) in PBS/Tween (1:2000)/BSA (1%) solution. Plates were washed four times with PBS/Tween (1:2000) and incubated for 1 h with streptavidin-alkaline phosphatase (1:2,000 dilution; Dako, Carpinteria, CA) in PBS/Tween (1:2000)/BSA (1%) solution. The plates were washed again four times with PBS and developed with freshly prepared nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) solution (BioRad, Richmond, CA). The reaction was stopped by rinsing several times with distilled water and the spots were counted on a AID ELISpot READER (Autoimmun Diagnostika GmbH, Strassberg, Germany). Results were expressed as number of spots observed per 2×10^5 cells in response to each antigen. A response was considered positive if the average number of spots seen for a particular peptide pool was greater than the mean plus 3 standard deviations of the response observed with 10 healthy blood donors for the same peptide pool.

Statistical analysis was performed with GraphPad Prism Version 5.0a software (GraphPad Software, La Jolla, CA). Mann-Whitney U tests were employed to compare the frequency of HCV-specific IFN- γ producing PBMC between groups. A $p < 0.05$ was considered significant.

RESULTS

Study Population

The average age of the RIBA indeterminate, spontaneously recovered and chronic HCV groups were 62.9 years, 47.8 years and 53 years, respectively. Eight (53.3%) of the 15 indeterminate, 4 (50%) of the 8 spontaneous recovered and 3 (42.9%) of the 9 chronic HCV patients were male. The mean duration of follow up was 13 years (range 3–19) with a median duration of 9 years. The exact duration of HCV infection is unknown and can only be estimated for those who were transfused prior to 1990 or those who admitted to a time-limited use of shared-needle IDU. In cases where the probable onset of infection could be estimated based on an identified parenteral exposure, the interval between the onset of infection and testing was greater than 30 years in all groups. There was a disparity in the risk factors for acquisition of HCV infection between persistent RIBA indeterminates and the other groups; only 2 of 15 (13%) indeterminates had a history of IDU or blood transfusion before 1990, compared to 8 of 9 (89%) chronic carriers and 7 of 8 (87%) spontaneously recovered subjects. Among RIBA indeterminates who were not transfused or did not share intravenous drugs, 4 of 13 reported either occupation-related needle sticks, acupuncture, tattooing or cocaine snorting. No risk factor was identified in the remaining 9 RIBA-indeterminate subjects. Of the 9 chronically infected patients, 8 were infected with HCV genotype 1a and one was infected with genotype 2b.

Humoral Immune Responses

Recombinant Immunoblot Assay 3.0 (RIBA 3.0)—Table 1 shows the frequency of single antigen reactivity in the 15 persistent RIBA 3.0 indeterminate donors; five (33%) were reactive against c33c antigen (NS3 region), 4 (27%) against NS5 antigen and 6 (40%) against C-22 core antigen. None of the patients were reactive to 5-1-1/c100 (NS4 region). Although, the reaction strength ranged from 1+ to 4+ only 4 of 15 indeterminates had reactivity of 3+ or greater. Among RIBA-indeterminates, the specific reactive antigen and the strength of the reaction remained constant over a mean of 6 determinations during an average follow-up duration of 13 years.

All spontaneously recovered patients reacted to 2 or more RIBA antigens and remained HCV-PCR negative throughout the study period (mean number of determinations=15.5). Among the 8 spontaneously recovered subjects 3 (37%) were positive for all four antigens and 7 of 8 had reactivity strength of 3+ for at least one antigen. RIBA data were available for only 7 of 9 chronically infected patients among whom 5 (71%) were positive for all four RIBA antigens and 7 of 7 had reactivity of 3+ to at least one antigen.

Luciferase Immunoprecipitation System (LIPS)—Figure 1 shows the LIPS heatmap representation of the antibody response against HCV antigens in 9 chronic HCV carriers, 8 spontaneously recovered and 15 RIBA indeterminate patients. Titer values greater than the mean of the 16 normal controls plus 3 standard deviations were color-coded from clear to dark blue to signify the relative number of standard deviations above these reference values. In addition to the core and non-structural proteins in the RIBA, the LIPS assay also includes two envelope antigens.

Among the 9 chronically infected donors, all were moderate to strongly positive to at least 3 LIPS antigens including the envelope antigens. The one patient lacking reactivity to the envelope antigens was the only patient infected with HCV genotype 2b.

The pattern of LIPS reactivity among the spontaneously recovered subjects was distinctly different, and generally weaker than that observed with chronic carriers (Fig.1). Although NS3 was the dominant reactive antigen, as it was among chronic carriers, the strength of reactivity was markedly less as indicated in the heat map.

The antibody pattern in the LIPS assay among RIBA indeterminates was very different from that of both chronic carriers and spontaneously recovered subjects. Only 4 of 15 (27%) of RIBA indeterminates showed reactivity in the LIPS assay. The correlation between the HCV genomic region recognized by antibodies in the RIBA assay and that detected by the LIPS assay was poor. Three of 15 RIBA-indeterminates who reacted to only a single band in the RIBA assay, showed reactivity to two HCV antigens in the LIPS assay. None of the indeterminate subjects showed reactivity against NS4 or either of the envelope proteins. NS3 was the most reactive antigen among all 3 groups.

Figure 2 shows that for each of the LIPS antigens, there is a stepwise gradient with strong antibody reactivity in chronic HCV carriers, moderate activity in spontaneously recovered subjects and weak or no reactivity in the RIBA indeterminate group. Figure 2G shows the cumulative differences in antibody responses to the 6 HCV antigens used in the LIPS assay and demonstrates highly significant (Mann Whitney U-test) quantitative differences in anti-HCV antibody levels between RIBA-indeterminates and both chronically infected ($p<0.001$) and spontaneously recovered subjects ($p<0.009$). The quantitative level of antibody in the indeterminate population was higher, but not significantly different than unexposed controls ($p<0.06$).

Cellular Immune Responses (IFN- γ ELISpot)—The cell-mediated immune response was assessed in an IFN- γ ELISpot assays using peptides that covered the entire HCV polyprotein sequence. The graphs in figure 3 show the sum of the IFN- γ response to all the HCV antigens used in the assay for each patient. Forty-sevenpercent of RIBA indeterminates exhibited >200 IFN- γ spots/200,000 PBMC compared to 62.5% of recovered subjects, and none of the HCV carriers or healthy controls. One patient in the indeterminate group (Fig. 3, patient #10) did not respond to any HCV antigens, but the highest ELISpot activity was seen in two indeterminate donors (Fig. 3, #3 & 4).

The overall pattern indicates that some RIBA-indeterminate subjects exhibit strong IFN- γ production in response to HCV antigens (Fig. 3E). CMI responses to HCV in indeterminate subjects are very similar to those in spontaneously recovered subjects and higher, though not significantly different, from those in chronically infected patients. CMI responses were significantly elevated in all three groups compared to uninfected controls.

DISCUSSION

Although an average of 230,000 new HCV infections were estimated to occur annually in the US in the 1980s the number had declined to 36,000 per year by 1996.^{14, 15} In 2007, CDC estimated 17,000 new infections for that year after adjusting for asymptomatic infection and underreporting.¹⁶ Chronically infected individuals serve as a source of transmission to others and are at risk for the development of chronic liver disease, cirrhosis or hepatocellular carcinoma in the long-term.

The primary mode of transmission of HCV is through percutaneous exposure, particularly IDU and blood transfusion prior to 1990.^{17, 18} Since individuals infected with HCV remain asymptomatic and are generally unaware of their infection, they may present as healthy blood donors. Although highly sensitive assays for anti-HCV and HCV RNA are used to screen all blood donations and have dramatically reduced the risk of transfusion-transmitted HCV infection, rare cases have been reported in the post-testing era.¹⁹

In the original anti-HCV EIA introduced in 1990, a recombinant protein, C-100-3, derived from the NS-4 region of the virus was used as the solid-phase antigen. A large number, but not all, infectious blood donations were interdicted by this assay. Second generation EIAs, introduced in 1992, added recombinant antigens from the core and NS-3 regions and improved sensitivity.²⁰ Subsequently, a 3rd generation EIA assay added NS-5 antigen and modification of the NS-3 antigens to provide a further small increase in sensitivity.⁵ Although these antibody assays can detect the majority of HCV-infected individuals, early infections may not be detected due to the delay in antibody seroconversion. In addition, remote past infections may not be detected due to decreased antibody titer over time.

