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Hepatitis C virus *quasispecies* in plasma and peripheral blood mononuclear cells of treatment naïve chronically infected patients

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SUMMARY. Peripheral blood mononuclear cells (PBMCs) from 45 treatment naïve, HIV-negative, chronically hepatitis C virus (HCV)-infected patients were analyzed for the presence of HCV RNA. Viral RNA was detected in 73% of the studied patients. Single-strand conformation polymorphism assays and sequence analysis of the HCV 5′untranslated regions amplified from RNA recovered from both Plasma and PBMCs suggested virus compartmentalization in 57.6% of patients studied. In summary, our study presents evidence that HCV RNA can be found in PBMCs of treatment naïve chronically infected patients that are not immunocompromised or co-infected with the human immunodeficiency virus.

Keywords: compartmentalization, HCV, PBMC.

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped RNA virus, classified in the Hepacivirus genus within the *Flaviviridae* family, that infects more than 170 million people worldwide [1]. In most infected patients HCV persists indefinitely leading to chronic hepatitis, cirrhosis and hepatocellular carcinoma [1].

Hepatitis C virus is primarily hepatotropic, but there is mounting evidence showing extrahepatic virus replication [2]. Virus extrahepatic replication was suspected as early as 1985, as plasma-free leukocytes from patients with post-transfusional non-A, non-B hepatitis were able to transmit the disease to chimpanzees [3]. Meanwhile, HCV genomic RNA has been detected not only in liver of chronically infected patients but also in perihepatic lymph nodes and peripheral blood mononuclear cells (PBMCs), among other tissues [2,4]. Some reports suggest that HCV persists, albeit at very low levels, in circulating lymphoid cells for years after apparently complete clinical and virological resolution of chronic hepatitis C, suggesting that PBMCs might be a natural reservoir for HCV [5,6]. Interestingly, even for a single individual, virus isolated from plasma and from PBMCs does not necessarily match, suggesting compartmentalization of HCV and viral replication in these non-hepatic cells [4,7–10]. The case for HCV replication in lymphoid cells is further supported by data showing that different types of primary lymphoid cells and cell lines are not only susceptible to *ex vivo* infection, but the virus derived from these *ex vivo* cultures are infectious [11–15]. Furthermore, HCV producing cell lines have been derived from naturally infected primary lymphoid cells [16,17], and both positive and minus-strand RNA was detected when PBMCs from HCV carriers were injected in a group of severe combined immunodeficiency mice [18]. In the latter set of experiments, virus production was monitored over time, strongly suggesting the persistent replication of HCV in the xenograft [18].

In Chile, as indeed worldwide, hepatitis C is one of the most important causes of liver-related mortality and morbidity [19,20]. Recent studies document that HCV genotype 1b is predominant among the infected Chilean population [19–21]. The implications of this predominance may be vast as genotype 1b infections shows higher resistance to antiviral therapy and confer greater risk of development of hepatocellular carcinoma [22].

Abbreviations: PBMCs, peripheral blood mononuclear cells; HCV, hepatitis C virus; SSCP, single-strand conformation polymorphism assays; HBV, hepatitis B virus.

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In this study we evaluated the prevalence of HCV-RNA in PBMCs from 45 treatment naïve, HIV-negative, chronically HCV-infected patients. Of these, 33 (73%) presented detectable amounts of viral RNA in PBMCs. Single-strand conformation polymorphism assays (SSCP) combined with sequencing analysis revealed that HCV was compartmentalized in 57.6% of the patients harbouring HCV in both plasma and PBMCs.

MATERIALS AND METHODS

Patients

Forty-five patients recruited from the outpatient clinic of the Hepatology Unit of the Clinical Hospital, Pontificia Universidad Católica de Chile (PUC) were included in this study. Protocols and consent forms used in this study were approved by the Ethical Review Board of the Faculty of Medicine, PUC. Donors completed a written consent before donating blood. Patient inclusion criteria were the following: adults with chronic hepatitis C, which was defined as positive for serum anti-HCV antibodies and positive for plasma HCV RNA for more than 6 months. HCV RNA levels in serum were determined using the Cobas Amplicor HCV Monitor Test, version 2.0 (Roche Diagnostic Systems, Inc., Branchburg, NJ, USA). Patients with anaemia (hematocrit <20%), pregnancy, HIV or hepatitis B virus (HBV) coinfection, undergoing anticancer chemotherapy, or which had received previous antiviral treatment were excluded from the study. Normal healthy controls were screened with a similar battery of tests to confirm their HCV-, HIV- and HBV-negative status.

Mononuclear cell separation

Peripheral blood was collected into EDTA-Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and plasma samples recovered from donors was stored at −80 °C until needed. PBMCs were isolated by centrifugation over a density gradient (Lymphocyte separation medium, Cellgro®; Mediatech Inc., Manassas, VA, USA) according to manufacturer's protocols.

