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New evidence of replication of hepatitis C virus in short-term peripheral blood mononuclear cell cultures

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ABSTRACT

Background: Even though hepatocytes are the main site for hepatitis C virus (HCV) replication, peripheral blood mononuclear cells (PBMC) have also been proposed as a suitable site for HCV replication. However, this issue still remains under discussion. We have previously developed an innovative system where HCV-RNA can be recovered during PBMC culture from HCV infected patients. Thus, the aim of this work was to use this novel approach in order to observe the evolution and replication of HCV genotype 1b in the PBMC of an HIV-HCV coinfected patient.
Methods: HCV-RNA was extracted from serum, uncultured PBMC and PBMC culture at day 6, 20 and 33. The evolutionary analysis was performed using the direct sequences of three viral regions: 5′UTR, E2 and NS5A. Additionally, E2 region was cloned in order to extend the evolutive analysis.
Results: In the present work, the molecular characterization of HCV along the culture showed a clear dynamic evolving process with the appearance of several nucleotide or amino acid changes in the three regions analyzed. Furthermore, the population analysis of E2 clones showed emerging and loss of lineages which indicate the fast evolutive dynamics of this system.
Conclusions: Since evolution can take place only if the virus is replicating in the culture, this finding constitutes an important evidence of viral replication in PBMC. Moreover, this extrahepatic compartment could be very important due to the presence of distinctive variants that could be responsible for resistance to treatment, viral pathogenesis and other clinical implications.

1. Introduction

About 180 million people are infected with hepatitis C virus (HCV) worldwide. Infection with HCV is a major cause of chronic liver disease, with more than 75% of patients developing chronic hepatitis and 20% of chronically infected people developing cirrhosis and hepatocellular carcinoma (Thomas and Seeff, 2005). While hepatocytes are the main site for viral replication, a broad clinical spectrum of extrahepatic complications and diseases, including non-Hodgkin's lymphoma and lymphoproliferative disorders are associated with chronic HCV infection, which suggests the existence of some other target cells for viral replication (Blackard et al., 2006; Giordano et al., 2007; Mele et al., 2003). Peripheral blood mononuclear cells (PBMC) have been proposed as a suitable site for HCV replication (Baré et al., 2005; Blackard et al., 2007; Fujiwara et al., 2013). Additionally, CD81, an entry factor for HCV, is expressed by many cell types in the blood and could mediate PBMC adsorption of HCV from the environment. Moreover, detectable levels of HCV RNA and/or proteins have been found in PBMC, B and T cells, monocytes/macrophages, dendritic cells and other extrahepatic tissues of infected individuals (Baré et al., 2005; Castillo et al., 2005; Fournillier et al., 2004; Goutagny et al., 2003; Hu et al., 2003; Pawełczyk et al., 2013; Radkowski et al., 2005; Wilkinson et al., 2009). However, since particles may be bound or taken up by blood cells without undergoing a complete infectious cycle, detection of HCV RNA associated to PBMC is not by itself proof that the virus replicates in these cells. Even though the mechanism of HCV replication is not fully understood, it is assumed that virus replication involves the synthesis of a minus strand RNA molecule that acts as a template for production of positive strand or genomic

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HCV-RNA (Cohen, 1999). Thus, the detection of the HCV-RNA minus strand is indicative of viral replication. However, although it has been achieved, multiple artifacts complicate the detection of this replicative intermediate (Boisvert et al., 2001; Castillo et al., 2005; Hu et al., 2003; Lanford et al., 1995; Lerat et al., 1996). Therefore, although different strategies have been used to demonstrate the HCV entry and replication, the issue has not been completely elucidated yet and the results should be interpreted with extreme caution (Boisvert et al., 2001; Castillo et al., 2005; Pal et al., 2006; Stamataki, 2010).

To confirm the extrahepatic replication of HCV, a different approach would be necessary in order to add another piece to this puzzle. Our group previously found that HCV-RNA is consistently recovered during PBMC culture from HCV monoinfected or HIV-HCV coinfected patients (Baré et al., 2005). For this reason, in the present work, the HCV viral sequences were characterized along the time in PBMC culture with the aim of demonstrating the presence of viral evolution and, consequently, the existence of virus replication in this system.

2. Materials and methods

2.1. Subjects and samples

A patient with hemophilia coinfected with HIV-HCV (genotype 1b) who was followed during 15 years at 'Fundación Argentina de Hemofilia' was studied. The patient was a male, 44 years old at the time of PBMC sampling, positive for HCV antibodies detected by ELISA (third generation) and RIBA HCV 3.0 (both Ortho diagnostic systems) and with evidence of past infection with Epstein Barr Virus. This patient was naïve for therapy against HCV and the highly active anti-retroviral therapy was interrupted a year before PBMC sampling.

