Continuous release of hepatitis C virus (HCV) by peripheral blood mononuclear cells and B-lymphoblastoid cell-line cultures derived from HCV-infected patients

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In order to investigate hepatitis C virus (HCV) persistence and replication in peripheral blood mononuclear cells (PBMC) from a group of haemophilic individuals, HCV production and release to PBMC culture supernatants (SNs) from HCV singly infected patients and HIV/HCV co-infected patients was studied. HCV RNA+ SNs were found more frequently from HIV/HCV co-infected individuals (89 ± 5 %) with poor reconstitution of their immune status than from singly HCV-infected patients (57 %) or from HIV/HCV co-infected individuals with a good response to highly active anti-retroviral therapy (50 %). The presence of the HCV genome in culture SNs was associated with lower CD4+ T-cell counts and with a more severe clinical picture of HIV infection. In spite of prolonged negative HCV viraemia, PBMC from HIV/HCV co-infected patients released the HCV genome after culture. HCV permissive PBMC allowed generation of HCV productive B cell lines with continuous HCV replication. These findings add further weight to the involvement of PBMCs in persistence of HCV infection and emphasize the role of B lymphocytes as HCV reservoirs.

INTRODUCTION

Hepatitis C virus (HCV) was originally thought to be strictly hepatotropic, but there is mounting evidence that it can also replicate in peripheral blood mononuclear cells (PBMC) from chronically infected HCV+ patients (Moldvay et al., 1994; Lérat et al., 1998; Laskus et al., 2000). It has been shown that HCV can infect lymphoid cells, preferentially B lymphocytes and monocytes/macrophages (M/M) (Caussin-Schwemling et al., 2001; Boisvert et al., 2001; Sung et al., 2003). Although HCV replication occurs at very low levels and the number of particles released may be too low to influence plasma viral loads (VL) (Rodríguez-Inigo et al., 2000; Boisvert et al., 2001), these cells might represent an extra-hepatic reservoir that can be implicated in virus recurrence and persistence, especially in immuno-suppressed individuals (Laskus et al., 2000).

Some reports suggest that human immunodeficiency virus (HIV) or its associated immunodeficiency state may enhance HCV replication in co-infected patients (Eyster et al., 1994; Beld et al., 1998; Bonacini et al., 1999; Thomas et al., 2000). The interplay of both viruses is difficult to demonstrate due to the lack of reliable in vitro culture systems or experimental models that can be used to analyse virus–cell interactions.

We described previously a technique that is useful to obtain prolonged cultures of normal (N) and HIV+ PBMC in the absence of exogenous stimuli (cytokines, allogeneic cells, etc.). This is achieved by allowing undisturbed interaction of lymphocytes, M/M and other accessory cells present in the PBMC suspension over 5–45 days (Ruibal-Ares et al., 1997). During the first week of PBMC culture, cell aggregation involving small clumps of T and B lymphocytes and M/M occurred. Between 7 and 21 days, the size of the aggregates increased, M/M divided and differentiated, becoming actively involved in the removal of dead cells and apoptotic debris. After approximately 3 weeks, the fate of the PBMC culture was defined: either Epstein–Barr virus positive (EBV+) lymphoblasts overcame immune
control and continuous B cell lines were generated or B-lymphoblast proliferation was abolished by CD8 T cells and the remaining PBMC died (Ruibal-Ares et al., 2001b). In HIV\(^+\) patients with CD4 cell counts above 200 CD4\(^+\) cells per mm\(^3\) the evolution of non-stimulated PBMC culture was similar to that of normal controls. HIV replication took place mainly in M/M (Ruibal-Ares et al., 2001a). The frequency of B-lymphoblastoid cell line (B-LCL) generation was higher than for N-PBMC non-stimulated cultures (60 vs 6 %). In contrast, in HIV\(^+\) patients with low CD4 counts (<200 CD4\(^+\) cells per mm\(^3\)) and high HIV viraemia, few B-LCL were formed because overall PBMC viability was low and prolonged PBMC cultures were not maintained (Ruibal-Ares et al., 2001b).