RNA purification and RT-PCR

Total RNA was extracted using a protocol adapted from Chomczynski and Sacchi [23] using a pellet of 5×10^6 cells. Recovered RNA was resuspended in 30 μL of nuclease-free water. RNA concentrations were determined by spectrophotometry (GeneQuant, GE Healthcare, Piscateway, NJ, USA). HCV-RNA was detected by a one step reverse transcription (RT)-polymerase-chain reaction (PCR) using the SuperScript™ III one step RT-PCR system with Platinum® Taq DNA Polymerase kit (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA). Amplification of the HCV 5′UTR was achieved using the sense primer: 5′-TTG GGG GCG ACA CTC CAC CAT AGA TC-3′ and the anti-sense primer: 5′-GTT ACG TTT GGT TTT TCT TTG AGG T-3′ generating a 370 bp amplicon. The RT-reaction was conducted at 50 °C for 45 min. The RT-enzyme was then inactivated and the Platinum® Taq DNA polymerase activated by heating the mix at 95 °C for 5 min. PCR amplifications were carried out for 40 cycles with each cycle at 95 °C for 45 s, 58 °C (5′UTR) for 45 s, and 68 °C for 45 s. *In vitro* transcribed RNA (T7-RNA polymerase; Fermentas, Vilnius, Lithuania) generated from plasmid pFK-I_{377}neo/NS3-3′/wt (AJ242652) [24] (kindly provided by Dr Ralf Bartenschlager, University of Heidelberg, Germany), was used as a positive control while water and an unrelated *in vitro* synthesized RNA were used as negative control. All amplicons were sequenced (Macrogen Corp, Rockville, MD, USA). A sense primer was synthesized containing an additional 18 nucleotide tag sequence (GCTCATGGTGGCGAATAA) for a Tth-based strand-specific RT-PCR assay for the detection of HCV negative RNA strand according to previously described methods [25,26].

HCV genotyping

Initial HCV genotyping of virus present in plasma and PBMCs was performed using the reverse-hybridization line probe assay, INNO-LiPA HCV II kit (Innogenetics, Ghent, Belgium) according to the manufacturer's instructions. Genotyping was confirmed by sequencing (Macrogen Corp) and phylogenetic analysis. All sequences identified herein can be found in GenBank (EU635507 to EU635563]. The HCV nucleotide sequences were aligned using the CLUSTAL W program [27]. Matrix distances for the Kimura two-parameter model were then generated [28] and used to compute neighbour-joining phylogenetic trees. The robustness of each node was assessed by bootstrap re-sampling (1000 pseudoreplicates). These methods were implemented by using software from the MEGA program [29]. The HCV sequences of the following strains were included in the analyses: 1a-HCV1 (M62321), 1b-HCV (AJ242654), 2a-HCJ6 (D00944), 2b-HCJ8 (D10988), 2c-BEBE1 (D50409), 3a-NZL1 (D17763), 3b-HCVTr (D49374), and 5a-EUH1480 (Y13184).

Single-strand conformation polymorphism (SSCP) analysis of HCV quasispecies

One volume (1/5 of the PCR reaction) of the HCV 5′-UTR amplicons was mixed with one volume of denaturing solution (950 mL/L formamide and 400 mg/L bromophenol blue), heated for 10 min at 95 °C, and chilled on ice for 10 min. Electrophoretic analysis was carried out with the total volume of this mixture on a nondenaturing 0.5X MDE™ Gel (Mutation Detection Enhancement, Cambrex Bio Science Rockland, Inc. ME, USA) cast in a standard

sequencing apparatus (3 watts, for 16 h). Gels were silver stained and dried prior to band migration analysis.

RESULTS

HCV-RNA in plasma and PBMCs of patients with chronic HCV infection

Plasma and PBMCs from 45 treatment naïve, HIV-negative, HCV chronically infected patients were screened for the presence of HCV RNA. Table 1 summarizes the clinical features of the enrolled patients highlighting genotype distribution found in plasma and PBMCs. Total RNA isolated from both compartments was used as templates in a specific single step RT-PCR targeting the highly conserved HCV 5′untranslated region (5′UTR). The 5′UTR was successfully amplified from all plasma samples, whereas 12 out of 45 (27%) HCV infected patients showed no detectable amounts of viral RNA in PBMCs (Table 2). Phylogenetic analysis of the 5′UTR sequences (Fig. 1) confirmed the prevalence of genotype 1 (82%) in plasma of the studied population [19,21]. Most patients (88%) with detectable amounts of virus RNA in plasma and PBMCs presented the same HCV

Table 1 Clinical features of the 45 enrolled patients

Variable	
N	45
Male [*n* (%)]	22 (49)
Age [years (IQ range)]	54 (46–63)
BMI [kg/m^2 (IQ range)]	26 (23–27)
Duration of infection [years (IQ range)]	31 (23–40)
Suspected route of infection [*n* (%)]	
Transfusion	24 (53)
Intravenous drug use	1 (2)
Vertical (Mother to Child)	1 (2)
Sexual transmission	1 (2)
Unidentified risk factor	18 (40)
Genotype in plasma [*n* (%)]	
1	37 (82)*
2	1 (2)
3	7 (15)
5	1 (2)*
Genotype in PBMCs [*n* (%)]	
1	31 (94)
3	2 (6)
Viral load (IU/mL, median (IQ range)]	451,842 (204.521 → 850.000)
ALT [(U/L, median (IQ range)]	85 (58–141)
AST [(U/L, median (IQ range)]	98 (63–131)
Cirrhosis [*n* (%)]	22 (48)

IQ, interquartile range; ALT, alanine-aminotransferase; AST, aspartate-aminotransferase; BMI, body mass index.
*Patient 43 (Table 2) is co-infected with genotypes 5a and 1b.