2.2. PBMC cultures

This PBMC culture system is achieved by allowing undisturbed interaction of lymphocytes, monocytes/macrophages and other accessory cells present in the PBMC suspension over 5–45 days, in the absence of exogenous stimuli (Ruibal-Ares et al., 1997). In the present study, the PBMC culture derived from the patient was carried out as described previously (Ruibal-Ares et al., 2001a). Briefly, PBMC were obtained by Ficoll Paque Plus (GE Healthcare, Umea, Sweden) density gradient of EDTA anti-coagulated blood. These cells were washed three times with Mg^{2+} and Ca^{2+}-free PBS and resuspended to 1×10^6 cell/ml in RPMI 1640 tissue culture medium (Gibco, Grand Island, NY, USA) containing 10% fetal calf serum and antibiotics (penicillin/streptomycin, 10 mg/ml) (RPMI-FCS). Then, 2×10^6 PBMC were suspended in 2 ml RPMI-FCS using round-bottom 5 ml polystyrene tubes and left undisturbed in a 5% CO_2 incubator. For this patient, seven tubes (A–G) were set up at the same time. From the sixth day of culture on, half of the supernatant was replaced twice a week by fresh medium by gentle aspiration, avoiding cell pellet disturbance. In order to detect RNA release, the supernatants from each independent culture were collected at different time points (6, 20 and 33 days of culture) and frozen for further analyses. As an example: for tube A, samples were collected after 6, 20, and 33 days and they were named A6, A20 and A33, respectively. To know the initial viral population at the culture beginning, the RNA was extracted from uncultured PBMC. Additionally, simultaneous serum samples were also processed, to characterize HCV in this compartment.

2.3. HCV RNA extraction, amplification, cloning and sequencing

Viral RNA collected from all sources (serum, uncultured PBMC and seven culture supernatants), were extracted using QIAamp Viral RNA Mini Kit (Qiagen, Gmbh, Hilden, Germany). cDNA corresponding to each segment was synthesized using a specific primer and Superscript® III RT reverse transcriptase (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. The resulting cDNA was subjected to PCR using the *Platinum Taq* DNA Polymerase enzyme (Invitrogen, Carlsbad, CA, USA). The evolutionary analysis was performed using the partial sequences of three viral regions: E2 (510 nucleotides), NS5A (743 nucleotides) and 5′UTR (209 nucleotides), which have been previously reported to be important for viral compartmentalization (Blackard et al., 2007), viral growth in vitro (Maekawa et al., 2004) and viral growth in blood cells (Lerat et al., 2000), respectively. The E2 genes were amplified by a hemi-nested PCR with the following primers: ES: (5′ GGA TAT GAT GAT GAA CTG GTC 3′, sense) and EA: (5′ RAA RCA RTC CGT GGG GCA 3′, antisense) and the internal primers: IS: (5′ TCC ATG GTG GGG AAC TGG GC 3′, sense) and EA: (5′ RAA RCA RTC CGT GGG GCA 3′, antisense). Additionally, amplified E2 samples were cloned by using pGEM-T Easy kit (Promega, Madison, WI, USA) according to the manufacturer's conditions. For each time point, an average of 20 cloned sequences was obtained (range, 16–23 sequences).

The 5′UTR and NS5A regions were amplified as previously described (Baré et al., 2005; Di Lello et al., 2008). The clones and amplicons were sequenced in both senses by an ABI automatic sequencer.

2.4. Phylogenetic analysis

The nucleotide sequences were aligned using ClustalX 2.12 program (Larkin et al., 2007). Phylogenetic trees were estimated using the maximum-likelihood method (PhyML 3.0) online program (Guindon and Gascuel, 2003), under the HKY substitution model. The transition/transversion ratio, proportion of invariable sites and gamma distribution shape parameter were estimated from the sequence data. Base frequencies were adjusted to maximize the likelihood. Their reliability was assessed by bootstrap resampling.

2.5. Signature sequence analysis

Alignment from direct/clonal amino acid (aa) sequences was used to determine, by visual inspection, signature patterns that distinguish, uncultured PBMC viral variants from those in corresponding serum sample and three PBMC cultures. Additionally, the VESPA program (Korber and Myers, 1992) was used to search a signature pattern between uncultured PBMC and the PBMC samples from cultures in clonal sequences. The VESPA program detects signature patterns (atypical aa or nucleotide residues) in a set of query sequences in relation to a set of reference sequences. VESPA calculates the frequency of each amino acid (or nucleotide) at each position (column) in an alignment for the query and reference set, and selects the positions for which the most common character in the query set differs from that in the background set.

2.6. Diversity and entropy of quasispecies

The evolutionary nucleotides mean genetic distances within group was estimated using maximum Composite Likelihood. Standard errors were calculated using 200 boostrapped replicates. These analyses were performed in MEGA v.4. Genetic complexity (number of different variants within each sample) in serum, PBMC and PBMCs cultures, was calculated with E2 amino acid

sequences and expressed as normalized Shannon's entropy (S_N), being $S_N = S/\ln N$, where $S = -\sum_i (p_i \ln p_i)$. In this case, p_i is the frequency of each clone in the quasispecies and N is the total number of clones in each sample. S_N theoretically varies from 0 (only one variant detected) to 1 (maximum quasispecies complexity).