Because most HIV-infected haemophilic patients were also HCV\(^+\), we investigated whether, in addition to HIV replication, HCV replication occurred after prolonged non-stimulated PBMC culture of HIV/HCV-PBMC and compared the results with those of HCV singly infected patients. Our results indicate that HCV can be recovered after culture of PBMCs from HCV\(^+\) patients, and that HIV co-infection enhances in vitro HCV replication. We have also demonstrated continuous HCV replication in some of the B-LCL that spontaneously overgrew the HIV/HCV-PBMC cultures. These results indicate that HCV infection of PBMCs is a general phenomenon associated with natural HCV infection, suggesting a role for these cells as HCV reservoirs after treatment. In addition our results provide further support for the role of HIV co-infection as an enhancer of HCV replication.

### METHODS

**Patients.** A total of 52 haemophilic, HCV chronically infected patients who were being periodically assisted at the 'Fundación Argentina de Haeemofilia' were studied; 21 were HCV singly infected (HCV group) without any HCV treatment during our study period and the other 31 had been infected with HIV in addition to HCV (HIV/HCV group). All patients were positive for HCV antibodies as detected by ELISA (third generation) and RIBA test. These patients were under highly active anti-retroviral therapy (HAART) since 1997. Only 3 of 21 patients from the HCV group had undetectable HCV RNA in plasma. The prevalent genotype was 1 (69 %) and the median value of HCV-VL was 5-61 log (IU ml\(^-1\)) (range: 2-80–5-93). In the HIV/HCV group, 7 of 31 patients (22 %) had persistently negative HCV RNA detection in plasma and the other 24 were HCV\(^+\) with a median HCV-VL of 5-93 log (IU ml\(^-1\)) (range: 2-78–5-93). The prevalent genotype was also 1 (67 %). Other clinical data are included in Table 1.

As controls, and for HCV infection assays, PBMC obtained from volunteer blood donors with negative serology for HIV, hepatitis B virus, HCV, human T-lymphotropic virus 1/IL, Chagas disease, Syphilis and Brucellosis were used (N-PBMC). Plasma and white blood cells for all the experiments were collected during routine evaluation visits. Informed consent was obtained from the patients studied.

**PBMC cultures.** PBMC were obtained by Ficoll-Hypaque density gradient of EDTA anti-coagulated blood. Cells were washed three times with Mg\(^++\)- and Ca\(^++\)-free PBS and resuspended to \(1 \times 10^6\) cell ml\(^-1\) in RPMI 1640 tissue culture medium (Gibco) containing 10 % fetal calf serum and antibiotics (penicillin/streptomycin, 10 mg ml\(^-1\)) (RPMI-FCS). PBMC cultures were carried out as described previously (Ruibal-Ares et al., 2001a). Briefly, \(2 \times 10^6\) PBMC were suspended in 2 ml RPMI-FCS using round-bottom 5 ml polystrene tubes and left undisturbed in a 5 % CO\(_2\) incubator. For each patient, 4–30 different tubes (depending on the lymphocyte yield) were set up. Beginning on day 5–6 of culture, half of the supernatant was replaced twice a week by fresh medium by gentle aspiration, avoiding disturbance of the cell pellet. The SNs collected were frozen for future studies. In order to detect HCV RNA release, SN samples were drawn sequentially from a single tube. For each series, 4–16 SN samples were tested at different culture periods (i.e. tube 1: day 7, 15, 21, 28 and 35). To assess viability and apoptosis, cells were stained by acridine orange and ethidium bromide, and examined by fluorescence microscopy (Ruibal-Ares et al., 2001b).

**Phenotype of purified PBMC and cultured PBMC.** In order to determine the number of CD4\(^+\) and CD8\(^+\) T cells, cytometric analysis was carried out on whole blood after lysis with fluorescence-activated cell sorter (FACS) lysing reagent (Becton-Dickinson). Anti-CD45/CD14, anti-CD20, anti-CD3, anti-CD4 and anti-CD8 monoclonal antibodies (mAb; Becton-Dickinson) were used to stain lymphocytes and the results were evaluated by flow cytometry using a FACSScan cytomter and the Simulset or CellQuest software (Becton Dickinson). The antibodies were used at the concentrations recommended by the suppliers. For phenotypic analysis of viable PBMC, the same mAbs were used to stain T and B lymphocytes and anti-CD14, anti-CD4, anti-CD64, anti-CD38, and anti-HLA-DR mAbs were used to characterize M/M. The results were analysed with the CellQuest software.