Table 2 HCV-RNA in plasma and PBMCs samples of chronically infected individuals

Patient number	5′UTR RNA in plasma	5′UTR RNA in PBMC	Genotype in plasma (P)/PBMC (C)	SSCP Group
1	+	–	1b	1
2	+	–	3a	1
3	+	–	1b	1
4	+	–	1b	1
5	+	–	1b	1
6	+	–	1	1
7	+	–	3a	1
8	+	–	3a	1
9	+	–	1b	1
10	+	–	1b	1
11	+	–	1	1
12	+	–	1b	1
13	+	+	1	2
14	+	+	1b	2
15	+	+	1b	2
16	+	+	3a (P)/1b (C)	2
17	+	+	3a	2
18	+	+	1a	2
19	+	+	1b	2
20	+	+	1	2
21	+	+	1b	2
22	+	+	1b	2
23	+	+	1b	2
24	+	+	1	2
25	+	+	1	3
26	+	+	1b	3
27	+	+	1b	3
28	+	+	1b	3
29	+	+	1	3
30	+	+	1	3
31	+	+	1b	3
32	+	+	1b	3
33	+	+	1b	3
34	+	+	1	3
35	+	+	1b	3
36	+	+	1b	3
37	+	+	3a	3
38	+	+	1b	ND
39	+	+	1b	ND
40	+	+	1	ND
41	+	+	1	ND
42	+	+	2a (P)/1b (C)	ND
43	+	+	5a and 1b (P)/1b (C)	ND
44	+	+	3 (P)/1b (C)	ND
45	+	+	1b	ND

