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 3.0) is used to confirm the specificity of antibodies to hepatitis C virus (HCV) detected by enzyme immunoassay (EIA). The result is interpreted as positive, indeterminate or negative and the counseling message for RIBA indeterminate donors is vague. Herein, we analyze data from a retrospective-prospective study of anti-HCV+ blood donors to interpret and estimate clinical relevance of persistent RIBA indeterminate reactions in the context of the natural history of HCV infection. **Materials and methods**: Donor demographics, HCV exposure history, humoral immunity and cell-mediated immune responses were compared in 15 RIBA persistent-indeterminates, 9 chronic HCV carriers (RIBA+/HCV RNA+) and 8 spontaneously recovered (RIBA+/HCV RNA-neg) subjects. Serum samples were tested for antibodies to multiple HCV antigens by a liquid phase Luciferase Immunoprecipitation System(LIPS) assay. Cell mediated immune response (CMI) was assessed by IFN-γ ELISpot assay. **Results**: The majority of RIBA 3.0 indeterminate patients did not have identified high risk behavior for HCV infection. The average age of patients in the indeterminate group (62.9yrs) was higher, but not statistically different than the other two groups. Among the 15 RIBA indeterminates, 6(40%) reacted only to C22 core antigen. The LIPS assay confirmed that antibody strength was weak and narrowly directed against NS3, core or NS5a and distinctly different from the broad reactivity observed in chronic carriers. Importantly, the quantitative LIPS assay showed a significant step-wise decline in antibody level, with chronic carriers>spontaneouslyrecovered> indeterminates> negative controls. In contrast, CMI was very strong in the indeterminate group and similar to persons with spontaneous recovery. **Conclusion**: The single-band RIBA pattern in persistent indeterminate donors indicates waning humoral immunity to remote HCV infection as also suggested by older age. Similar CMI response to those known to have spontaneously recovered, but significantly lower antibody levels suggests that RIBA indeterminacy is a late stage in spontaneous recovery from HCV infection and a possible transition phase to RIBA negativity.

INTRODUCTION

Approximately 200 million people are estimated to harbor HCV worldwide[1]. In the US, the estimated prevalence is 3.9 million with 2.7 million people found to have chronic infection [2,3]. The incidence of HCV infection by 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, asymptomatic HCV-infected donors continue to be identified mainly due to prevalent intravenous drug use.

The widely used screening test for HCV infection is a third generation enzyme immunoassay (EIA) for anti-HCV and molecular amplification for HCV-RNA. The presence of antibody, in those who are HCV RNA negative, is confirmed by RIBA. A positive RIBA shows reactivity to at least 2 of the 4 antigens displayed while a negative RIBA shows no reactive bands. Only one RIBA band is considered an indeterminate pattern. Majority of blood donors with indeterminate RIBA result are HCV-RNA negative by polymerase chain reaction (PCR), though indeterminate reactions have been observed rarely in HCV-RNA positive subjects who are immunocompromised[5].

Currently available solid phase assays, such as EIA and RIBA, are unable to identify pathogen specific antigens or epitopes. The LIPS assay, a recently described liquid-phase immunoprecipitation assay, can quantitatively detect antibody response to multiple pathogen-specific antigens purified under non-denaturing conditions[8] .

Cell mediated immune (CMI) response to HCV has been observed in individuals with reduced antibody response[9]. Measurement of CMI in RIBA indeterminates could 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 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 anti-HCV antibody.

MATERIALS AND METHODS

Study population

Donors to Transfusion Medicine Department, NIH and 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. Donors were also followed if they had evidence of recovery from HCV infection and if they were RIBA-indeterminate.

Within this study population, we identified 15 RIBA indeterminates. Laboratory results were compared to 9 chronic HCV carriers and 8 spontaneously recovered patients. Thirteen and 16 anti-HCV and HCV RNA negative healthy volunteer blood donors were included as negative controls in the IFN-γ ELISpot and the LIPS assays, respectively. There are no clinical data available for the anonymized healthy volunteer control donors.