**Outgrowth of B-LCL.** After 30–40 days, in some cases, the PBMC culture could progress to yield an EBV\(^+\) lymphoblastoid cell line

### Table 1. Clinical data from the population studied

<table>
<thead>
<tr>
<th></th>
<th>HCV(^+) group</th>
<th>HIV/HCV(^+) group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD4(^+) (cells per mm(^3))</strong></td>
<td>953 ± 484-3</td>
<td>459–2024</td>
</tr>
<tr>
<td><strong>CD8(^+) (cells per mm(^3))</strong></td>
<td>653 ± 264-8</td>
<td>282–1183</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>29 ± 12</td>
<td>15–52</td>
</tr>
<tr>
<td><strong>HIV-VL (copies ml(^-1))</strong></td>
<td>NA</td>
<td>3-09 ± 1-26</td>
</tr>
<tr>
<td><strong>HCV-VL (IU ml(^-1))</strong></td>
<td>5-18 ± 1-03</td>
<td>5-39 ± 1-10</td>
</tr>
</tbody>
</table>

NA, Not applicable.
HCV replication in PBMC and B-LCL

When B-lymphoblast aggregates replaced the other cell series, the cell suspensions were transferred to 50 ml tissue culture flasks and maintained in culture as continuously growing cell lines in RPMI-FCS. Twice a week the cells were fed with fresh RPMI-FCS.

HCV RNA extraction and detection. To monitor HCV positivity in the SN throughout the days of culture, we used a nested PCR. HCV RNA extraction from plasma samples or culture SN (140 μl) were carried out with the QIAamp Viral RNA minikit (Qiagen) following the manufacturer’s instructions. Reverse transcription was performed on 10 μl RNA with 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega) and the outer antisense primer. The nested PCR was performed with specific primers for 5’ UTR (external primers: EA, 5’-ATACTCGAGGTGCAC-GGTCTACGAGCACCCTATCAGG-3’; ES, 5’-CTGTGAGGAACCTACTGTCTT-3’ and internal primers: IA, 5’-CAGTCTCAGACCCCTATCAGG-CAGT-3’; IS, 5’-TTACCCACCAGAAAGCTCTAG-3’ and 5 μl cDNA in a total volume of 50 μl containing 1 μl each primer at 250 ng μl⁻¹, 10 mM Tris/HCl (pH 8-3), 50 mM KCl, 1 mM MgCl₂, 6 deoxynucleoside triphosphate at a concentration of 200 μM and 1-25 U Taq DNA polymerase (Promega). After an initial denaturation step at 95 °C for 5 min, 40 cycles of PCR at 95 °C for 45 s, 53 °C for 45 s and 72 °C for 45 s were carried out with a final extension of 10 min. The second round of PCR was performed with 2 μl of the first-round amplification product with the same conditions described above. The PCR products were analysed on a 10 % polyacrylamide gel stained with ethidium bromide. The sensitivity of the assay reaches 50 IU ml⁻¹ plasma or culture supernatant. Appropriate procedures were employed to avoid contamination. All PCR runs included positive and negative controls and every positive result was confirmed, at least, in three independent PCR experiments. All HCV RNA studies were performed in cell-free culture SN.

HIV- and HCV-VL measurement. To quantify HCV viraemia in the culture SN and in plasma samples, we used Amplicor HCV monolayer version 2.0 (Roche Diagnostics) (range a detection HCV: 600–850 000 IU ml⁻¹). All HCV-VL in plasma samples were performed with Amplicor H1-3 monolayer version 1.5 (Roche Diagnostics) (range of detection: 400–750 000 copies ml⁻¹ or 50–500 000 copies ml⁻¹ with the ultrasensitive method). Recommendations of the suppliers were followed.

Determination of HIV replication in culture SNs. The concentration of the core HIV protein p24 was determined by ELISA (Vironostika HIV-1 antigen; Biomériux). Only p24 values above 25 pg ml⁻¹ were considered positive.