2.7. Bayesian coalescence analysis

To study the relationship between the different lineages observed for E2 region Bayesian Coalescence analyses were carried out. The population dynamic and the phylogeny were jointly estimated in a Bayesian framework as implemented in BEAST v 1.7.5 (Drummond et al., 2012). The analyses were performed using the HKY+G model of nucleotide substitution. The dates of sampling were used to calibrate an uncorrelated lognormal relaxed molecular clock (Drummond et al., 2006). The population dynamic was modeled by the non-parametric Bayesian Skyline Plot model (Drummond et al., 2005). Convergence was assessed by visual examination of traces and ensuring all effective sampling size values of >200 in the program Tracer v.1.5. The results of the analysis were summarized into a maximum clade credibility tree (with branches scaled in time) with the TreeAnnotator utility of the BEAST package.

Lastly, the parsimony score (PS) and the association index (AI) were tested by using the program BaTS v0.1 (Parker et al., 2008) in order to test for the presence of statistically significant differences between serum and PBMC cultures clones. As input for BaTS, the posterior distribution of trees arising from the Bayesian analysis was used.

2.8. Nucleotide sequences accession numbers

Nucleotide sequences for the HCV strain have been deposited in GeneBank under accession numbers JF416945, JF749816–JF749817, JF749819–JF749824, JF749826–JF749827, KJ469134–KJ469264.

2.9. Ethics statement

Written informed consent to participate in this study was obtained from the patient. The study protocol was approved by the ethics committees of the "Academia Nacional de Medicina" and "Facultad de Farmacia y Bioquímica de la Universidad de Buenos Aires" (record number 732575/2010) in accordance with the 1975 Helsinki Declaration.

3. Results

3.1. PBMC cultures

In order to characterize viral evolution, seven cultures (A–G) were initiated from PBMC obtained from the patient. Each independent culture was analyzed for HCV-RNA detection in supernatants at 6, 20 and 33 days. Two cultures supported HCV-RNA viral detection up to six days (C6 and G6) and one culture up to 33 days (B6, B20 and B33). In the other four cultures, the HCV-RNA viral was not detected at any time point.

3.2. Phylogenetic analysis

Phylogenetic analysis of the E2 and NS5A regions from samples belonging to serum, uncultured PBMC and virus recovered from three PBMC cultures demonstrated a monophyletic origin (Fig. 1). 5′UTR has not a sufficient amount of phylogenetic signals to assess relationships at this level.

3.3. Signature sequence analysis

To investigate if HCV evolved during PBMC culture, direct and clonal viral sequences were compared in the course of PBMC culture. Alignment from direct aa sequences from E2 and NS5A regions determined an aa signature pattern that distinguished uncultured PBMC viral variants from those in the corresponding serum sample and the three PBMC cultures samples. Additionally, VESPA program and visual inspection of clonal sequences alignment were used in order to determine signature patterns from clonal E2 sequences. However, no additional aa were detected with the use of VESPA program with respect to the visual inspection.

3.4. Direct sequences

3.4.1. 5′UTR

As a conserved region, the study of the 5′ UTR region showed that only the viral sequence of the sample B33 presented two nucleotides changes (C204A, G243A), with regard to the rest of the investigated sequences.

3.4.2. E2

After six days of cell culture, the E2 region showed identical aa sequences for PBMC, B6 and C6 cultures. Nevertheless, mixed populations were observed from chromatogram analysis of these samples. On the other hand, the G6 culture showed two distinct aa compared to the PBMC. The culture B20 showed two aa changes with respect to the B6 culture and lastly, B33 showed 13 differences with respect to the B20 culture. These differences observed in B culture were located mainly, 57% (8/14), in the hypervariable regions 1 and 3. Table 1 shows the aa changes at each time point.

3.4.3. NS5A

The analysis of the NS5A region, demonstrated the presence of several aa changes between viruses recovered from uncultured PBMC and those obtained from B and C cultures (Table 2). Interestingly, both viral sequences from samples B6 and B20, showed an aa insertion (H) at position 2218/9 and five aa changes with respect to all other analyzed sequences. Noteworthy, comparison of virus recovered from culture B6 and B20, showed four additional aa differences (Table 2).

3.5. Characterization of quasispecies of E2 region

To further analyze the diversification process in the E2 region, a genetic population study of molecular clones recovered from uncultured PBMC and PBMC cultures were performed using Coalescence analyses in a Bayesian framework.

3.5.1. Diversity and entropy of quasispecies

Multiple measures of HCV diversity and complexity were assessed in each sample, including genetic distance and entropy. PBMC showed major diversity and complexity of HCV quasispecies compared with simultaneous serum sample (Table 3). Similar results were observed when culture B was analyzed. Thus, complexity and diversity globally increased in these samples with respect to serum and were similar to PBMC. On the other hand, C and G cultures, showed homogenization of HCV quasispecies after six days of culture with respect to plasma and PBMC (Table 3).