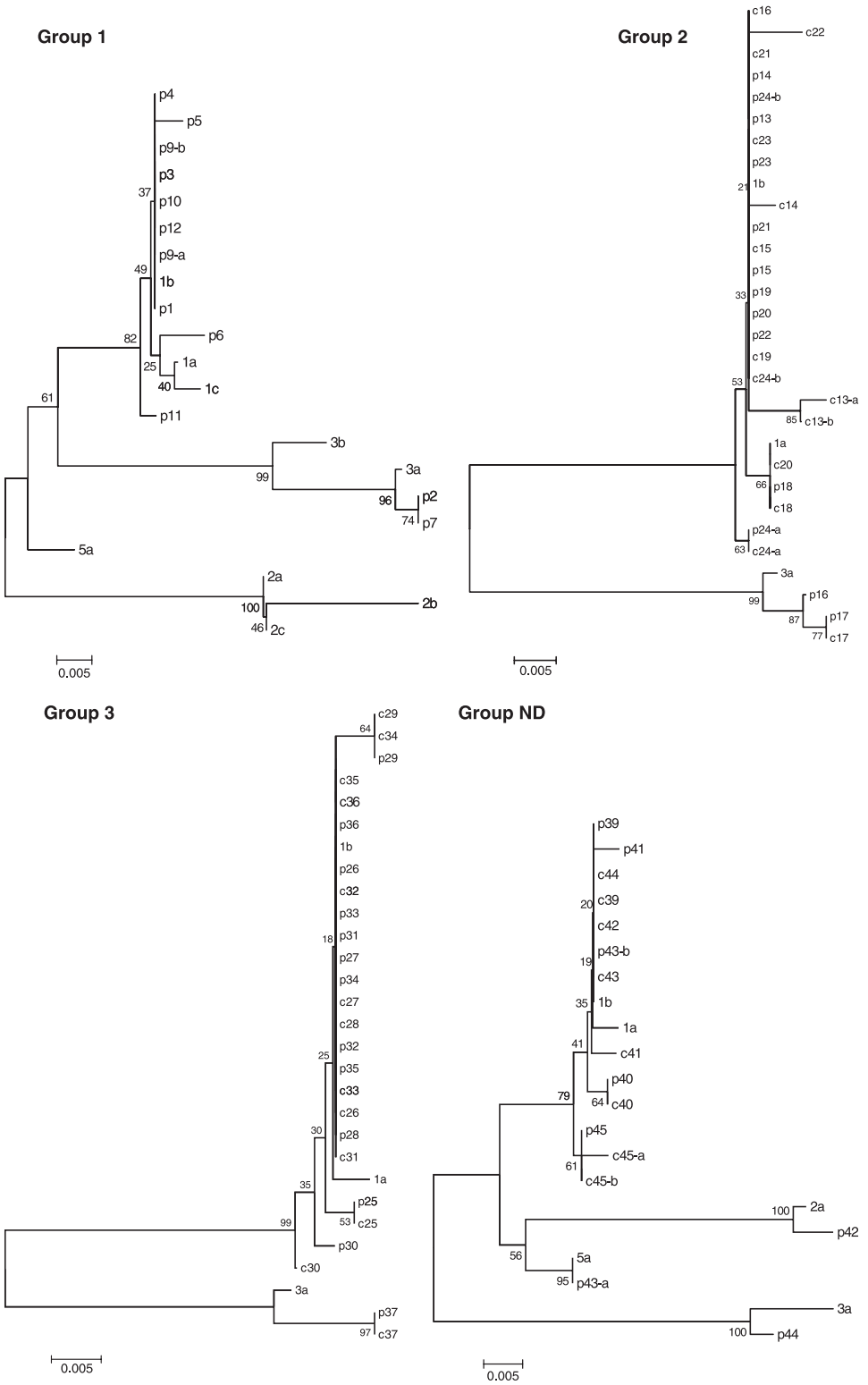


Fig. 1 Phylogenetic trees based on the HCV 5′UTR sequences. The 5′UTR of the HCV genome was amplified from total RNA recovered from plasma (P) and PBMCs (C) samples for HCV chronically infected patients. The amplified products were sequenced and trees were built using the MEGA 3.1 software as described in the section Materials and Methods. Group 1 included patients with detectable amounts of HCV RNA only in plasma. Group 2 includes patients displaying dissimilar SSCP band patterns between both compartments (see Fig. 4). Group 3 incorporated patients with presenting indistinguishable SSCP patterns (see Fig. 4). Group ND includes samples that did not exhibit consistent SSCP patterns in independent experiments (Table 2).

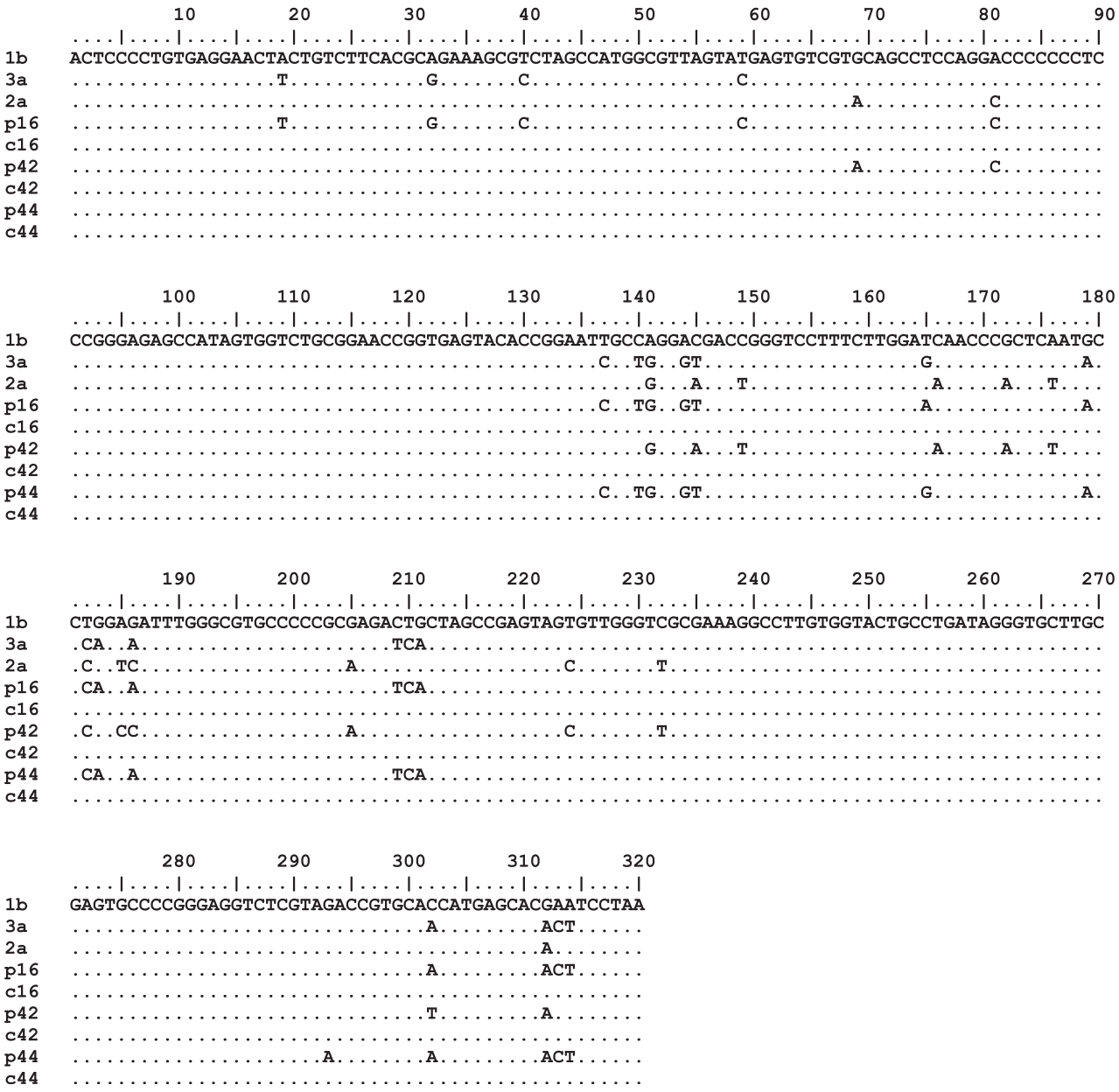


Fig. 2 Sequence analysis of the HCV 5′UTR recovered from patients exhibiting disparate genotypes in plasma and PBMC. The nucleotide sequence of the 5′UTR of the HCV genome (nucleotides 39–358 according prototype AJ242654) recovered from plasma (P) or PBMC (C) from patients 16, 42 and 44 was aligned against the 5′UTR of the here considered prototype lb (AJ242654), 2a (D00944), and 3a (D17763) HCV sequences. The first nucleotide in the sequence corresponds to nucleotide 39 according to numeration adopted for the here considered prototype 1b HCV (AJ242654) sequence.