HCV infection assays. After 4–6 days in culture, the N-PBMC SN was replaced by 1 ml filtered HCV SN (14 600 IU ml⁻¹, 0-45 μl Microclar filter) without disturbing the pellets. The cells were incubated for 16 h and then washed with PBS (2 ml). After centrifugation and removal of all the SN, fresh medium was added and the cells were maintained in culture tubes for 40 days, replacing half of the SN with fresh medium twice a week. The collected SNs were stored at −80 °C for HCV detection. This experiment was done in duplicate for two different normal blood donors. Mock infection was performed incubating cells with virus-free filtered SNs obtained from a normal donor B-LCL. The presence of HCV genome in B-LCL was performed incubating cells with virus-free filtered SNs obtained from a normal donor B-LCL. The presence of HCV genome in B-LCL. The presence of HCV genome in B-LCL was performed incubating cells with virus-free filtered SNs obtained from a normal donor B-LCL. The presence of HCV genome in B-LCL was performed incubating cells with virus-free filtered SNs obtained from a normal donor B-LCL. The presence of HCV genome in B-LCL was performed incubating cells with virus-free filtered SNs obtained from a normal donor B-LCL.

RESULTS

Non-stimulated prolonged culture of HCV, HIV, HCV and N-PBMC

The organization and morphological characteristics of non-stimulated PBMC culture from HIV⁺ individuals and normal controls have been detailed previously (Ruibal-Ares et al., 1997). The most important difference with the methods used by others, in order to detect HCV replication, is that in those studies mitogen-, cytokine-activated or fixed and permeabilized in a 2 % paraformaldehyde-buffered solution containing 0-25 % Triton X-100 for 15 min at 4 °C. After washing in PBS buffer, a dual immunofluorescence technique was performed. First, cells were incubated with primary antibodies for 1 h at room temperature. The primary antibodies used were mouse monoclonal anti-pCore (1:150; Abcam, distributed by Novus Biological, cat. # ab2740) and goat polyclonal anti-CD20 or goat polyclonal anti-CD64 (1:100; Santa Cruz, cat. # sc-7733 and cat. # sc-7642, respectively). Subsequently, the cells were incubated for 1 h at room temperature with appropriately diluted, fluorescein isothiocyanate-labelled rabbit anti-mouse or rhodamine-labelled rabbit anti-goat secondary antibodies (1:250; Vector Laboratories and 1:50; Santa Cruz). The slides were mounted in 4’,6-diamidino-2-phenylindole (DAPI)-containing antifade solution (Vector Laboratories) and were examined under a fluorescence microscope. At least 150 cells per slide were scored for the presence of intracellular staining. Two independent experiments were performed. N-PBMC cultures and N-PBMC-derived B-cell lines were used as negative controls. In addition, appropriate isotype IgG negative controls were carried out to discard non-specific binding.

HCV genotyping. The HCV genotype was determined by restriction fragment length polymorphism (RFLP) of the 5’ UTR of the genome.

RFLP analysis. Restriction digests were carried out on 10 μl secondary PCR product for 1 h at 37 °C in the presence of 10 U each of RsaI and HaeIII, HindIII and MspI, Sall or BstUI (at 60 °C) as described by Davidson et al. (1995). Digestion products were visualized under UV light after electrophoresis through a 10 % acrylamide-bisacrylamide gel stained with ethidium bromide. Molecular mass marker (pGEM DNA markers; Promega) and undigested PCR product were included in every analysis. The genotype was deduced from the fragmentation pattern of the amplified DNA.

HCV sequence analysis. The 5’ UTR products were purified and the fragments were sequenced using internal primers. The sequences were aligned in accordance with their position similarity using the CLUSTALX program (Thompson et al., 1997). The phylogenetic tree was constructed from the nucleotide sequences according to the neighbour-joining and maximum-likelihood methods, using the MEGA2 program and PHYLIP package (Felsenstein, 1993), respectively. Their reliability was assessed by bootstrap resampling.

Statistical analysis. Median values of continuous variables without normal distribution were compared with the use of non-parametric tests (Mann–Whitney U test). The analysis of qualitative data were done with a chi square test or Fisher’s exact test. A level of P<0.05 was accepted as being statistically significant. VL results were log-transformed for analysis. Values with normal distribution were expressed as mean ± standard deviation (SD). All statistical comparisons were made taking into account cultures from different patients. Multiple cultures from the same patient were not considered for this purpose.