3.5.2. Coalescence analysis

The coalescence analysis of HCV culture showed a strongly temporal structure with limited diversity at any time point. In the initial population of uncultured PBMC, the existence of two viral lineages was determined, L1 (18/22: 82% of clones) and L2 (4/22: 18% of clones). The same analysis was carried out on the virus recovered

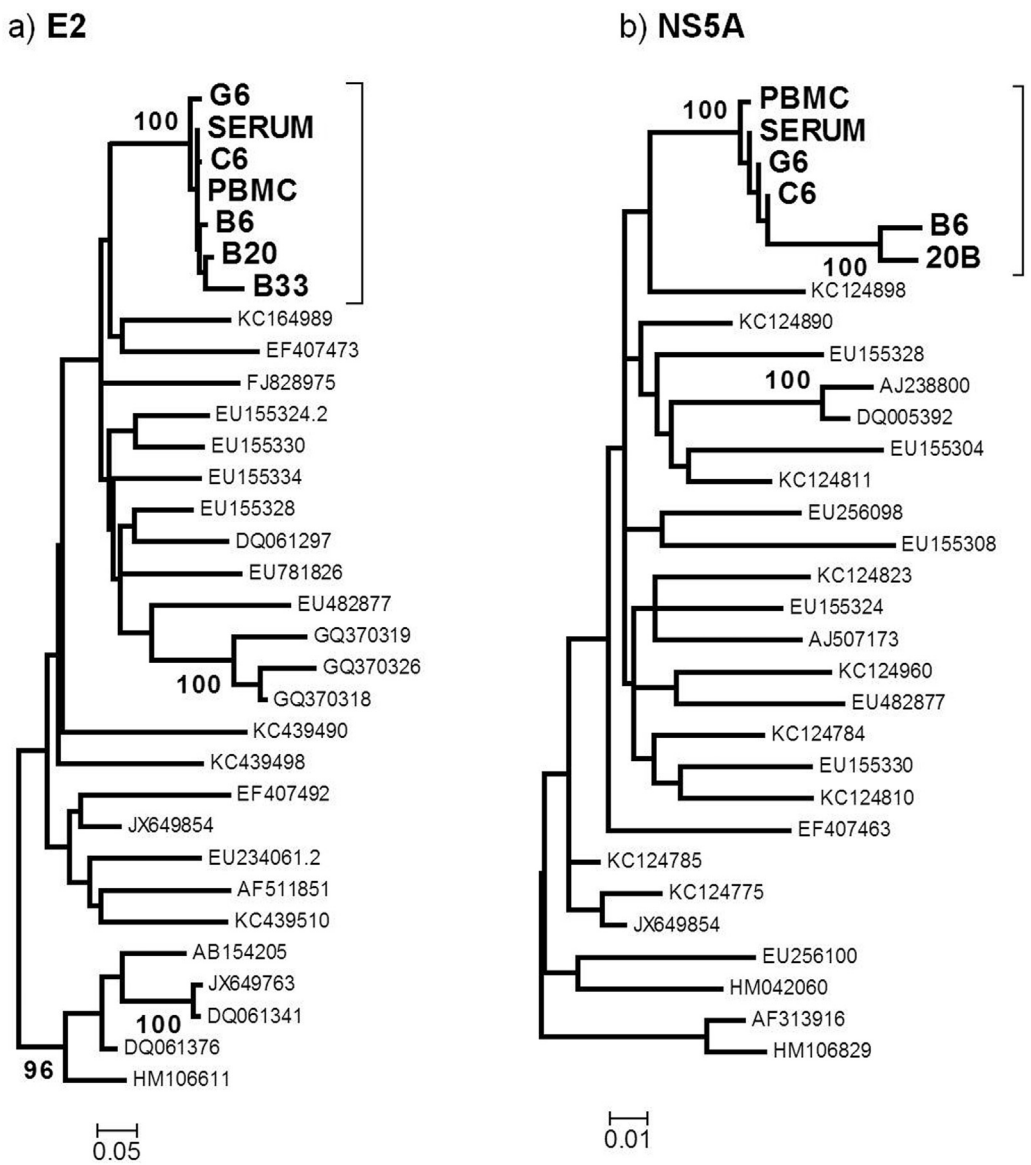

Fig. 1. Phylogenetic tree according to the maximum-likelihood method derived from nucleotide sequences of virus recovered from serum, uncultured PBMC, PBMC culture and other HCV-1b world sequences. (a) E2 region, (b) NS5A region. The numbers at each node correspond to bootstrap values obtained with 100 replicates. Only bootstraps values higher than 70% are shown. Samples of this study are in brackets.

from three independent cultures (B, C and G) derived from PBMC naturally infected with HCV (Fig. 2). Even though the serum and the uncultured PBMC sequences were very similar, the presence of differences in the spatial distribution in the serum and PBMC cultures clones was demonstrated by labeling the sequences according to their compartment and using the PS and AI statistics (observed PS of 6.72, expected PS of 45.16, $p < 0.001$; observed AI of 0.48, expected AI of 8.53, $p > 0.001$). Additionally, it was found that the viral populations observed along the B cultures were more related to the viral population present in PBMC than that in the simultaneous

Table 1
Amino acids of E2 direct sequences.

Sample	E2 amino acid position													
	HVR-1						HVR-3							
	397	398	400	401	407	410	414	416	422	440	453	460	492	524
PBMC	L	R	T	S	S	R	V	T	I	S	P	R	Q	V
SERUM	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B6	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B20	.	.	.	.	A	.	.	S	.	.	.	.	.	.
B33	R	G	V	G	.	K	I	.	V	A	S	H	.	A
C6	.	.	.	.	.	.	.	.	.	.	.	.	.	.
G6	.	.	.	.	.	.	.	.	.	A	.	.	R	.

Amino acid of E2 numbering according to isolated H77 genome (GenBank accession no. NC_004102).
Amino acids are indicated at one letter code. Dots indicate amino acid identical to those of PBMC.

Table 2
Amino acids of NS5A direct sequences.

Sample	NS5A amino acid position										
	ISDR										V3
	2181	2216	2218/9	2224	2234	2237	2251	2282	2283	2285	2372
PBMC	I	T	–	V	R	M	V	P	R	M	S
SERUM	.	.	–	.	.	.	.	.	.	.	.
C6	L	.	–	.	.	.	.	.	.	.	.
G6	.	.	–	.	.	.	.	.	.	.	.
B6	L	N	H	L	W	K	I	Q	.	.	L
B20	L	N	H	L	W	K	I	.	Q	I	P
B33	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Amino acid of NS5A numbering according to isolated H77 genome (GenBank accession no. NC_004102).
Amino acids are indicated at one letter code. Dots indicate amino acid identical to those of PBMC.
nd: no determined.
ISDR, interferon sensitivity-determining region.

serum sample. In this culture, the phylogenetic analysis showed the presence of both lineages at every time point. Nevertheless, this behavior was not static and the lineages changed its frequency through time. Clones from the first time point (B6) derived from PBMC clones and grouped in two different lineages: L1 [15 clones (83%)] and L2 [3 clones (17%)]. The clones belonging to lineage L1 formed two sub-lineages (A and B). The sub-lineage 1A continued to evolve over time while sub-lineage 1B was not detected in B20 or B33. At 20 days of culture (B20) all clones had as ancestor clones from B6 and the lineage L1 began to decrease its frequency [14 clones (61%) vs. 9 clones (39%) for lineage L2]. Additionally, clones belonging to L2 initiated a diversification that was reflected in the formation of several sub-lineages with statistical support. Lastly, and in accordance with the previous observation, the clones from 33 days of culture (B33) also derived from the previous B clones (B20). In this time point, the lineage L2 turned more frequent and diversified than the lineage L1 18/21 (86%) vs. 4/21 (14%) respectively, which is almost the reverse situation to that observed in B6. On the other hand, all clones from culture C and G at six days belonged to lineage L1 and also derived from PBMC clones.

3.5.3. Amino acids analysis

To identify specific aa differences associated with HCV replication in PBMC cultures, signature sequence analysis was performed at each time point. The aa sequences confirmed the phylogenetic analysis and showed the presence of two distinctive lineages in E2, as it was observed for the direct sequences. The relevant aa for the analysis of E2 evolution, including those aa that changed according to the positions chosen by VESPA program and those ones selected after the visual inspection of the alignment, appear in Table 4. Again, as it was observed for direct sequences, most of the observed changes [8/14 (57%)] were located within the hypervariable regions 1 and 3. However, there have been other positions with amino acid changes, which were at a very low frequency and therefore are not shown in Table 4. The initial viral population in the culture was represented by the aa found in the uncultured PBMC sample, which also showed the dominance of two distinctive lineages (L1 and L2), previously described. The lineage L1 (similar to the direct sequences) appeared represented by 4 aa sequences and the lineage L2 by two aa sequences. After the first six days of PBMC incubation, the lineage L1 predominated in the three cultures analyzed (B, C and G). The time B20 showed less diversity in clones with only three different aa sequences. Lastly, in the latest time analyzed, 33 days (B33), the diversity increased substantially showing twelve different aa sequences, mostly belonging to lineage L2. Besides, it is important to note that the direct sequence was in most cases the most abundant among clones at each time.

Table 3
Diversity and complexity of HCV quasispecies.

Sample	Diversity[a] (SE)	Complexity[b]
Plasma	0.010 (0.003)	0.62
PBMC	0.016 (0.003)	0.89
C6	0.003 (0.001)	0.34
G6	0.002 (0.001)	0.28
B6	0.018 (0.004)	0.85
B20	0.016 (0.006)	0.48
B33	0.027 (0.005)	0.93

[a] Intra-sample diversity of the E2 amplified region was estimated using maximum Composite Likelihood model of nucleotide sequences. Standard errors (SE) were calculated using 200 boostrapped replicates.
[b] Intra-sample complexity of the E2 amplified region was calculated by the normalized Shannon entropy of amino acid sequences.

4. Discussion

In this study, the dynamic diversification during PBMC culture in three regions of HCV has been shown. The coalescence analysis and the observed change of aa sequences demonstrated that HCV evolves over time in PBMC culture. As evolution only could be observed when virus is replicating, these results represent clear evidence that PBMC is not only a reservoir for the virus but also an extrahepatic compartment where replication occurs. More importantly, the in vitro system used in this work takes advantage of the possibility of obtaining viable long-term HCV from PBMC cultures in the absence of exogenously added stimuli or HCV contamination from other compartments such as serum or HCV bound to cells (Hamaia et al., 2001). Thus, the natural infection provided by this system could promote the understanding of the molecular processes involved in the evolution and replication of the virus in this particular compartment.

In a previous work, it has been shown that this cell culture system represents a dynamic process in which the destiny of the PBMC culture was defined after three weeks (Ruibal-Ares et al., 2001b). Then, the PBMC culture may have two destinations: (a) lymphoblasts infected with Epstein Barr virus overcome the immune control, resulting in continuous B-cell lines (4–5% of the cases), or (b) lymphoblast proliferation is abolished by CD8T cells and the remaining PBMC die (Ruibal-Ares et al., 2001b). The second option was the case for most culture tubes, including C and G, in which the culture was not able to persist beyond day 6. Even that, in cultures C and G was possible to observe the HCV evolution in 6 days. Only in culture B, cells were capable to establish a B-lymphoblastoid cell line that could support HCV replication and diversification of the viral genomes during 33 days.

Herein, we analyzed the HCV direct sequences and quasispecies in PBMC culture of a HIV-HCV coinfected patient. The examination of the direct sequences of 5′UTR and NS5A regions showed