Because EIA assays are inherently prone to false positive reactions, a solid phase strip RIBA has been used as a confirmatory test for blood donors who test repeatedly reactive by EIA. The third-generation RIBA (RIBA 3.0) assay is more sensitive and specific than the second generation assay (RIBA 2.0), particularly in resolving and reclassifying RIBA-2.0 indeterminate reactions.^{5, 21}

A RIBA indeterminate result has generally been considered to represent a false positive reaction. This has created controversies in blood utilization and donor counseling. Additional information regarding HCV infection status can now be derived from routine HCV-RNA testing of donors such that RIBA testing is increasingly limited to those who test HCV-RNA negative. Nonetheless, for those who have had an indeterminant RIBA test in the

past or for those HCV-RNA negative subjects now found to be RIBA indeterminate, it is important to understand the meaning of this result.⁷

In this analysis, we selected patients from a large prospective study of HCV infected donors detected since anti-HCV testing was initiated in 1990.²² We selected 15 donors who were RIBA indeterminate on at least two occasions, remained indeterminate until the time of this study, and were available for recall sampling for PBMC. Humoral and cellular immune responses to HCV in the indeterminate population were compared to chronic HCV carriers, spontaneously recovered subjects and to those lacking any HCV serologic markers. Although not statistically significant ($P=0.11$) demographic analysis revealed that RIBA-indeterminate donors were older (mean 62.9 years) than spontaneously recovered subjects (mean 54.6 years) or chronic HCV carriers (mean 53 years). The older age of this group suggests that their HCV exposure might have been in the remote past allowing time for some anti-HCV antibody responses to have waned. It is also of interest, that only 2 of 15 (13%) persistent RIBA indeterminates had a history of IDU or blood transfusion compared to 89% of spontaneously recovered and 87% of chronic carriers. This raises the question of whether persons who ultimately lose their serologic responses to HCV are more likely to have had subtle exposures to HCV where perhaps the initial viral inoculum was very small. The small number of subjects studied and the ambiguity of risk posed by non-parenteral HCV exposures does not allow this speculation to be confirmed.

While it was previously assumed that most RIBA-indeterminates represented false positive reactions, it now appears from our work and others^{7, 23} that the majority represent waning antibody responses in persons who have recovered from a distant HCV infection. This is particularly true if the RIBA-indeterminate result is persistent. We have shown this to be the case through two main lines of evidence. First, RIBA indeterminates have strong cell mediated immune responses, very similar to those who have spontaneously recovered from HCV infection and very different from controls who have never been HCV infected (Fig. 3). These findings are similar to those reported by Bes, et al²³ and Hitzinger, et al⁷ further supporting the concept that RIBA indeterminacy reflects past exposure rather than a false positive EIA. Second, we assessed antibody to HCV quantitatively using the liquid phase LIPS assay and have shown a stepwise diminution in antibody level as one moves from being a chronic carrier (highest antibody level), to spontaneously recovered (intermediate level) to RIBA indeterminate (low level). These quantitative determinations of anti-HCV antibody are unique to this study and support the concept that, in at least some individuals, antibody gradually wanes after HCV infection is cleared. Although not demonstrated in this study, the logical extension of this supposition is that antibody would completely disappear over time in some HCV recovered subjects as has already been shown in the study of Seeff et al.²⁴

The decreased humoral response manifest as both RIBA indeterminacy and weak LIPS reactivity suggests the loss of anti-HCV antibody over time. Circulating HCV-specific antibodies have been reported to be undetectable in some subjects decades after recovery from HCV infection while HCV-specific helper and cytotoxic T-cell responses with IFN- γ are sustained.^{9, 25} Thus, combining CMI and LIPS responses, RIBA-indeterminates appear to represent persons who are at a later stage in the process of spontaneously recovered from HCV infection as manifest by a lower level and more restricted specificity of their anti-HCV antibody. This is indirectly supported by the older age of the RIBA indeterminate population (mean 62.9 years) compared to recovered subjects (mean 47.8 years) suggesting they may have had a more remote infection.