Relevant demographic and clinical data, including a history of parenteral exposures and high-risk sexual activity were obtained from study subjects. All subjects, except anonymized healthy volunteers 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. Enrolled patients were seen annually for clinical assessment and every six months for laboratory evaluation.

Humoral immune response

RIBA3.0, a solid phase assay and LIPS, a liquid phase assay were used to asses humoral immune response in patients included in the study.

Recombinant Immunoblot Assay (RIBA 3.0)

Blood units collected from healthy volunteer donors were routinely screened for the presence of serum anti-HCV using a commercial enzyme linked immunosorbent assay (EIA 2.0; ORTHO-Clinical Diagnostics, Raritan, NJ). Repeat EIA reactive samples were confirmed with an *in vitro* qualitative strip immunoblot assay, RIBA HCV 3.0 SIA (Chiron Corp., Emeryville, CA).

According to the manufacturer's description, 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. A sample is considered positive when at least two HCV bands have $\geq$1+, indeterminate when a single HCV band has $\geq$1+ and negative when no band reactivity are present.

Luciferase Immunoprecipitation System (LIPS)

pREN2 was used to generate all plasmids for expression in mammalian cells and production as Ruc-antigen fusion proteins[8,10]. Genomic DNA and cDNA templates for the HCV antigens were obtained from HCV-1a genotype and all Ruc-HCV antigens have been prepared as previously described[8,11].

LIPS with Ruc fusion proteins: Cos1 cells lysates were prepared as described. Total luciferase activity in 1μL of each crude extract was determined by adding 9 μL of PBS in a 1.5 ml microfuge tube, followed by the addition of 100μL 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[10]. In a total volume of 100μL in a 96-shallow-well microtiter plate, 10μL of human sera was incubated with 90μL Buffer A (20mM Tris, pH 7.5, 150mM NaCl, 5mM MgCl2, and 1%Triton X-100) and 10 x 10^6 light unit equivalents of Ruc-antigen Cos1 cell extract at 4°C with tumbling for 1hr. Then 5μL of a 30% suspension of Ultralink protein A/G beads (Pierce Biotechnology Inc, Rockford, IL) in PBS was added for an additional 1hr 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 100μL buffer A and twice with 1.0ml of phosphate-buffered saline (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 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 beads plus extract.

Cell Mediated Immunity

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 20mg/mL in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO), pooled, and further diluted with PBS (Mediatech, Manassas, VA). 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 RIBA-

indeterminate, 8 spontaneously recovered, 9 chronic HCV carriers and 13 healthy, blood donors lacking serological markers of HCV infection. PBMC were separated from plasma, thrombocytes and erythrocytes by density gradient centrifugation as previously described[12] and washed three times with PBS. Cells were used immediately or cryopreserved for later use.

IFN-γ Elispot assays: IFN-γ Elispot assays were performed as previously described[9] with minor modifications. Ninety-six-well plates (Millipore, Bedford, MA) were coated with 0.5μg/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 1hr at 25°C, washed twice more with PBS and blocked for 0.5hr with complete cell culture medium (Roswell Park Memorial Institute 1640; RPMI1640) containing 5% fetal bovine serum (Serum Source International Inc, Charlotte, NC), 100U/ml penicillin, 100μg/ml streptomycin, 2mM 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 1μg/ml per individual peptide. Stimulation with 1μg/ml phytohemagglutinin (PHA, Sigma, St. Louis, MO) served as positive control. After a 30hr incubation the plates were washed three times with PBS and four times with PBS/Tween (1:2000) and incubated overnight with 100μl of 0.25μg/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 1hr with streptavidin–alkaline phosphatase (1:2,000 dilution; Dako, Carpinteria, CA) in PBS/Tween (1:2000)/ BSA (1%) solution. The plates were washed 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 +3 standard deviations of the response observed with 10 healthy blood donors for the same peptide pool.