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co-cultured cells were added to obtain virus release and/or to demonstrate PBMC infection with HCV. In contrast, using non-stimulated prolonged PBMC culture as described here, the virus genome was released spontaneously from the in vivo infected cells. The HCV genome was detected in PBMC culture SNs from 35 of 52 patients: 12 (57%) belonging to the HCV group and 23 (74%) to the HIV/HCV group (Table 2) and no morphological or phenotypical differences could be established between PBMC from these two groups. As the level of HCV RNA found in the SN cell cultures was low, PCR results were positive and negative at different time points from a sequential tube culture. The frequency of HCV positive tubes per patient was higher in patients from HCV group (41-34 vs 27-71%, \(P=0.01\)) while the number of tubes tested was similar for both groups. Continuous EBV+ B-LCLs were generated from eight of 21 HCV patients and from 19 of 31 HIV/HCV patients, but in six patients from this group the B-LCLs harboured the HCV genome and more than one HCV+ B-LCL was obtained from each patient (total number of HCV+ B-LCL = 16). The frequency of B-LCL generation in the HCV group was higher than in the control group (\(P=0.0005\)). Although none of these B-LCLs were HCV+, HCV RNA was detected before B-LCL generation in the PBMC cultures that gave rise to B-LCLs. In contrast, in the HIV/HCV group, B-LCL generation was not related to HCV release in PBMC culture before B-LCL establishment (Table 2). The phenotypical and morphological characteristics of the B-LCLs from both the HCV and the HIV/HCV group were those generally described for EBV+ B-LCLs (Ruibal-Ares et al., 2001b).

**Recovery of HCV RNA from PBMC culture SNs before B-LCL establishment**

During the first week of culture, the rate of HCV+ SN recovery was similar for the HCV and HIV/HCV groups (60% for HCV group and 50% for HIV/HCV group, \(P>0.05\)). During the second week, recovery of HCV+ SN reached 57% in the HIV/HCV group while the frequency of HCV+ SN diminished gradually in the HCV group until the fourth week, when no HCV+ SN was detected (Fig. 1a and b). The increased frequency of HCV+ SN observed in the sixth week for the HIV/HCV group may be due to the influence of B lymphoblasts that started to divide and generate HCV+ B cell lines. HCV RNA+ persisted for longer periods in SNs from the HIV/HCV group. In contrast, in those obtained from the HCV group, HCV RNA resulted positive until day 21 and became negative during the fourth week (Fig. 1b). It is noteworthy that 20 patients were cultured and analysed repeatedly (two or three times) and consistent results were observed in their culture SNs. Nevertheless, multiple cultures from one individual patient were not considered for the calculations.

**Table 2.** HCV+ cultures and HCV+ B-LCL derived from PBMC of HCV and HIV/HCV patients

<table>
<thead>
<tr>
<th>Sample group</th>
<th>n</th>
<th>HCV+ cultures</th>
<th>B-LCL</th>
<th>HCV+ B-LCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (normal donors)</td>
<td>52</td>
<td>0</td>
<td>2/52 (3.8%)</td>
<td>0</td>
</tr>
<tr>
<td>HCV group</td>
<td>21</td>
<td>12/21 (57%)</td>
<td>8/21 (38%)</td>
<td>0</td>
</tr>
<tr>
<td>HIV/HCV group</td>
<td>31</td>
<td>23/31 (74%)</td>
<td>19/31 (61%)</td>
<td>6/19 (31.6%)</td>
</tr>
</tbody>
</table>

**Fig. 1.** Recovery of HCV RNA from PBMC culture SNs before B-LCL establishment. (a) Percentage of HCV+ results after different weeks of culture. The total number of patients analysed per week is stated above the bars. (b) Sequential HCV-VL in different patients of HCV group (\(n=5\)) and HIV/HCV group (\(n=10\)) during days of culture.
In five patients, cultured PBMCs were stained for HCV pCore antigens and for CD64 or CD20. CD64/HCV+ and CD20/HCV+ cells displayed cytoplasmic pCore antigen immunostaining (Fig. 2a). HCV pCore antigens were found in variable proportions of M/M (CD64+ cells) and B lymphocytes (CD20+ cells). The percentage of CD64+ or CD20/HCV+ cells varied from patient to patient (Fig. 2b). Notably, the percentage of HCV+ B lymphocytes was higher in patients that later on gave rise to B-LCL (Lo and Rob), whether the B-LCLs that developed thereafter were or not HCV-producers.

**HCV and HIV viraemia, CD4, CD8 cell counts and in vitro HCV recovery**

In the HCV group, HCV production after culture was associated with HCV plasma viraemia ($P=0.03$) since patients with negative plasma HCV RNA (3 of 21) did not

![Fig. 2. Immunofluorescence staining during culture. (a) PBMC CD20+ (i, iii) and PBMC CD64+ (iv, vi) showing immunofluorescence pattern of HCV core protein deposits (ii, iii and v, vi) from patient Lo after 1 week of culture. Staining procedures as described in Methods. (vii–ix) Isotype controls. Original magnification ×1000. (b) Percentage of HCV M/M (CD64/HCV+ cells) or HCV+ B lymphocytes (CD20/HCV+ cells) in individual patients (Ib, Tra, Na, Lo and Rob) during 2 weeks of culture.](http://vir.sgmjournals.org)
originate HCV + cultures. The level of HCV viraemia did not predict the frequency of HCV production in culture. As for CD4 + or CD8 + T-cell counts, they were neither related to HCV viraemia condition (detectable or non-detectable) nor to the frequency of HCV + cultures.

Considering the HIV/HCV group, higher frequency of HCV production after culture was associated with lower CD4 + T-cell counts (P = 0.02) and detectable HIV RNA in plasma (P = 0.009). HCV presence in plasma was not a factor predicting an HCV + culture. In reference to patients with undetectable HCV RNA in plasma, five of seven gave rise to PBMC cultures with HCV + SN. Moreover, it is noteworthy that four patients with long lasting undetectable HCV viraemia (4 years) yielded HCV + SN from day 7 to 35 (Baré et al., 2003). Only two of seven originated non-HCV productive cultures. Furthermore, the group of patients with better clinical status [CD4 + T-cell counts higher than 300 cells per mm 3, (range: 302–973)] and undetectable HIV-VL (12 of 31), good HAART responders, showed a lower frequency of HCV productive PBMC cultures, only 6 of 12 (50 %) generated cultures with HCV + SN (Table 3). These results were similar to those of the HIV/HCV group (12 of 21, 57 %), whose CD4 + T-cell counts were significantly higher (median value: 752 cells per mm 3, range: 459–2024). In contrast, 89-5 % (17 of 19) of non-HAART responders generated cultures with HCV + SN. It should be considered that p24 antigen production was undetectable along different days of culture in all of the PBMC cultures studied. This is not unexpected since all HIV/HCV patients had been under HAART for more than 3 years and the frequency of HIV replication in non-stimulated cultures (probably reflecting continuous low-level HIV replication) is reduced after 3 years of successful HAART (Belmonte et al., 2002).

### HCV genotyping and sequence analysis

HCV genotypes were analysed from 10 plasma samples and compared with the virus recovered from the culture SN. The same HCV genotype was found in the different types of samples (plasma, PBMC culture SN and B-LCL SN).

HCV genotype in patients Ru, Cou, Lo and Va corresponded to genotype 2a; genotype 1b was observed in patients Ca, Se, Ro; genotype 1a in patients Na and Rob and genotype 4 in patient Ib.

Plasma samples and culture SNs after 1 and 2 weeks were sequenced further for patients Ib, Lo and Va. Phylogenetic analysis indicates that the viruses present in cell cultures were those found in patients and not the result of experimental contamination (Fig. 3).

#### Table 3. CD4 T-cell counts, HIV-VL and HCV positivity after culture in HIV/HCV co-infected patients

The frequency of HCV + PBMC cultures obtained from HIV/HCV co-infected patients was analysed in patients with good response to HAART (undetectable HIV-VL and CD4 + T-cell counts above 300 cells per mm 3) compared with those with non-HAART responders (detectable HIV-VL and/or low CD4 + T-cell counts).

<table>
<thead>
<tr>
<th>n</th>
<th>HIV-VL log (copies ml⁻¹)</th>
<th>CD4 + T-cell counts (cells per mm ³)</th>
<th>HCV + cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>12</td>
<td>3·78 ± 1·09</td>
<td>3·54</td>
<td>2–5·87</td>
</tr>
<tr>
<td>19</td>
<td>288 ± 182</td>
<td>286</td>
<td>41–670</td>
</tr>
</tbody>
</table>

#### Fig. 3. HCV sequence analysis. The virus recovered from the cultures was analysed on samples collected during the first and/or second week of culture. Phylogenetic tree according to the neighbour-joining method derived from nucleotide sequences of serum (1) and SN (2) of patients Lo, Ib and Va and of representative HCV genotypes. Bootstrap values higher than 70 % are indicated.
Recovery of HCV RNA from B-LCL

Previous reports have demonstrated the establishment of HCV-producing B cell lines after experimental EBV and/or HCV infection (Giannini et al., 2002; Sung et al., 2003; Hu et al., 2003). In this study, we show that HCV-infected B-LCL were obtained by spontaneous outgrowth in six HIV/HCV patients that were studied repeatedly over 5–7 years.

Three of these B-LCLs could be studied in detail. One of the B-LCL, RuF, gave persistently positive results for the presence of the HCV genome during 20 months from 11/2001 to 8/2003 and the SNs became negative thereafter. However, HCV RNA was detected in the B-LCL cell samples. The other two B-LCLs are still producing HCV particles since 12/2002 and 7/2003. The virus recovered was genotype 1b as shown by RFLP analysis. Although their HIV and HCV profile and immunological status were dissimilar (Fig. 4), all of the individuals have adhered to HAART since 1997. In one of the patients (RoC), negative HCV viraemia persisted for more than 4 years. This patient had reduced plasma HIV-VL after HAART, but the CD4⁺ T-cell count remained below 300 cells per mm³. HCV production was observed during this PBMC culture before B-LCL appearance and the continuously growing B lymphoblasts of the B-LCL were able to maintain and support HCV replication while HIV genome detection (proviral DNA genome and HIV RNA) and p24 release were negative. Patients RuF and CaA, who also produced an HCV⁺ HIV⁻ B-LCL, were considered good HAART responders because their immunological and virological parameters improved greatly. In contrast, their HCV plasma viral burden maintained high levels throughout the study period.

Rate of HCV replication in spontaneous B-LCL from HIV/HCV patients

The HCV replication rate was analysed throughout 11 days of culture without tissue culture medium replacement after establishment of a continuous B-LCL in patient CaA. An initial amount of 2 × 10⁵ cells ml⁻¹ was cultured in 9 ml fresh medium. A sequential increase in HCV RNA levels was observed from day 0 (non-detectable HCV genome) until day 11 (Fig. 5a). Considering a constant number of approximately 1 × 10⁷ cells and once the culture was left undisturbed, without addition of fresh tissue culture medium for 10 days, cumulative HCV production of the three B-LCL reached different values. In the case of RoC the VL reached 14 600 IU ml⁻¹ (4.16 log). Comparatively, when the same experiment was carried out with RuF, the HCV-VL reached 1815 IU ml⁻¹ (3.26 log). With CaA B-LCL the level obtained was 29 540 IU ml⁻¹ (4.47 log) of HCV. Thus, the relative efficiency for the generation of HCV RNA was not equal between the B cell lines. Immunofluorescent pattern of HCV pCore antigen detection in B-LCL is shown in Fig. 5(b). The immunostaining assessment of HCV⁺ replicative cells showed the presence of cytoplasmic staining pCore⁺ cells; pCore antigen was detected in a high proportion of cells (15–20 %). Cells with apoptotic nuclear morphology showed the most intensive staining pattern. To determine whether the HCV produced after culture by B-LCL were infectious; HCV⁺ SN were used to infect N-PBMC. Consequently, HCV⁺ SN containing 14 600 IU ml⁻¹ (obtained from RoC B-LCL SN) was added at day 5 of N-PBMC culture. The presence of HCV RNA was sought in SNs after culture. The HCV genome was detected intermittently (Fig. 5c). During the initial days of culture, the probability of detecting HCV from the original inoculum exists, but after 2 weeks of culture with several medium replacements, it would be diluted below detection limits. The HCV genotype of the recovered virus was the same used for cell infection (genotype 1b). Mock-infected cells SN resulted negative for HCV RNA detection all along the days of culture.

DISCUSSION

These results are in agreement with those reported by others (Daar et al., 2001; Laskus et al., 2000, 2004) indicating that HCV replication in PBMC occurs and is more likely with concomitant HIV infection.

Taking advantage of the possibility of obtaining viable long term PBMC cultures in the absence of exogenously added stimuli, we now demonstrate that HCV can be recovered after culture of PBMCs from HIV/HCV co-infected and from HIV singly infected patients.

Compatible with other reports (Torres et al., 2000; Baré et al., 2003; Pham et al., 2004; Radkowski et al., 2005) HCV RNA was detected in extra-hepatic reservoirs in patients with negative viraemia. Our findings highlight the role of PBMC in the persistence of HCV infection after apparently spontaneous clearance. Interestingly, HCV RNA may be detected not only in mitogen-stimulated PBMC (Pham et al., 2004; Radkowski et al., 2005) but also in non-stimulated cultured PBMC and in the B-LCLs that spontaneously overgrew long term PBMC cultures from patients who had remained HCV RNA negative in serum for several years.

Long term extra-hepatic reservoirs of HCV and low-level replication in patients with negative viraemia should be considered because the absence of HCV viraemia does not exclude the possibility of reappearance of the virus in HIV/HCV co-infected persons.

In the present study, the HCV recovery demonstration was based on the persistent detection of HCV RNA in cell-free SNs throughout the culture (for more than 30 days in some cases) with occasional quantification by Amplicor HCV monitor. The genotype and phylogenetic analysis of HCV detected in the PBMC culture SNs and in patient’s plasma revealed that the viruses present in cell cultures were those found in patients and not the result of experimental contamination. In addition, as shown previously, both M/M and B lymphocytes harboured HCV viral proteins during the culture period (Cauissin-Schwemling et al., 2001; Boisvert et al., 2001; Sung et al., 2003).
**Fig. 4.** HCV\(^+\) B-LCL were obtained from three HIV/HCV patients on HAART for 6–7 years. CD4\(^+\) and CD8\(^+\) T-cell counts (cells per mm\(^3\)), HIV-VL \(\log\) (copies ml\(^{-1}\)) and HCV genome detection (+ or −) are shown for each date of analysis.
The outgrowth of EBV+ B-LCL occurred at low frequency after culture of N-PBMC (3-8%). The present findings show that chronic HCV and HCV/HIV infection are directly or indirectly involved in B-LCL occurrence with values of 38 and 61%, respectively. The higher frequency of outgrowth of EBV+ B-LCL occurred after culture of HIV/HCV PBMC has been previously demonstrated (Ruibal-Ares et al., 2001b). In PBMC cultures from non-immunosuppressed individuals, EBV+ B-LCL outgrowth is controlled by the immune response. CD4+ T-cell effectors are known to inhibit EBV-induced B-cell proliferation recognizing early expressed lytic and latent EBV antigens and CD8+ T-cell effectors specific for latent antigens are able to kill proliferating EBV+ B-lymphoblasts (Nikiforow et al., 2001).

Since CD4 lymphocytes affect the magnitude and breadth of the CD8 cytotoxic-response against HCV (Kim et al., 2005), it can be postulated that in the HIV/HCV group, the
observed low CD4+ T-cell counts (a consequence of HCV infection) could be related to a deficient control of HCV replication in culture. Regarding the occurrence of HCV producer B cell lines in HIV/HCV co-infected patients, longer HCV RNA persistence in cultures in addition to failure of the anti-EBV and HCV immune response can be proposed, favouring the escape of continuously replicating EBV+ or EBV+/HCV+ clones of B-lymphoblasts. The experiments that demonstrate that the HCV-VL increases in culture B-LCL SNs, the detection of HCV proteins by immunofluorescence techniques and successful HCV infection assays confirm in vitro replication in these cells.

In summary, although HCV replication after PBMC culture was observed in both groups of patients analysed (HCV singly infected group and HIV/HCV co-infected group), HIV might be involved in HCV RNA persistence in our culture system. Long-term persistence of HCV in PBMC cultures allowed the outgrowth of HCV RNA+ B-LCL containing HCV genome apparently capable of infecting primary PBMC cultures. These results highlight the role of PBMC, mainly that of B lymphocytes, in the maintenance of persistent HCV infection in extra-hepatic sites, even after negativization of HCV plasma VL. The role of HIV co-infection in maintaining the PBMC/B-lymphocyte reservoir is also emphasized, suggesting that reducing continuous HIV replication could benefit the ability of HIV/HCV co-infected patients to control the persistence of extra-hepatic HCV reservoirs.

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