Although not demonstrated in this study, the next step in the progression would be the complete loss of detectable anti-HCV antibody. Such complete loss was shown in a

retrospective-prospective study of Seeff et al²⁴ wherein 7% of subjects who had anti-HCV in their original stored sample, no longer had antibody when recalled 23 years later. Hence, the rate of spontaneous recovery from HCV infection is probably higher than previously estimated from the observed pattern of anti-HCV antibody (RIBA+) in the absence of HCV RNA.

When considering the natural history of HCV infection, it is customary to focus on the outcomes of those chronically infected. However the Seeff study²⁴ and this current study, showing quantitative reduction in antibody, suggest that the natural history of HCV infection has to be viewed in the context of the totality of the infection. The relatively large percentage of infected individuals who spontaneously recover proportionately diminish the percent who have severe outcomes.

Based on these findings, the message to blood donors found to be RIBA indeterminate, particularly if persistent, should be changed to indicate that while the test could represent a false positive result, it more likely indicates recovery from a distant, silent HCV infection. Additionally, donors could be counseled that such a remote HCV infection has no implications for their current health and represents no current risk to their close contacts.

In summary, persistent RIBA indeterminate reactions generally indicate recovery from a remote HCV infection. This suggests that the frequency of spontaneous recovery from HCV infection is higher than previously estimated.

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Figure 1. Heatmap analysis of anti-HCV antibody profiles

Anti-HCV antibody titers to the 6 antigens are shown for each of the 9 patients with chronic HCV infection, 8 spontaneously recovered, 15 RIBA indeterminate, and 16 normal volunteers. The titer values greater than the mean of the 16 normal volunteers plus 3 standard deviations were color-coded from green to black to signify the relative number of standard deviations above these reference values.

Figure 2. LIPS detection of antibodies against HCV antigens

Each symbol in the scatter plots represents an individual sample corresponding to the 16 normal volunteer (NV), 15 RIBA indeterminate, 8 spontaneously recovered or 9 chronic HCV patient samples. Antibody titers in light units (LU) are shown for HCV core (A), NS3 (B), NS4 (C), NS5a (D), Env1 (E), Env2 (F), as well as the sum of the anti-HCV antibody titers to these 6 antigens (G). For determining sensitivity and specificity, the solid line represents the cut-off level derived from mean plus 3 SD of the antibody titer of the 16 normal volunteers. *P*-values were calculated using the Mann Whitney *U*-test.

Figure 3. Frequency of HCV-specific IFN- γ -producing PBMC

(A–D) The frequency of HCV core, envelope, p7, NS2/3, NS4 and NS5-specific IFN- γ -producing PBMC of subjects with indeterminate RIBA result (A), spontaneously HCV-recovered patients (B), patients with chronic hepatitis C (C) and healthy, antibody-negative blood donors (D) was determined by Elispot analysis using overlapping HCV peptides covering the complete HCV polyprotein. Responses against peptides are indicated by the different colors. (E) Frequency of all HCV-specific IFN- γ -producing PBMC. Horizontal bars indicate the mean response per group, vertical bars indicate the standard deviation. P-values were calculated using the Mann-Whitney U test.

Table 1

Frequency of single antigen reactivity in the 15 persistent RIBA 3.0 indeterminate donors

RIBA 3.0 Antigen	Antigen source	Indeterminate patients
c33c (NS3)	NS recombinant	5 (33.3%)
NS5	NS recombinant	4 (26.7%)
5-1-1/c100 (NS4)	NS synthetic	0
C-22	Core	6 (40%)
Total		15 (100%)

NS: non-structural protein