RESULTS

Study Population

The average age of the RIBA indeterminate, spontaneously recovered and chronic HCV group was 62.9 years, 47.8 years and 53 years, respectively. The mean duration of follow up was 15.5 years (range 3–28). The exact duration of HCV infection 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 all groups where the potential interval since infection could be estimated according risk factors, it was greater than 30. There was a disparity between RIBA indeterminates and the other groups in that only 13% of indeterminates had a history of IDU or blood transfusion before 1990 compared to 89% of chronic carriers and 87% of spontaneously recovered subjects. Eight of the chronic carriers were infected with HCV genotype 1a and one with genotype 2b.

Humoral immune response

Recombinant Immunoblot Assay 3.0 (RIBA 3.0)

Table 1 shows that none of the patients were reactive to 5-1-1/c100 (NS4 region). All spontaneously recovered patients reacted to 2 or more RIBA antigens and remained HCV-PCR negative throughout the study period (mean # of determinations 15.5). Among the spontaneously recovered subjects 37% reacted to all four antigens, 71% of chronically infected patients were positive for all four RIBA antigens.

Luciferase Immunoprecipitation System (LIPS)

Figure 1 shows the LIPS heatmap representation of 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. LIPS reactivity among the spontaneously recovered subjects was distinctly different, and generally weaker than that among chronic carriers

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 patient infected with HCV genotype 2B.

The antibody pattern in the LIPS assay among RIBA indeterminates was very different from the chronic carriers and spontaneously recovered subjects. Only 4 of 15 (27%) of RIBA indeterminates

showed reactivity in the LIPS assay with poor correlation between the HCV genomic region detected by RIBA and that detected by LIPS. Three of 15 who reacted to a single band in the RIBA assay, showed reactivity to two HCV antigens in the LIPS assay. NS3 was the most reactive antigen among indeterminates as it was among chronic carriers and spontaneously recovered subjects. No indeterminate subjects showed reactivity against NS4 or the envelope proteins.

Figure 2 shows that for each of the LIPS antigens, there is a stepwise gradient showing strong reactivity in chronic HCV carriers, medium level activity in the spontaneously recovered and weak or no reactivity in the RIBA indeterminate group and Figure 2G shows the sum of antibody responses to the 6 HCV antigens used in the LIPS assay and demonstrates highly significant quantitative differences in anti-HCV antibody levels between each of the four groups tested.

Cellular immune response (IFN-γ Elispot)

In Figure 3 the graphs represent the EliSpot score sum of INF-γ response to all the antigens used in the assay. Mann-Whitney U tests were employed to compare the frequency of HCV-specific IFN-γ producing PBMC between groups. The overall pattern makes it evident that CMI responses in patients with RIBA indeterminate results are similar to those of persons who spontaneously recovered from HCV infection and very distinct from persons with chronic HCV infections or those never exposed to HCV. One patient in the indeterminate group (patient #10) did not respond to any HCV antigens analyzed.

Figure 3E shows the frequency of all HCV-specific IFN-γ-producing PBMC. Horizontal bars indicate the mean response per group and vertical bars indicate the standard deviation. P-values were calculated using the Mann-Whitney U test. The key observation in Figure 3E is that the mean sum response of indeterminate subjects is almost identical to spontaneously recovered subjects.

DISCUSSION

Although an average of 230,000 new HCV infections were estimated to occur annually in the US in the 1980s, that number had declined to 36,000 per year by 1996[13,14]. In 2007 CDC estimated 17,000 new infections for the year after adjusting for asymptomatic infection and underreporting [15]. The Third National Health and Nutrition Examination Survey (NHANES III), conducted during 1988–1994, indicated approximately 3.9 million (1.8%) Americans have been infected with HCV.[2,3] .