genotype in both compartments (Table 2, Fig. 1). However, patients 16, 42 and 44 exhibited diverse genotypes in plasma (P) and PBMCs (C) (Table 2, Fig. 2). Strikingly in these three cases, only genotype 1b was isolated in PBMCs. Patient 43, the only injection drugs user (IDU) enrolled in this study, exhibited genotypes 5a and 1b simultaneously circulating in plasma (Fig. 3), yet despite successive attempts only genotype 1b could be isolated from PBMCs (Table 2, Fig. 3). In an effort to demonstrate HCV extrahepatic replication we evaluated the presence of the negative-strand of HCV RNA in all PBMC samples yet the viral replication intermediate RNA could not be detected in cells, even when a well described Tth-based strand-specific assay using tagged-primers and a hot start strategy was employed [25,26].

Compartmentalization of HCV quasispecies in plasma and PBMCs

Hepatitis C Virus is characterized by a high degree of genetic heterogeneity resulting from the absence of proofreading

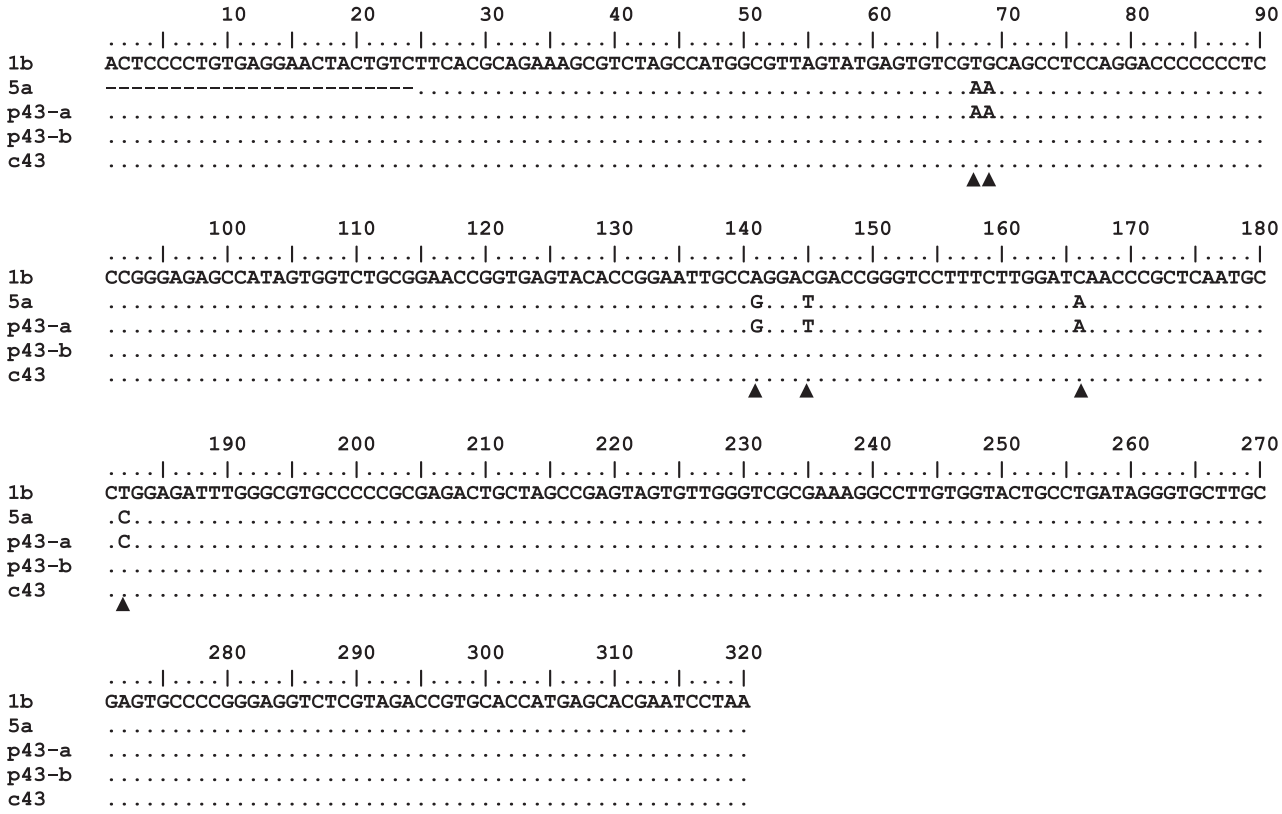
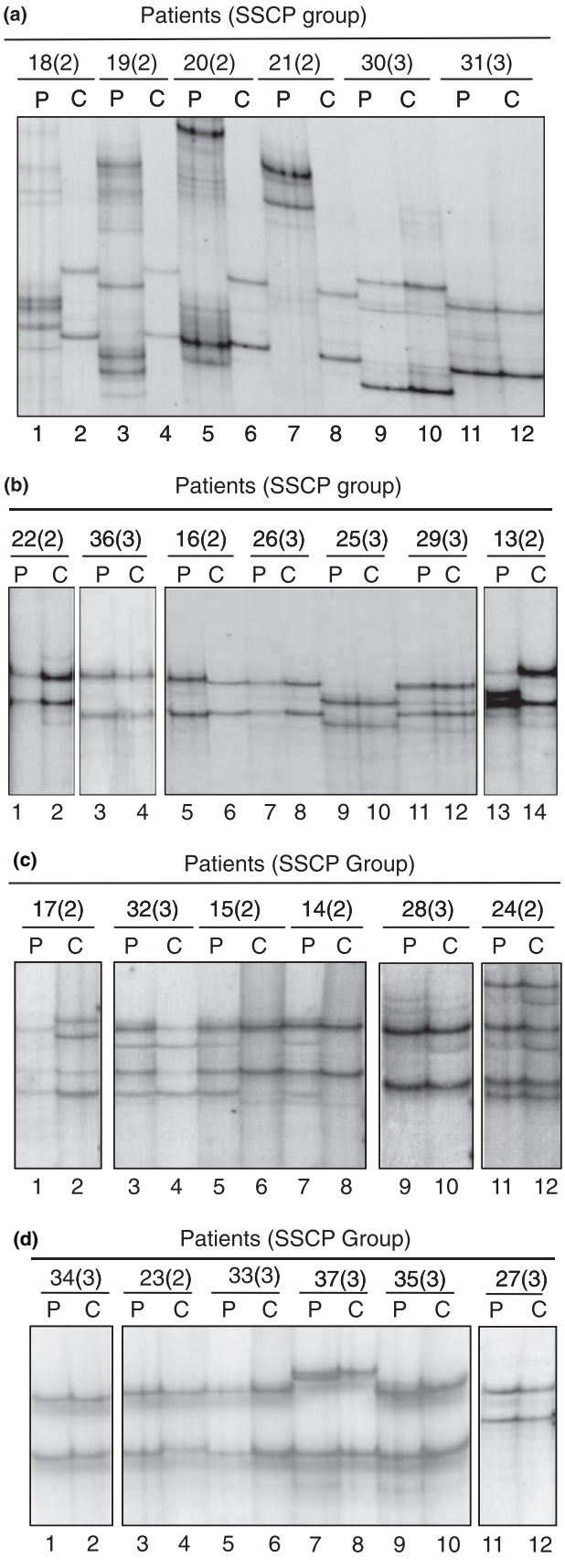


Fig. 3 Sequence analysis of the HCV 5′UTR recovered from patient 43. Sequence analysis of the 5′UTR of the HCV genome (nucleotides 39–358 according prototype AJ242654) recovered from plasma (P) or PBMC (C) from patient 43 were aligned against the 5′UTR of the here considered prototype lb (AJ242654) and 5a (Y13184) HCV sequences. The first nucleotide in the sequence corresponds to nucleotide 39 according to numeration adopted for the here considered prototype 1b HCV (AJ242654) sequence. Black arrows highlight the nucleotide difference between the sequences.

activity in its RNA polymerase. As a consequence HCV circulates as a population of closely related but nonidentical genomes, referred to as *quasispecies* [30]. Several reports show that the different *quasispecies* compartmentalize in PBMCs, serum, and liver of infected individuals [7,8,10,31–36]. These observations prompted us to evaluate HCV *quasispecies* distribution in plasma and PBMCs using SSCP, a technique that is widely used in the analysis of HCV *quasispecies* polymorphism [8,35–40].

Single-strand conformation polymorphism assays patterns for the 5′UTR were established for all 45 patients. To lower the risk of false polymorphisms that would lead to data misinterpretation, SSCP analysis was duplicated in an independent experiment using new RNA templates. RT-PCR products were checked and used only if a single sharp product band was observed on a regular agarose gel. Data were considered in our analysis if the results of the control experiment were consistent with the initial findings. This experimental restriction reduced our study population to 37 patients (Table 2) as patients 38–45 did not exhibit consistent SSCP patterns in independent experiments (ND in Table 2). Based on the presence of HCV RNA in PBMCs and on the resulting SSCP patterns infected patients were divided into three distinct groups (Table 2, Fig. 4). Group 1 comprised patients with detectable amounts of HCV RNA only in plasma (12/37). Group 2 comprised individuals harbouring HCV-RNA in both plasma and PBMCs, but that displayed dissimilar SSCP band patterns between both compartments, clearly reflecting HCV compartmentalization (12/37) (Fig. 4). Plasma samples from group 2 exhibited a highly heterogeneous SSCP pattern when compared with their PBMC counterparts suggesting the presence of different HCV *quasispecies* (Fig. 4). Group 3 incorporated patients with HCV-RNA in plasma and PBMCs that presented visually indistinguishable SSCP patterns between both compartments (13/37) (Fig. 4). Strikingly, the SSCP patterns for samples isolated from PBMCs from group 2 and 3 were highly homogeneous, which clearly contrasts to the high heterogeneity observed in plasma samples from group 2 (Fig. 4). This observation suggests that *quasispecies* distribution in PBMC is limited.

We next sought to confirm *quasispecies* distribution in patients belonging to group 3. For this purpose sequences from the 5′UTR of the HCV genome amplified from plasma or



PBMCs were aligned (data not shown). Sequence analysis revealed that the 5′UTR sequences isolated from patients 30 and 34 exhibited nucleotide differences between RNA recovered from plasma and PBMCs (Fig. 5a). As samples from patients 39, 40, 41 and 45 (Table 2) did not exhibit consistent SSCP patterns in independent experiments, we opted to expand our analysis by aligning nucleotide sequences from the major viral species found in plasma and PBMCs (Fig. 5b). Sequence analysis revealed that the 5′UTR of the HCV genome isolated from patients 41 and 45 exhibited nucleotide differences between plasma and PBMCs (Fig. 5a). Therefore, based on both SSCP and sequence analysis we conclude that a total of 19 out of 33 patients (57.6%) harbouring HCV-RNA in plasma and in PBMCs exhibited HCV compartmentalization.

DISCUSSION

The presence of extrahepatic replication in PBMCs is still widely debated because negative-strand HCV RNA, which is a viral replicative-intermediate, is generally not detected in these cells when proper strand-specific assays are employed [25,26]. In agreement with these findings we were incapable of detecting HCV negative-strand RNA in PBMCs from 33 patients that harboured detectable amounts of viral RNA in this compartment (Tables 1 & 2). Even though negative, these data are not in variance with reports showing HCV replication in PBMCs, as active extrahepatic HCV replication has been demonstrated mainly in immunosuppressed individuals [41–44]. Some exceptions however do exist in the literature [4]. Patients selected in our study were not co-infected with immunosuppressive viruses, nor were they undergoing any immunosuppressive medical therapy, such as chemotherapy at the time of sampling. Therefore, the inability to detect the negative-strand may relate to the described capacity of HCV to replicate in PBMCs of immunocompetent individuals only if these cells are previously stimulated [15,45]. In non-immunosuppressed individuals where PBMCs were apparently negative for HCV RNA, the use of mitogen cocktails supplemented with interleukin-2 (IL-2) and IL-4 to stimulate T and B lymphocytes and monocytes increased HCV replication in the respective cells leading to enhanced detection of the residing virus [15,45]. Reports of the positive- to negative-strand ratio in HCV-

Fig. 4 Single strand conformation polymorphism (SSCP) analysis of the HCV 5′UTR. Total RNA recovered from plasma from patients that presented detectable amounts of HCV-RNA in plasma (P) and PBMCs (C) were amplified by RT-PCR and analyzed by SSCP. In panel a–d patient number is followed by the assigned SSCP group in brackets. Group 2 includes patients displaying dissimilar SSCP band patterns between both compartments, while group 3 incorporated patients with presenting indistinguishable SSCP patterns.

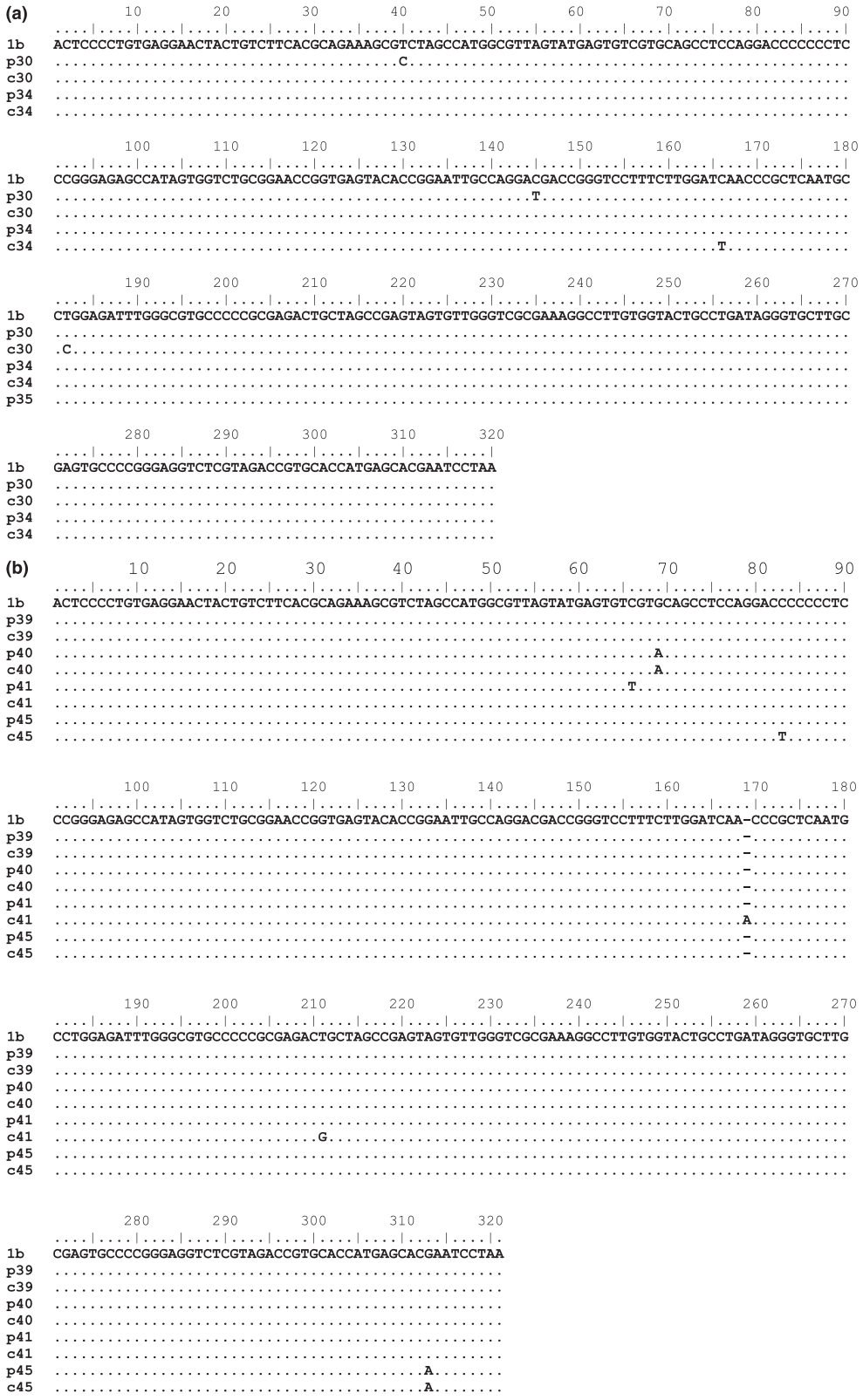


Fig. 5 Sequence alignment of two discordant patients from SSCP Group ND. Nucleotide sequence alignments of the 5′UTR region of the main circulating HCV *quasispecies* from plasma (P) and PBMCs (C) from (a) patients 30 and 34 of SSCP Group 3, which presented indistinguishable SSCP patterns between P and C, and (b) from patients classified within the SSCP ND group, which includes samples that did not exhibit consistent SSCP patterns in independent experiments, against genotypes 1b-HCV (AJ242654). Nucleotide one corresponds to position 39 of the HCV genome (in respect to AJ242654).

RNA-positive livers vary widely, from 1:1 to 1100:1 [46–48]. This variability may reflect the difficulties of precise and specific RNA quantification that occur with low copy numbers of negative-strand RNA in such a complex biological sample. A similar phenomenon might be expected when using PBMCs as RNA source for detecting HCV negative-strand RNA. This is consistent with observations associating HCV replication with B-cells; cells which represent a very small subpopulation within the total PBMCs [4,16,17]. Thus, an alternative explanation to our inability to detect the HCV replicative-intermediate RNA in PBMCs might be the low concentration of this RNA species in cells that have not been stimulated with mitogens prior to RNA isolation [15,45].

Even though we where incapable of directly demonstrating HCV replication in PBMCs, this study does however provide evidence in support of HCV genotype compartmentalization [8,33]. Patients 16, 42, 43 and 44 presented different HCV genotypes in plasma *vs* in PBMCs (Table 2, Figs 2 & 3). Strikingly, in each case only genotype 1b could be detected in cells (Table 2, Figs 2 & 3). These data agree with previous reports suggesting that genotype 1 is better adapted to PBMCs than other genotypes [10,33]. Nonetheless, our data indicate that the genotype is not the sole factor that determines the infection of PBMCs by HCV. Results show that 37 out of 45 (82%) patients harbour genotype 1 in plasma, yet nine of these patients classified within group 1 (Table 2) with no detectable viral RNA in PBMCs. Infection by genotype 1 thus does not necessarily imply that PBMCs are targeted by the virus. To complement our studies we conducted SSCP assays of the HCV 5′UTR. Plasma samples from patients herein classified within SSCP groups 1 and 2 presented a highly variable SSCP pattern (Fig. 4). When compared with plasma, viral RNA isolated from PBMCs presents a relatively homogenous SSCP pattern (Fig. 4). Similar findings were previously reported for other extrahepatic sites that harbour HCV RNA [38,39,49]. These relatively homogeneous SSCP patterns observed in cells may be because of the low concentration of viral RNA within the PBMCs that have not been stimulated with mitogens prior to RNA isolation [15,45]. Thus, RT-PCR would preferentially amplify the main viral species over the less represented *quasispecies*. Alternatively, the homogeneous SSCP patterns may reflect lymphotropic HCV *quasispecies*. In agreement with this last possibility here we report that 57.6% of the individuals that harboured HCV-RNA in plasma and PBMCs exhibited virus compartmentalization.

The clinical significance of HCV infection and compartmentalization within PBMCs is currently a matter of discussion [2]. However, consistent with a putative lymphotropism a high prevalence of lymphoproliferative disorders is described in HCV-infected individuals [2]. Approximately 20% of patients relapse after an apparently successful course of treatment with pegylated interferon in combination with ribavirin. Relapse is also seen after treatment with newer drugs specifically targeted against HCV. The mechanism underlying relapse is not completely understood, but it is tempting to speculate that extrahepatic HCV reservoirs may play a role in this very relevant clinical outcome. Therefore, the existence of alternative biological compartments for the generation of competent infectious virus, such as PBMCs may have profound clinical and pathogenic implications in HCV natural history. In this context, our findings are relevant as they support previous reports [10], showing that infection of PBMCs by HCV is not exclusively restricted to immunocompromised individuals such as IDU, liver transplant recipients, or HCV patients co-infected with the HIV.

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