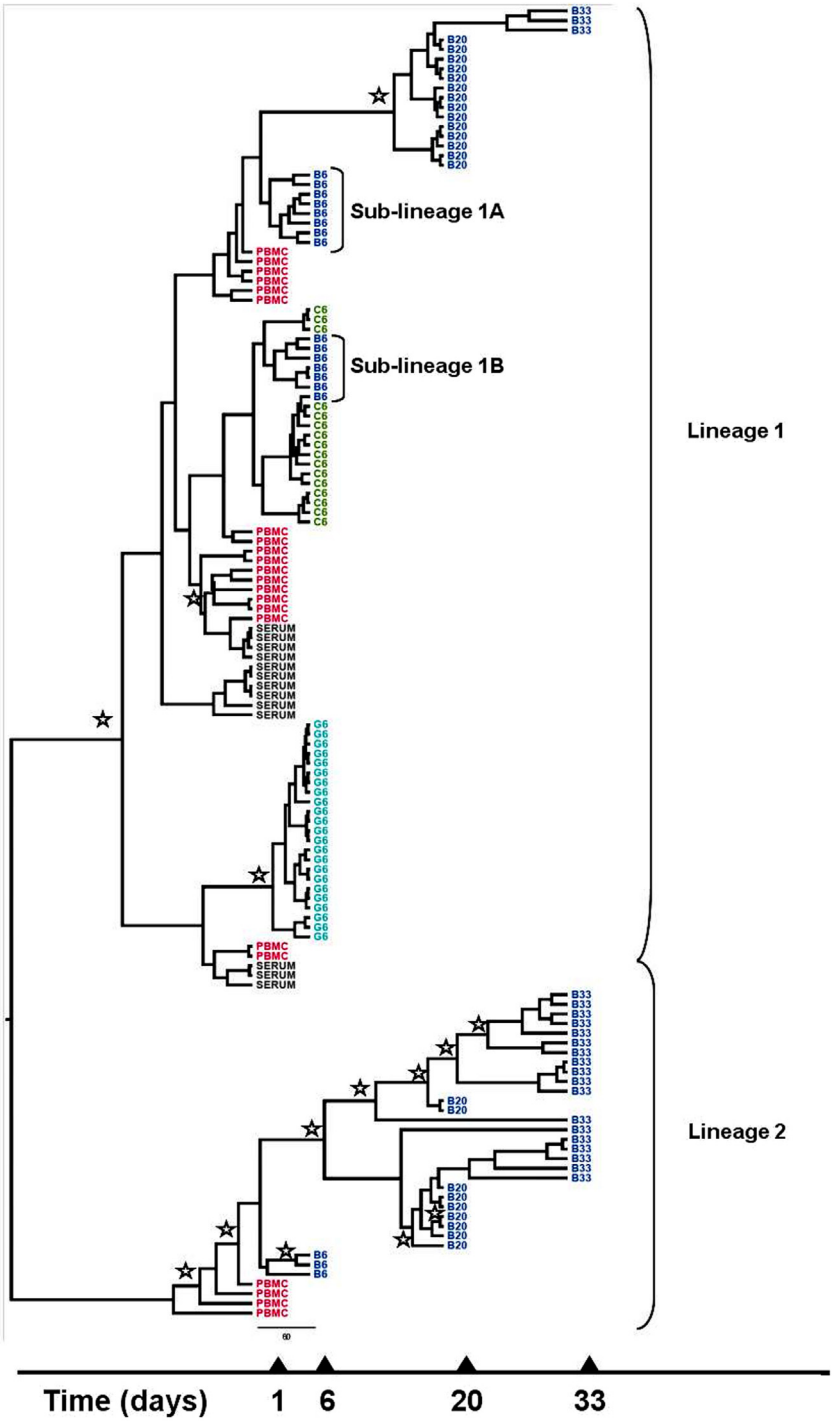

Fig. 2. Bayesian phylogenies. Tree is shown for Serum, uncultured PBMC and PBMC cultures B, C and G, where branches are scaled in time (days). Terminal taxa are colored according to the type of sampling: gray, Serum; red, uncultured PBMC; blue, culture B; green, culture C and sky blue, culture G. The stars at nodes correspond to posterior support values >0.9. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

interesting results. In the 5′UTR region, two nucleotide changes were observed at positions 204 and 243 in the viral sequence of tube B33. Viral variants at positions 204 and 243 have been previously associated with the adaptation process to grow in lymphoid long-term cell cultures (Nakajima et al., 1996). Additionally, the nucleotide substitutions within IRES may enhance the internal initiation of translation of the viral RNA (Durand et al., 2010; Lerat et al., 2000). In this sense, HCV has been found to have important interactions with numerous miRNAs that modulate HCV replication. One of them, miR-122, is highly expressed in liver where it binds to two target sites in the 5′ UTR of the HCV genome and is essential for enhanced HCV RNA accumulation (Jopling et al., 2008; Machlin et al., 2011). However, miR-122 was not differentially expressed in PBMCs in other study (Nogales-Gadea et al., 2014). Then, the cellular factors associated with HCV replication in PBMC cells, deserves further investigations.

On the other hand, several aa changes were also observed in NS5A region of PBMC culture. It is important to note that most of them were limited to the interferon sensitivity-determining region (ISDR). The substitutions in the NS5A region, and particularly, in the

Table 4
Amino acids sequences of E2 clones.

Sample	Lineage (%)	No. clones	E2 amino acid position													
			397	398	400	401	407	410	414	416	422	440	453	460	492	524
PBMC	L1 (82%)	10[a]	L	R	T	S	S	R	V	T	I	S	P	R	Q	V
		5	.	.	.	.	.	.	.	S	.	.	.	.	.	.
		2	.	.	.	.	.	.	.	.	.	A	.	.	.	.
		1	.	.	.	.	.	.	.	.	.	A	.	.	.	.
	L2 (18%)	3	R	G	V	G	.	.	I	.	.	A	.	.	.	.
		1	R	G	V	G	.	.	I	.	.	A	.	.	.	A
B6	L1 (83%)	6	.	.	.	.	.	.	.	S	.	.	.	.	.	.
		5[a]	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		2	.	.	.	.	.	.	.	.	.	.	.	.	.	A
		1	.	.	.	.	.	.	.	S	.	A	.	.	.	.
	L2 (17%)	1	.	.	.	.	.	.	.	S	.	.	.	.	.	A
		3	R	G	V	G	.	.	I	.	.	A	.	.	.	A
B20	L1 (61%)	14[a]	.	.	.	.	A	.	.	S	.	.	.	.	.	.
	L2 (39%)	7	R	G	V	G	.	K	I	.	V	A	S	.	.	A
		2	R	G	V	G	.	K	I	.	.	A	S	H	.	A
B33	L1 (18%)	2	.	.	.	.	A	.	.	S	.	.	.	.	.	A
		1	.	.	.	.	A	.	.	S	.	.	.	.	.	A
	L2 (82%)	4[a]	R	G	V	G	.	K	I	.	V	A	S	H	.	A
		4	R	G	V	G	.	K	I	.	.	A	S	H	.	A
		3	.	.	.	.	.	K	I	.	V	A	S	.	.	A
		2	R	G	V	G	.	K	I	.	V	A	S	.	.	A
		1	R	G	V	G	.	K	I	.	V	A	S	.	.	A
		1	.	.	.	.	.	K	I	.	V	A	S	H	.	A
		1	.	.	.	.	.	K	I	.	.	A	S	H	.	A
		1	R	G	V	G	.	K	I	.	.	A	S	H	.	A
		1	R	G	V	G	.	K	I	.	.	A	S	H	.	.
C6	L1 (100%)	15[a]	.	.	.	.	.	.	.	.	.	.	.	.	.	.
G6	L1 (100%)	23[a]	.	.	.	.	.	.	.	.	.	A	.	.	R	.
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Amino acid of E2 numbering according to isolated H77 genome (GenBank accession no. NC_004102).
Amino acids are indicated at one letter code. Dots indicate amino acid identical to those of PBMC.
[a] Represent amino acid sequence identical to the one observed in the direct sequence.