The primary mode of transmission of HCV is through percutaneous exposure, particularly shared-needle IDU and blood transfusion prior to 1990[16,17]. The development of highly sensitive laboratory screening assays for anti-HCV and HCV-RNA for all blood donations has dramatically reduced the risk of transfusion-transmitted HCV infection.

In the original anti-HCV enzyme immunoassay (EIA), a single recombinant protein, C-100-3 of the virus was used as the solid-phase antigen. Second generation EIAs, added recombinant antigens from the core and NS-3 regions with improved sensitivity[18]. Current third generation EIA uses recombinant c22, C200 and NS5.

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 repeat reactive to EIA. Antigens from the core, NS3, NS4 and NS5 regions of HCV are displayed on a nitrocellulose strip to confirm antibody responses to HCV-specific proteins. The RIBA result is interpreted as positive (≥2 reactive antigens), indeterminate (1 reactive antigen) and negative (no reactive antigen). The third-generation RIBA (RIBA 3.0) assay is more sensitive and specific than the second generation (RIBA 2.0), particularly in providing additional information on indeterminate specimens[5,19]. Additional information regarding HCV infection status can now be derived from routine HCV RNA testing of donors and RIBA testing is increasingly limited to those who test HCV RNA negative.

We selected patients from a large prospective study of HCV infected donors detected since 1990. We selected 15 RIBA indeterminate donors. Humoral and cellular responses to HCV in the indeterminate population were compared to chronic carriers, spontaneoulsy recovered and those lacking HCV serologic markers. Although not stastically 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 the exposure might have been in the remote past allowing time for anti-HCV antibody responses to have waned. It is also of interest, that only 13% persistent RIBA indeterminates had a history of IVDU or blood transfusion compared to 87% of those spontaneously recovered and 89% of chronic carriers. This raises the question whether persons who ultimately lose their serologic responses to HCV are more likely to have had subtle

exposures to HCV where the initial viral inoculum was very small. The small number of subjects studied and the vagueness of non-parenteral HCV exposures does not allow this speculation to be confirmed.

Table 1 shows RIBA results of the indeterminates where 6(40%) reacted to the most immunogenic core antigen. This is in contrast to Melve et al [6] prospective study where RIBA reactivity against the nonstructural NS5 antigen was the most frequent finding. This study suggested that cross-reactivity with other viruses could account for indeterminate HCV RIBA reactivity. As shown in Figure 1 and 2 there is a significant decline in LIPS antigen detection from strongly positive in the chronically infected to weaker reactivity in the spontaneously recovered followed by RIBA indeterminates. This suggests that RIBA indeterminate patients have very low undetectable antibody level as they are in the process of complete recovery. Circulating HCV-specific antibodies have been shown to be undetectable 18 to 20 years after recovery while HCV specific helper and cytotoxic T-cell responses with IFN γ are sustained [9,20].

While it was previously assumed that most RIBA indeterminates were false positive reactions, it is now clear from our work and others[1,7] that the majority represent waning antibody responses in persons who have recovered from a distant HCV infection. There are two main lines of evidence for this conjecture. First, RIBA indeterminates have strong CMI responses, similar to those who have spontaneously recovered from HCV infection and different from those chronically infected and normal controls (Figure 3). Second, direct quantitation using LIPS, we have shown that there is a stepwise diminution in antibody level from being a chronic carrier, to spontaneously recovered, to RIBA indeterminate. These quantitative determinations of anti-HCV antibody are unique to this study. Thus, combining CMI and LIPS responses, RIBA indeterminates represent persons who have spontaneously recovered from HCV infection, but now have a low level and more restricted specificity of anti-HCV antibody.

Although not demonstrated in this study, the next step in the progression would be the complete loss of detectable anti-HCV antibody. Such loss of detectable antibody was shown in a retrospective-prospective study of Seeff et al[21] wherein 7% of subjects with anti-HCV in their original stored sample, no longer had antibody when recalled 25 years later. Hence the rate of spontaneous recovery from HCV infection is higher than previously estimated from the observed pattern of anti-HCV antibody.

