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Cross-genotypic Polyclonal Anti-HCV Antibodies from Human Ascitic Fluid

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Abstract

Many anti-HCV antibodies are available, but more are needed for research and clinical applications. This study examines whether ascitic fluid from cirrhotic patients could be a source of reagent-grade antibodies. Ascitic fluid from 29 HCV patients was screened by ELISA for anti-HCV antibodies against three viral proteins: core, NS4B, and NS5A. Significant patient-to-patient variability in anti-HCV antibody titers was observed. Total ascitic fluid IgG purified by Protein-A chromatography reacted with HCV proteins in immunoblots, cell extracts, and replicon-expressing cells. Affinity-purification using synthetic peptides as bait allowed the preparation of cross-genotypic antibodies directed against pre-selected regions of HCV core, NS4B, and NS5A proteins. The performance of the polyclonal antibodies was comparable to that of monoclonal antibodies. Anti-NS4B antibody preparations reacted with genotype 1a, 1b, and 2a NS4B proteins in immunoblots and allowed NS4B to be localized in replicon-expressing cells. Summary: Ascitic fluid is an abundant source of human polyclonal cross-genotypic antibodies that can be used as an alternative to blood. This study shows the utility of selectively-purifying human polyclonal antibodies from ascitic fluid. Affinity purification allows antibodies to be selected that are comparable to monoclonal antibodies in their ability to react with targeted regions of viral proteins.

1. Introduction

Antibodies to hepatitis C virus (HCV) proteins have a number of potential uses in both research and clinical settings. Although antibodies can be made by immunizing animals, this approach has limitations. Polyclonal antibodies generated in response to viral antigen

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inoculation often have a low signal-to-noise ratio. Monoclonal antibodies can be difficult and costly to obtain, and often recognize a single HCV sub-genotype (Eng, et al. 2009). Additionally, many available antibodies are focused on single antigenic epitopes or regions. For example, nearly all commercially available antibodies to the core protein recognize the highly conserved region between amino acid residues 21–40 (Eng, et al. 2009). As a result of these limitations, new and easy methods to obtain antibodies would be useful.

Unlike the antibodies generated in animals, the anti-HCV antibodies in patients evolve through repeated cycles of antigenic stimulation and B cell selection. For many RNA viruses, the antigenic stimulus is provided by the viral quasispecies, which expresses a vast population of closely-related isoforms of the viral proteins (Fishman and Branch. 2009). This process is expected to yield a diverse population of antibodies with superior performance characteristics.

In addition to research applications, polyclonal human antibodies have the potential to reduce transmission by neutralizing infectious HCV particles (Knodel, et al. 1976, Sanchez-Quijano, et al. 1988, Piazza, et al. 1998). Polyclonal human antibodies may also have diagnostic utility. They are used in the most sensitive Immunohistochemical (IHC) assay that has been developed for the detection of human liver cells infected with HCV (Ballardini, et al. 2002, Grassi, et al. 2006). In order to make human antibodies available for use in research and in the clinic, sources of these antibodies need to be identified. Human antibodies may be obtained by venipuncture, but the amount of blood that may be safely drawn at one time is limited to about 600 mL. Plasmapheresis allows for collection of a much larger volume of plasma, but it is an invasive procedure that requires specialized equipment.

In about 10% of patients with cirrhosis, ascitic fluid accumulates as a result of portal hypertension (Epstein. 1989, Seeff. 2002). Large volume paracentesis is used to treat diuretic refractory ascites in HCV patients with end-stage liver disease (Garcia-Tsao. 2002), potentially providing an abundant source of anti-HCV antibodies from a diverse donor population. Ascitic fluid is typically discarded as waste, but can be collected under sterile conditions. This study tests the hypotheses that reagent-grade antibodies can be prepared from ascitic fluid using affinity purification to *select* antibodies with desired antigenic specificities (Garcia-Tsao. 2002). It demonstrates that ascites is a source of anti-HCV antibodies that can be used to address basic questions about HCV molecular biology and pathogenesis.

2. Materials and methods

2.1. Patient selection

Forty-one adults undergoing therapeutic large volume paracentesis at Mount Sinai Medical Center in NY, NY were enrolled with IRB approval. This study was approved by the Mount Sinai Ethics Committee and subjects were included only if they were able to provide informed consent for specimen collection and medical record review. Twenty-nine subjects were infected with HCV. One patient was also co-infected with HIV-1 (IgG #29). All had a positive anti-HCV clinical antibody test; 27 of 29 had HCV RNA in serum and/or ascitic fluid. The majority of patients were genotype 1a and 1b (79%) or genotype 3a (14%); the genotype of two patients was unknown. Among 12 control subjects who lacked anti-HCV antibodies, nine had alcoholic liver disease and three had hepatitis B virus (HBV) infection. Patients with evidence of spontaneous bacterial peritonitis were excluded. Ascitic fluid was collected under sterile conditions on ice, aliquoted and stored at -70°C .

2.2. Peptides and indirect anti-HCV enzyme-linked immunosorbent assays

A collection of HCV H77 (genotype 1a) peptides and a control peptide from PDC-E2 were obtained from 21st Century Biochemicals (Marlboro, USA) or the AIDS Reagent Program (Germantown, USA). The following six peptides were used in this study: Core-2 (7–25;PQRKTKRNTNRRPQDVKFPC-amide); Core-3 (28–41;Ac-CGQIVGGVYLLPRRG-amide); Core-5 (29–46;GQIVGGVYLLPRRGPRLGV-amide); NS4B (1901–1922;Ac-ILRRHVGPGEAVQWMNRLIAF-C-OH); NS5A (2297–2314;VETWKKPDYEPPVHGC-OH). A peptide corresponding to an immunodominant epitope from the pyruvate dehydrogenase complex E2 subunit or PDC-E2 (Ac-CGDLLEIETDKATI-amide) was used as a negative control. Ascitic fluid specimens were tested for activity against peptides using indirect ELISAs performed in Pierce 96-well maleimide (Rockford, USA) or NUNC IMMOBILIZER-AMINO plates (Kamstrupvej, Denmark). After peptide attachment and blocking, specimens were added and subsequently reacted with Jackson ImmunoResearch HRP-conjugated goat-anti human IgG (West Grove, USA). The HCV peptide or a control peptide (PDC-E2) were covalently attached to wells of microtiter plates and reacted in duplicate with each ascitic fluid specimen. The O.D. value of the PDC-E2 reaction was subtracted from the O.D. of the HCV reaction. Values of HCV specimens were normalized by dividing them by the cutoff value which was the mean of the control specimens plus three standard deviations. Optical Density (O.D.) was read at 450 nm, with a 650 nm reference subtraction. Normalized values greater than 1.0 were considered positive. All specimens were tested in duplicate on at least two occasions.

2.3. Preparation of crude IgG from ascitic fluid

To obtain IgG, ascitic fluid was microfiltered (0.45 μ) using a GE Healthcare Quixstand (Uppsala, Sweden) hollow fiber system (CFP-4-E-4X2MA column) followed by diafiltration with PBS (pH 7.4). The fluid was concentrated 10-fold using GE Healthcare Quixstand hollow fiber system (UFP-30-C-4X2MA column) followed by diafiltration with PBS. The fluid was then run over a MabSelect Protein-A from GE Healthcare column. The column was washed with PBS until baseline absorbance was obtained. Antibody was eluted at low pH. The antibody peak was collected in the presence of 10X quench buffer (50mM NaH₂PO₄, pH 8.6) and extensively dialyzed against PBS. Antibody concentration was determined by O.D. at 260nm and 280nm, and purity was determined using non-denaturing SDSPAGE.

2.4. Affinity purification of core, NS4B, and NS5A antibodies

Affinity purification of anti-core, anti-NS4B, and anti-NS5A antibodies was performed by binding peptides (Core-2, NS4B, or NS5A) to Pierce SulfoLink Coupling Gels (Rockford, USA) and then exposing ascites-derived IgG to the matrix-bound peptide according to the manufacture's protocol. Antibodies were eluted in 1ml fractions and the protein content was determined by O.D. at 260nm and 280nm.

2.5. Replicons: cell culture, immunoblotting and immunofluorescence (IF)

Huh-7.5 cells and Huh-7.5 cells with full-length (FL) and subgenomic (SG) bicistronic neomycin-expressing HCV replicons were cultured as described before (Blight, et al. 2002, Tscherne, et al. 2007). The replicons express HCV proteins of H77 (genotype 1a), Con1 (genotype 1b), J6/JFH and JFH (genotype 2a) under the control of the EMCV IRES and they express a p28 core-neomycin fusion protein under the control of the HCV IRES.

Immunoblots were performed as previously described (Walewski, et al. 2001). Briefly, proteins were fractionated in 12% Bis-Tris SDS-polyacrylamide gels and transferred to Invitrogen nitrocellulose membranes (Carlsbad, USA). Ascitic fluid, crude IgG, affinity-

purified polyclonal antibodies, and mouse monoclonal antibodies were generally used at a 1:1000 dilution. The primary antibodies were detected using BioRad (Hercules, USA) goat anti-human or anti-mouse IgG (catalog #170–6521/170–6520, respectively). Monoclonal antibodies included anti-core 2M (epitope, amino acids 7–50, Alexis), anti-E2 monoclonal 3/11 (Flint, et al. 1999) and anti-NS5A monoclonal 9E10 (Lindenbach, et al. 2005).

Direct and indirect IF microscopy was performed on cells grown on glass chamber slides. Cells were washed in PBS, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 5% goat serum, incubated with primary antibody, and then with secondary antibody. A 1:1000 dilution of ascitic fluid IgG and 1:100 dilutions of affinity-purified antibodies were used in most experiments. Coverslips were mounted with mounting media containing DAPI. Confocal microscopy was performed with a Leica (Bannockburn, USA) TCS-SP confocal laser scanning microscope with Ar-UV (350nm), Argon (488nm), and Diode (561nm) lasers. Epifluorescence microscopy was performed with a Nikon Eclipse E600 (Melville, USA). To demonstrate specificity, affinity-purified antibodies were incubated with either the HCV peptide used for purification or the PDC-E2 peptide.

2.6. Data analysis and statistical methods

Analyses were performed using the Mann-Whitney U test or the log-rank test, as appropriate, for continuous data; and using chi-square or Fisher's exact tests for binary and ordinal data. A p value of less than 0.05 was considered significant. Data analyses were performed using Excel and JMP software (Nutley, USA).

3. Results

3.1. Baseline patient data, immunoglobulin levels and anti-HCV ELISAs

Patients with HCV had a median age of 55.5 years. HCV patients and controls were well-matched with respect to stage of liver disease (Table 1). The ascitic fluid/serum IgG ratio was about 1:5. Patients with HCV had significantly higher levels of IgG in both serum and ascitic fluid than controls ($p < 0.05$). The median concentration of IgG in ascitic fluid from HCV patients was 549 mg/dL. Thus, a typical 6-liter large volume paracentesis provided approximately 33 g of IgG.

Based on published data about anti-HCV antibodies in serum (Desombere, et al. 2007, Takao, et al. 2007, Gabrielli, et al. 1996, Zhang, et al. 1995), we reasoned that the titer and profile of anti-HCV antibodies in ascitic fluid were likely to vary from patient-to-patient. Indirect ELISAs were used to identify ascitic fluid samples with high titers of antibodies against three HCV peptides: Core-2, NS4B, and NS5A. Among HCV mono-infected patients, 86% had anti-Core-2, 14% had anti-NS4B, and 24% had anti-NS5A antibodies (Fig. 1). Among HCV patients, reactivity to the Core 2 peptide was generally higher than to the other two peptides; however, the antibody profiles varied considerably. Subject 2, for example, had no detectable anti-Core 2 antibodies, but had the second highest titer of anti-NS4B antibodies. None of the 12 control subjects had anti-HCV antibodies. IgG from the ascites of patients 2, 7, 8, and 29 was purified by Protein-A chromatography. This process yielded 3.08 g (IQR = 2.41, 3.75) of IgG/liter of ascitic fluid, a recovery of approximately 60%.

3.2. Crude IgG from ascites fluid contains highly specific anti-HCV antibodies

Immunoblots and Immunofluorescence (IF) were used to test the performance of the IgG preparations. The IgG preparations differed from each other in their reactivity to HCV proteins in extracts of Huh-7.5 cells with sub-genomic (Fig. 2A–C, lanes b) and full-length

(lanes c) replicons. As expected based on the variation seen in the antibody survey presented in Fig. 1, each ascites fluid IgG preparation reacted with a characteristic set of HCV protein bands. IgG #7 reacted with core (arrowhead), the core-neomycin (neo) fusion protein (arrow), and the E2 envelope protein (asterisk). The core-neo fusion protein contains the N-terminal 12 amino acids of the HCV core protein. In contrast to its high reactivity with HCV proteins IgG #7 reacted with almost no cellular proteins (Fig. 2A, lane a). IgG #8 reacted with core, the core-neomycin fusion, and NS5A (Fig. 2B, dot). IgG #2 reacted with NS5A and other non-structural proteins, but showed little reactivity to core (Fig. 2C). The positions of core, E2 and NS5A were determined using mouse or rat monoclonal antibodies (Fig. 2D, E).

The IgGs of all three subjects produced intense IF signals when reacted with replicon-containing Huh-7.5 cells (Fig. 2F–H) and produced extremely low background signals when reacted with Huh-7.5 control cells (Fig. 2I–K). The immunoblots and IF analyses indicated that the ascites fluid-derived IgG preparations contained high titers of anti-HCV antibodies and showed that ELISAs and Western blots can be used to identify preparations with antibodies directed against specific HCV proteins.

3.3. Affinity-purification of polyclonal human anti-core antibodies

Polyclonal antibodies directed against specific regions of HCV proteins were selected from the IgG preparations by using affinity purification methods. Anti-Core-2 antibodies were separately purified from IgG #7, IgG #8 and IgG #29. The average peak fraction obtained from 50 mL of crude IgG contained 0.85 mg of affinity-purified antibody. ELISAs were used to determine the increase in specificity achieved by affinity purification. Titers of anti-Core-2 and anti-Core-5 antibodies were measured before and after affinity purification of ascitic fluid IgG #8. The titer of anti-Core-2 antibodies was 1.9-fold higher than the titer of anti-Core-5 antibodies in the starting material. After affinity purification with the Core-2 peptide, the titer of anti-Core-2 antibodies was 1035-fold higher than the titer of anti-Core-5 antibodies, a 534-fold enrichment. Conversely, the titer of anti-Core-2 antibodies in the flow-through fraction was lower than the titer of anti-Core-5 antibodies, demonstrating the efficiency of the purification process (Fig 3A–C).

The experiments in Figure 4 confirm the ability of affinity-purification to generate anti-HCV antibodies with the desired specificities. IgG #29 was the starting material in panels A–C. This sample came from a patient with HIV/HCV, and demonstrates that patients with co-infection also provide adequate starting material for HCV antibody purification. This preparation contains antibodies to both the core-neo fusion protein (arrow) and the core protein (arrowhead) (Fig. 4A). Antibodies purified using the Core-2 peptide reacted with both core and core-neo proteins (Fig. 4B); whereas antibodies purified using Core-3 (amino acids 28–41) reacted with core, but not with the core-neo protein (Fig. 4C). This demonstrates that it is possible to remove unwanted antibodies from crude IgG preparations, while retaining antibodies with desired narrow specificities. Detection of the core-neo protein shows that the affinity purified polyclonal human antibodies are able to react with epitopes in the *extreme* N-terminal portion of the core protein.

3.4. Affinity-purification and characterization of antibodies against non-structural proteins

A wide variety of biological effects have been attributed to HCV non-structural (NS) proteins (Egger, et al. 2002, Konan, et al. 2003, Tasaka, et al. 2007, Einav, et al. 2008, Brillet, et al. 2007, Randall, et al. 2007). Additional reagents are needed to characterize these proteins. An NS4B peptide from the C-terminal cytoplasmic tail (Lundin, et al. 2003) and an NS5A peptide from Domain II (Tellinghuisen, et al. 2004) were used to select

antibodies directed against these non-structural proteins. IgG #2 was used as the starting material.

Affinity-purified, anti-NS5A polyclonal antibodies reacted specifically with their target, as indicated by immunoblot analysis of Huh-7.5 cells with (Fig. 4D, lanes a and b) and without replicons (Fig. 4D, lane c). Performance of the human polyclonal anti-NS5A antibodies was comparable to that of the NS5A mouse monoclonal antibody, 9E10 (Fig. 4E).

Immunoblots (Fig. 5A) and immunofluorescence (Fig. 5B–E) demonstrated the specificity of the affinity-purified polyclonal human anti-NS4B antibodies. These antibodies were highly specific for the p27 NS4B protein (Yu, et al. 2006). They reacted with both genotype 1a (H77), 1b (Con1) and genotype 2a (JFH-1) NS4B (lanes a, b, and c, respectively), but did not react with NS5A, although anti-NS5A antibodies were present in the starting material, as demonstrated by the blots shown in Figures 2C and 4D. The anti-NS4B antibodies allowed a slight mobility difference between the H77 protein and the Con1 and JFH proteins to be detected in Western blots (Fig. 5A). These antibodies also produced a strong signal in cells expressing HCV replicons (Fig. 5B). Specificity of this signal was demonstrated in three ways: The IF signal was inhibited by pre-incubation with the NS4B peptide (Fig. 5C); the signal was absent from control cells lacking replicons (Fig. 5D); and it was not inhibited by pre-incubation with an irrelevant peptide (Fig. 5E).

4. Discussion

Waste ascitic fluid is an abundant source of antibodies. This study demonstrates that it contains high-affinity, cross-reactive, human polyclonal anti-HCV antibodies and thus is a new source of anti-HCV antibodies. Ascitic fluid is easily obtainable. An average large volume paracentesis generates more than 30 g of IgG. Monoclonal antibodies, in contrast, often have narrow specificities and frequently recognize proteins produced by a single sub-genotype. The methods used can be applied directly to the preparation of antibodies against other viruses that cause chronic infections, provided that blood or a fluid analogous to ascitic fluid is available.

ELISAs were used to characterize the antibody profiles of ascitic fluid specimens. These profiles illustrated how greatly the anti-HCV antibody population varies from patient-to-patient. Because of this natural variability, by screening a large collection of ascitic fluid preparations it may be possible to identify antibodies capable of recognizing HCV protein domains that do not readily stimulate antibody responses in animals.

Synthetic peptides were used to affinity-purify antibodies against core, NS4B, and NS5A as a proof-of-principle. Over a milligram of anti-Core-2 antibodies was generated on average from 50 mL of ascitic fluid IgG. If the desired specificity is available, equivalent commercial antibodies cost about \$3,000/mg, and monoclonal antibodies can be far more expensive if they need to be generated *de novo*. The anti-Core-2 affinity columns cost about \$300 in consumable supplies and could be re-used several times. Affinity-purified antibodies recognized their target viral proteins in both immunoblots and in cells harboring HCV replicons. Significantly, antibodies purified with the Core-3 peptide did not react with the core-neo protein (Fig. 3D), although antibodies against the core-neo protein were present in the starting material. Similarly, antibodies purified with the NS4B peptide did not react with the NS5A protein (Fig. 5A,) although antibodies against the NS5A protein were present in the starting material (Fig. 2C). These results show that affinity-purification can be used to generate preparations of polyclonal antibodies that recognize pre-selected portions of HCV proteins. This degree of specificity is required for the use of the affinity-purified antibodies in studies of viral protein topology and intracellular localization.

The anti-NS4B antibodies allowed investigation of the electrophoretic mobility and intracellular location of the NS4B protein, an important viral constituent that is reported to interact with both viral and cellular proteins (Yu, et al. 2006). A review of the literature suggests that these affinity-purified anti-NS4B antibodies are the first antibody reagents that react with NS4B from genotype 1a, 1b, and 2a. When used in immunoblots, they revealed that there is a mobility difference between the H77 protein and the Con1 and JFH-1 proteins produced by replicons. The molecular basis of this difference is unknown, but interestingly, the high mobility (H77) form of NS4B correlates with inefficient replication in subgenomic replicons (Blight, et al. 2002). Affinity-purified anti-NS4B antibodies will facilitate the further characterization of NS4B proteins.

Human polyclonal anti-HCV antibodies may also find use in clinical settings. Published data show that human antibodies are the most effective reagents for detecting cells infected with HCV in clinical specimens by immunohistochemistry (Fenwick, et al. 2006, Grassi, et al. 2006); although, current methods lack the sensitivity needed for use in a standard assay to detect cells infected with HCV in clinical specimens. A second potential clinical application of polyclonal human anti-HCV antibodies is in the prevention of HCV transmission (Knodel, et al. 1976, Sanchez-Quijano, et al. 1988, Piazza, et al. 1998, Vanwolleghem, et al. 2008) and in the reduction of graft infection following liver transplantation (Fera, et al. 1998). The ready availability of ascites fluid makes it a potential source of antibodies for optimization of immunohistochemical assays and for therapeutic purposes, as large quantities of ascites fluid can be obtained from patients undergoing large volume paracentesis on a routine basis.

5. Conclusion

Ascitic fluid from HCV patients contains high-affinity anti-HCV antibodies that can be used to identify HCV proteins immunoblots and in cell culture models of HCV infection. In addition, these antibodies have potential utility in clinical immunohistochemistry and in the prevention of HCV transmission.

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Abbreviations

AP	affinity-purified
FL	full-length
HCV	hepatitis C virus
HIV	human immunodeficiency virus type-1
IF	immunofluorescence
IHC	immunohistochemical
NS	non-structural
S.D	standard deviation

SG subgenomic

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Figure 1. Anti-HCV antibodies in ascitic fluid react with HCV peptides in ELISAs
Panels A–C. The ascitic fluid of 28 patients with HCV mono-infection and one patient with HIV/HCV (#29) infection were tested in an indirect antibody ELISAs using the Core-2 peptide (amino acids 7–25), NS4B (amino acids 1901–1922), and NS5A (amino acids 2297–2314). Normalized values greater than 1.0 were considered positive.

Figure 2. IgG fractions from ascitic fluid produce strong and specific signals from viral proteins in immunoblots and immunofluorescence assays of Huh-7.5 cells harboring HCV replicons
Panels A–E: Immunoblots reacted with total IgG populations purified from ascitic fluid are presented in Panels A–C. IgG preparations from three different subjects were reacted with proteins extracted from Huh-7.5 control cells (lane a), or cells producing HCV proteins (lanes b and c). Specifically, Huh-7.5 cells with SG JFH-1 replicons (lane b) and cells with FL J6/JFH replicons (lane c) are shown. Size markers are shown on the left. Panel A shows the protein bands that react with ascitic fluid IgG from subject #7; Panel B shows the proteins that react with ascitic fluid IgG from subject #8; and Panel C shows the proteins that react with ascitic fluid IgG from subject #2. The p21 core protein is identified by an arrowhead; the core-neomycin fusion protein is marked by an arrow; the NS5A protein is designated by a closed circle, and the E2 envelope protein is denoted by a star. A blot reacted with a mixture of anti-core and anti-NS5A mouse monoclonal antibodies is shown in Panel D, and a blot reacted with an anti-E2 rat monoclonal is shown in Panel E. Panels F–K: The IF signals produced by IgG preparations reacted with Huh-7.5 cells harboring FL H77 bicistronic replicons (Panels F–H) or Huh-7.5 cells without replicons (Panels I–K) are shown. Prior to IF microscopy, cells were incubated with goat-anti-human IgG conjugated to Alexa Fluor 594 and with DAPI, which stains nuclei blue. Cells exposed to IgG #7 are shown in Panels F and I; cells exposed to IgG #8 are shown in Panels G and J; and cells exposed to IgG #2 are shown in Panels H and K.

Figure 3. Enrichment of anti-Core-2 antibody

Levels of anti-Core-2 and anti-Core-5 antibodies in the total IgG population of ascitic fluid from subject #8 are compared in Panel A. The total IgG population was then exposed to a Core-2 peptide column and the flow-through was collected. Depleted levels of anti-Core-2 antibodies were noted compared to anti-Core-5 (Panel B). The peak elution from the Core-2 column contained a 534-fold enrichment of anti-Core-2 antibodies and undetectable levels of anti-Core-5 antibodies (Panel C).

Figure 4. Affinity-purified anti-Core, anti-NS4B, and anti-NS5A antibodies from ascitic fluid produce clear immunoblot and immunofluorescent signals

Panel A–D show proteins from Huh-7.5 cells harboring either FL J6/JFH replicons (lane a), SG JFH replicons (lane b), or no replicons (lane c). Proteins were reacted with the total IgG of subject #29 (Panel A). Antibodies were affinity-purified from IgG #29 using the Core 2 peptide (amino acids 7–25; Panel B), and Core 3 peptide (amino acids 28–41; Panel C). An arrow indicates the position of the core-neomycin fusion protein and an arrowhead marks the position of the p21 core protein. Panel D shows that antibodies affinity-purified using the NS5A peptide (amino acids 2297–2341) reacted with NS5A protein expressed by SG JFH (lane a) and by FL J6/JFH (lane b). These antibodies did not react with proteins of Huh-7.5 control cells (lane c). Panel E indicates the positions of the NS5A and p21 core proteins expressed by SG JFH (lane a) and FL J6/JFH (lane b) in blots reacted with mouse monoclonal antibodies. A closed circle marks the position of the NS5A protein and an arrowhead identifies the position of the p21 core protein. Size markers are presented on the left hand side of the figure.

Figure 5. Detection of NS4B protein using affinity purified anti-NS4B polyclonal antibodies
 In Panel A–E antibodies affinity-purified from IgG #2 are used. Panel A shows that human polyclonal anti-NS4B antibodies react with NS4B of genotype 1a (lane a), genotype 1b (lane b), and genotype 2a (lane c) proteins in Western blots, but did not react with proteins from Huh-7.5 control cells (lane d). A closed square shows the position of the NS4B protein. Panels B–E show that affinity-purified anti-NS4B antibodies produced an intense IF signal in Huh-7.5 cells harboring replicons (Panel B). This signal was blocked by incubation with 1 μ g/ml of the NS4B peptide (Panel C), but it was not blocked by incubation with 10 μ g/ml of PDC-E2, an irrelevant peptide (Panel E). No signal was produced when the anti-NS4B antibodies were incubated with control Huh-7.5 cells (Panel D). The NS4B protein in panels B and C is from H77 HCV (genotype 1a) and the NS4B protein in panel E is from Con1 (genotype 1b). DAPI-stained nuclei are blue.

Table 1

Patient Characteristics

	HCV % or Median (Q1, Q3)	Control % or Median (Q1, Q3)	P value
N	29	12	
Age (years)	55.5 (50.8, 61.5)	63 (55.8, 67.5)	0.33
% Males	71%	75%	0.72
MELD score	17	19.5	0.64
% with History of SBP	16%	11%	0.60
% on Dialysis	4%	11%	0.93
% with HCC	25%	25%	0.95
AFP (ng/ml)	4.5 (2.3, 12.8)	3.2 (2.7, 4.4)	0.23
Albumin (g/dl)	3.0 (2.6, 3.3)	3.0 (2.6, 3.6)	0.79
Total Bilirubin (mg/dl)	2.1 (1.1, 4.1)	2.4 (1.2, 5.3)	0.47
Creatinine (mg/dl)	1.0 (0.9, 1.2)	1.1 (0.8, 1.3)	0.52
INR	1.7 (1.4, 2.1)	1.5 (1.3, 2.0)	0.76
Platelet Count	103 (71.5, 149.0)	114 (66.8, 176.0)	0.40
Serum IgG (mg/dl) ^a	2270 (1760, 2790)	1430 (1140, 1950)	0.013
Ascites IgG (mg/dl) ^b	549 (345, 727)	240 (178, 258)	0.026

SBP (spontaneous bacterial peritonitis)

^a Twenty-four of the 28 patients with chronic HCV mono-infection were included in the analysis

^b Twenty-six of the 28 patients with chronic HCV mono-infection were included in the analysis