ISDR of HCV have been closely associated with response to IFN therapy and, for this reason, are of great clinical importance (Enomoto et al., 1996; Pascu et al., 2004). Remarkably, in this patient, the changes observed in PBMC culture of both, 5′UTR and NS5A had never been found among eight serum samples obtained during a 15-year period of follow up (data not shown). Therefore, this finding demonstrated not only the evolution of HCV, but also a distribution of viral variants that appeared to be compartment dependent.

One of the challenges of modern evolutionary studies is the integration of different data sources to address evolutionary hypotheses over the full range of spatial and temporal scales. In this sense, Bayesian methods allow the relatively simple implementation of extremely complex evolutionary models (Drummond and Rambaut, 2007). Given the evolutionary characteristic of the E2 region analyzed here and the temporal dynamics of quasispecies it was convenient to incorporate this method to the study. In the Bayesian analysis, a progressive lineage switching in the quasispecies of E2 region was observed, supporting the evolutionary process in this compartment and, showing a strong temporal variability with different diversity at any time point (Table 3 and Fig. 2). Moreover, the BaTS program showed that viral population has a spatial distribution consistent with every compartment which indicates that PBMC cultures come from uncultured PBMC. On the other hand, cultures B showed a diversification process, with an increase of diversity and entropy after 33 days of culture. Interestingly, these increments in genetic distance and entropies were associated with the absence of sampling bias and discarded long term sequestration of HCV debris in PBMC, which would result in a decrease of viral diversity over time (Blackard et al., 2007; Ducoulombier et al., 2004).

This evolutionary process in PBMC culture is an example of fast and dynamics diversification, where the infection of new cells should take place on a time scale of days.

As stated above, the aa sequences of E2 clones confirmed the presence of two distinctive lineages in which direct sequences were the most prevalent among this clones. The analysis of this sequences revealed that the observed differences in direct sequences were the result of a change in lineage proportion. The coalescence analysis showed an evolutionary process with a quasispecies shift characterized by the expansion of divergent sequences (L1A and L2) forming new lineages and the extinction of other ones (L1B). Moreover, the most prevalent lineage at culture B33 (L2), presented several additional aa changes with respect to the L2 in previous B cultures, indicating once again the fast HCV evolution and diversification in this system. Despite the large number of aa changes observed in the direct/clonal sequences of culture B33 with respect to the rest, the phylogenetic analysis showed that all culture times had a monophyletic common origin. Additionally to the evolution, HCV replication was evidenced because, without replication, the initial viral load and virus bound to PBMC would be diluted in 33 days below the detection limit since half of the medium supernatant was replaced twice a week in this system.

A limitation of this study is that the number of clones is small. However, although it is possible that viral variants at low frequencies may not be represented with 15–23 clones studied here, multiple measures of viral diversity were examined. Moreover, a previous study has shown that sequencing 10 viral variants is sufficient for quasispecies evaluation (Marukian et al., 2008). Additionally, the use of techniques as deep sequencing would produce an increase of the observed diversity (Gong et al., 2013) but would not affect the qualitative results of this work.

Another limitation is the lack of the analysis of a HCV monoinfected patient in order to elucidate whether the presence of HIV alters the replication of HCV culture derived from PBMC in this system. However, although there is information suggesting that HIV coinfection influenced the persistence and replication of HCV, we previously observed its replication in both, HCV monoinfected and HIV-HCV coinfected patients in the same experimental conditions (Baré et al., 2005). On the other hand, as mentioned above, the complexity of the system used make difficult to test several cultures in numerous time points. However, the evolution of six days in culture C and G, and 33 days in culture B was sufficient to assess the evolution of HCV.

Currently, although several studies have shown that HCV can replicate in PBMC (Castillo et al., 2005; Laskus et al., 2007) the contribution of this extrahepatic site as a significant viral reservoir and the importance of viral persistence is unknown (Baré, 2009). Consequently, the role of HCV lymphotropism in the natural history of HCV infection is not yet determined and reports remain uncertain. This system was also able to show the replication and the evolutionary dynamics that was not seen in any other system using HCV to infect PBMC subsets (Dahari et al., 2005).

4.1. Conclusions

Finally, since HCV is primarily hepatotropic, the contribution of extrahepatic sites to virus in circulation is estimated to be only about 3.1% (Torres-Puente et al., 2003). However, according to what was observed in this work, the importance of the PBMC compartment could lie not in the amount of virus produced but rather in the distinctive variants generated. Moreover, these variants could be responsible for resistance to treatment, viral pathogenesis and other clinical implications.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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