Based on these findings, the message to blood donors found to be RIBA indeterminate should indicate that they are recovering from a distant/silent HCV infection. Although there is no current evidence of HCV reactivation a RIBA-indeterminate result would only have negative implications, if seemingly recovered subjects harbor HCV in PBMCs or the liver that could reactivate with immunosuppression.

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TABLE AND FIGURE LEGEND

Table 1: RIBA 3.0 positive antigens in indeterminate patients.

Fgure 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 clear to dark blue 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), 13 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.

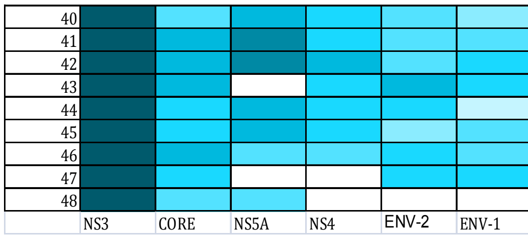
Figure 3: Frequency of HCV-specific IFN-γ-producing PBMC.
(A-D) The frequency of HCV core, envelope, p7, NS2/3, NS4 and NS5-specific IFN-γ-producing PBMC of subjects with indeterminate RIBA result (A), spontaneously HCV-recovered patients (B), patients with chronic hepatitis C (C) and healthy, antibody-negative 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:

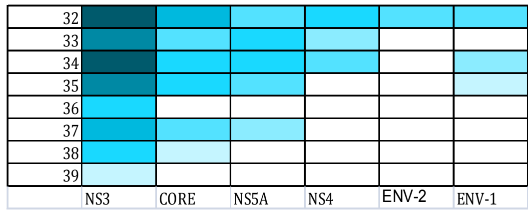
RIBA 3.0 Antigen	Antigen source	Indeterminate patients
c33c (NS3)	NS recombinant	5 (33.3%)
NS5	NS recombinant	4 (26.7%)
5-1-1/c100 (NS4)	NS synthetic	0
C-22	Core	6 (40%)
Total		**15 (100%)**

NS: non-structural

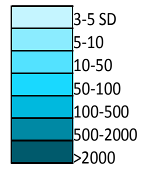
Chronic HCV



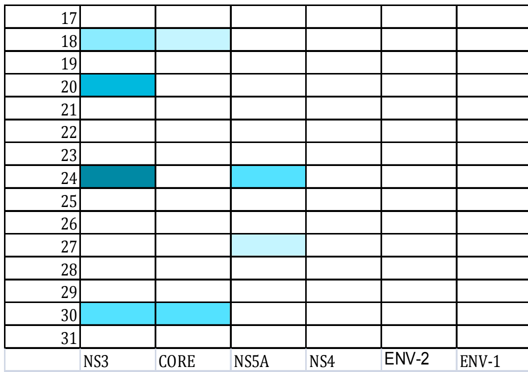
Spontaneous Recovery



SD above NV



Indeterminate



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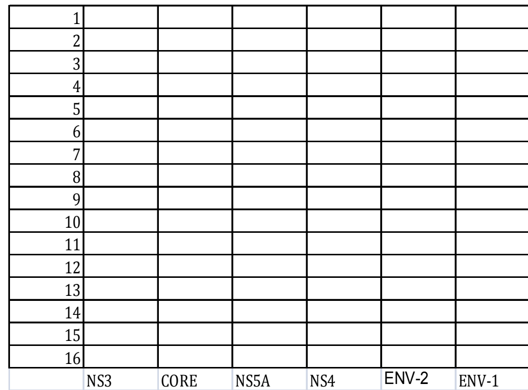

Figure 1:

A. Core

B. NS3

C. NS4

D. NS5A

E. Env1

F. Env2

G. Sum of antibody responses to 6 HCV antigens

P>0.06

P>0.009

P>0.001

LU

CTRL Indeterminate Spon Rec Chronic

Figure 2:

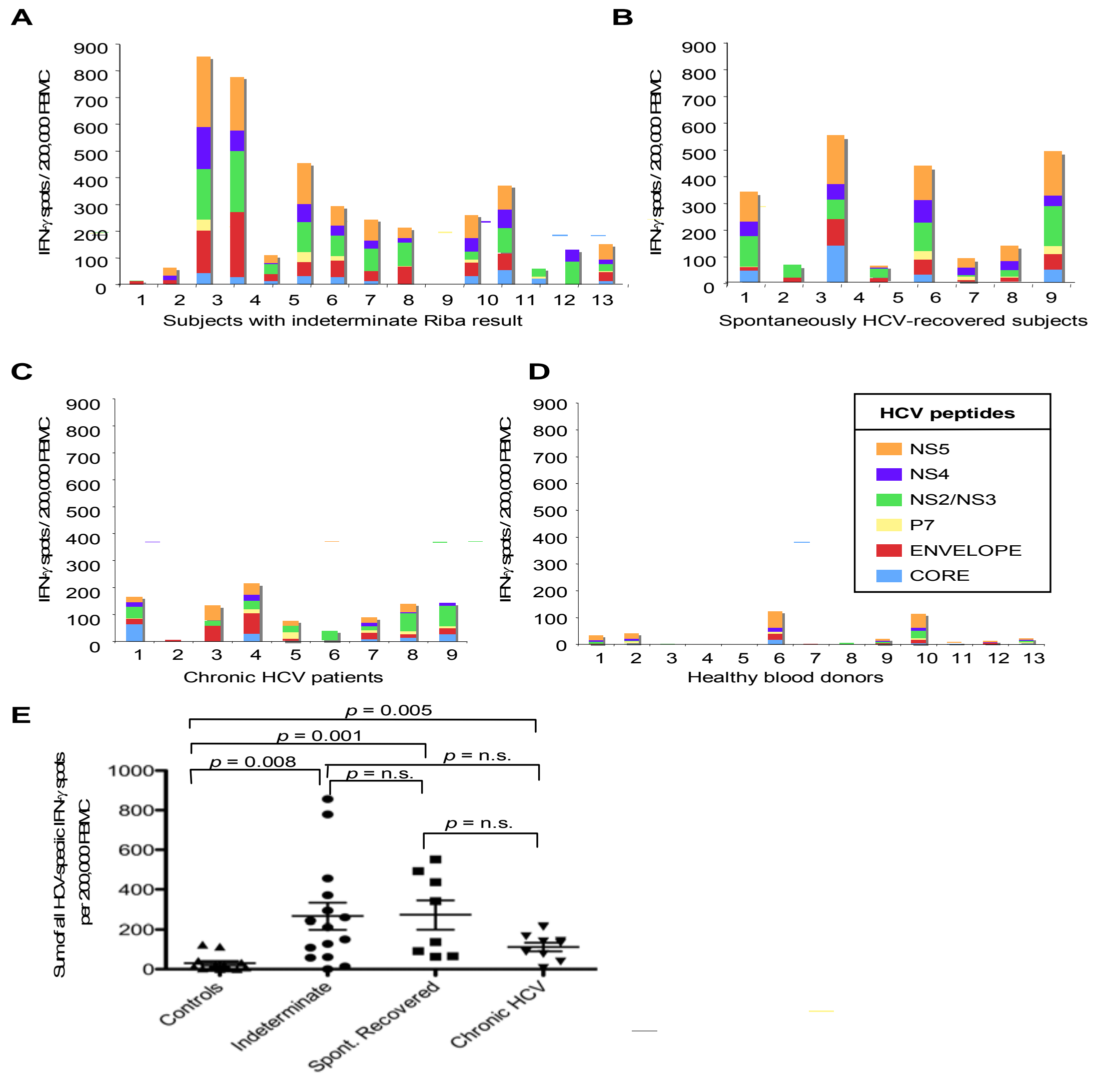

Figure 3: