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

Patricia Baré,[1] Ivana Massud,[1] Cecilia Parodi,[1] Liliana Belmonte,[1] Gabriel García,[2] Marcelo Campos Nebel,[1] Marcelo Corti,[3] Miguel Tezanos Pinto,[1,3] Raúl Pérez Bianco,[1,3] María M. Bracco,[1] Rodolfo Campos[2] and Beatriz Ruibal Ares[1]

Correspondence
Patricia Baré
pbare@hematologia.anm.edu.ar

[1]Instituto de Investigaciones Hematológicas "Mariano R Castex", Academia Nacional de Medicina, Pacheco de Melo 3081, Buenos Aires 1425, Argentina

[2]Cátedra de Virología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

[3]Fundación Argentina de la Haemofilia, Soler 3485, Buenos Aires 1425, Argentina

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-Iñigo *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-supressed 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 Haemofilia' were studied; 21 were HCV singly infected individuals (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 HCV 2·0 or 3·0 (both Ortho Diagnostic Systems). 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 I/II, 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^{2+}- and Ca^{2+}-free PBS and resuspended to 1×10^6 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, 2×10^6 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 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 FACScan cytometer 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, anti-CD16 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

NA, Not applicable.

	HCV^{+} group			HIV/HCV^{+} group		
	Mean ± SD	Median	Range	Mean ± SD	Median	Range
CD4^{+} (cells per mm[3])	953 ± 484·3	752	459–2024	401 ± 239	337	41–973
CD8^{+} (cells per mm[3])	653 ± 264·8	650	282–1183	954 ± 454	863	454–2928
Age (years)	29 ± 12	26	15–52	33 ± 11	30	17–64
HIV-VL (copies ml^{−1})	NA	NA	NA	3·09 ± 1·26	2·6	1·7–5·87
HCV-VL (IU ml^{−1})	5·18 ± 1·03	5·61	2·8–5·93	5·39 ± 1·10	5·93	2·78–5·93

(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. B-LCL were characterized by flow cytometry using mAbs for B cell antigens (anti-CD19, CD20, CD22 and CD23), anti-CD40, anti-CD30, anti-HLA-DR (markers of cell activation); anti-CD44, anti-CD54, anti-CD83, anti-CD86 and anti-CD38. Spontaneous release of HCV or HIV to the SN was surveyed as described below.

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-GGTCTACGAGACCT-3′; ES, 5′-CTGTGAGGAACTACTGTCTT-3′ and internal primers: IA, 5′-CACTCTCGAGCACCCTATCAGG-CAGT-3′; IS, 5′-TTCACGCAGAAAGCGTCTAG-3′) with 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 MgCl$_2$, each 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 monitor version 2.0 (Roche Diagnostics) (range of detection: 600–850 000 IU ml^{-1}). All HIV-VL in plasma samples were performed with Amplicor HIV-1 monitor version 1.5 (Roche Diagnostics) (range of detection: 400–750 000 copies ml^{-1} or 50–50 000 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; Biomérieux). 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 culture SNs was assayed by nested PCR as described above.

Immunofluorescence technique. Cells at 1×10^5 per slide were collected by cytospin centrifugation (5 min, 250 g). The cells were

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 *Rsa*I and *Hae*III, *Hin*fI and *Mva*I, *Scr*FI or *Bst*UI (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.

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

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

Sample group	*n*	HCV$^+$ cultures	B-LCL	HCV$^+$ B-LCL
Control group (normal donors)	52	0	2/52 (3·8 %)	0
HCV group	21	12/21 (57 %)	8/21 (38 %)	0
HIV/HCV group	31	23/31 (74 %)	19/31 (61 %)	6/19 (31·6 %)

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 HIV/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.
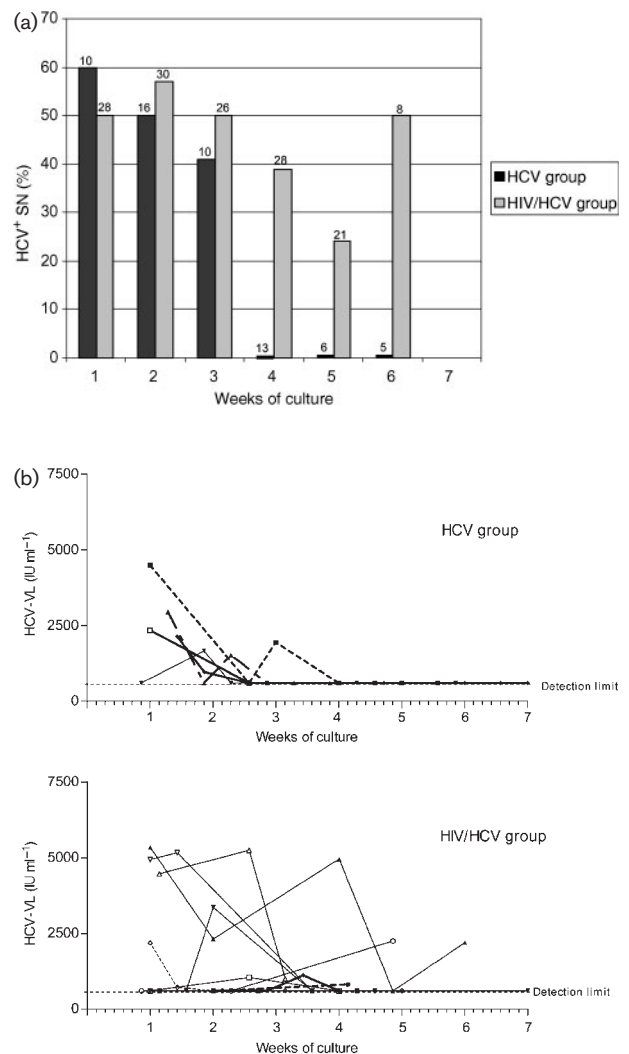

Fig. 1. Recovery of HCV RNA from PBMC culture SNs before B-LCL establishment. (a) Percentage of HCV$^+$ results after different weeks of culture. The total number of patients analysed per week is stated above the bars. (b) Sequential HCV-VL in different patients of HCV group ($n = 5$) and HIV/HCV group ($n = 10$) 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
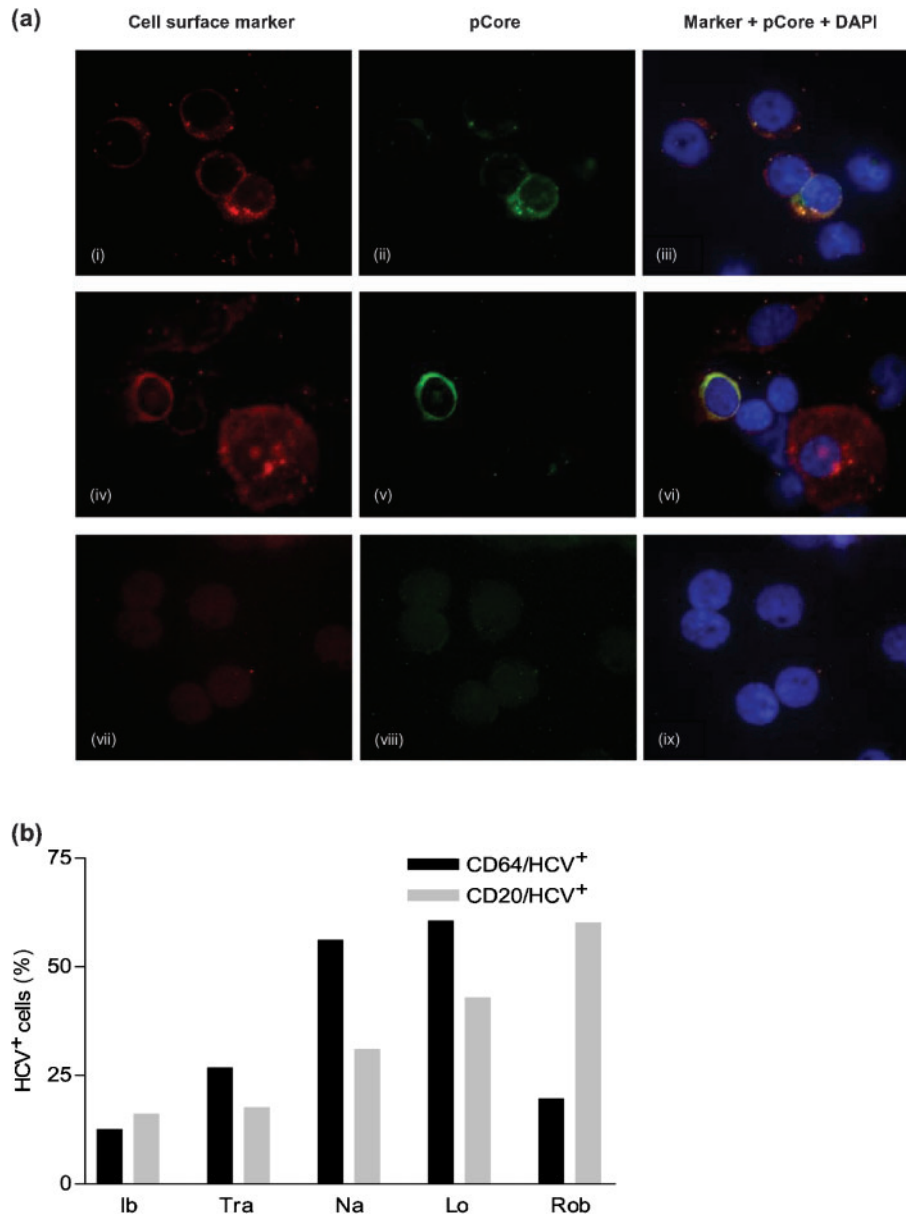
Fig. 2. Immunofluorescence staining during culture. (a) PBMC CD20$^+$ (i, iii) and PBMC CD64$^+$ (iv, vi) showing immunofluorescence pattern of HCV core protein deposits (ii, iii and v, vi) from patient Lo after 1 week of culture. Staining procedures as described in Methods. (vii–ix) Isotype controls. 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.

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).

n	HIV-VL log (copies ml^{-1})			CD4^{+} T-cell counts (cells per mm^3)			HCV^{+} cultures
	Mean ± SD	Median	Range	Mean ± SD	Median	Range	
12	Non-detectable			571 ± 227	540	302–973	6/12 (50 %)
19	3·78 ± 1·09	3·54	2–5·87	288 ± 182	286	41–670	17/19 (89·5 %)

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 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).
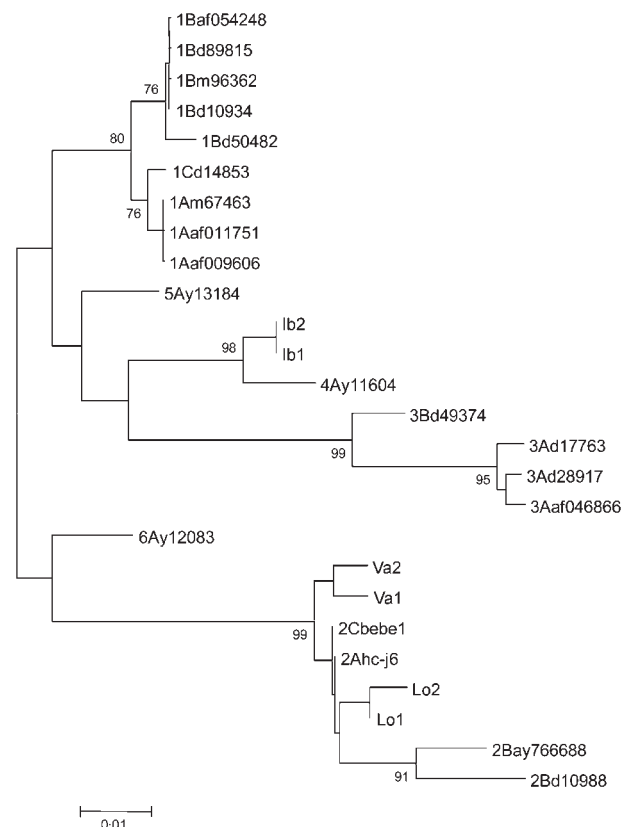
Fig. 3. HCV sequence analysis. The virus recovered from the cultures was analysed on samples collected during the first and/or second week of culture. Phylogenetic tree according to the neighbour-joining method derived from nucleotide sequences of serum (1) and SN (2) of patients Lo, Ib and Va and of 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 mm^3. 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 2×10^5 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 1×10^7 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 HCV 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 (Caussin-Schwemling *et al.*, 2001; Boisvert *et al.*, 2001; Sung *et al.*, 2003).
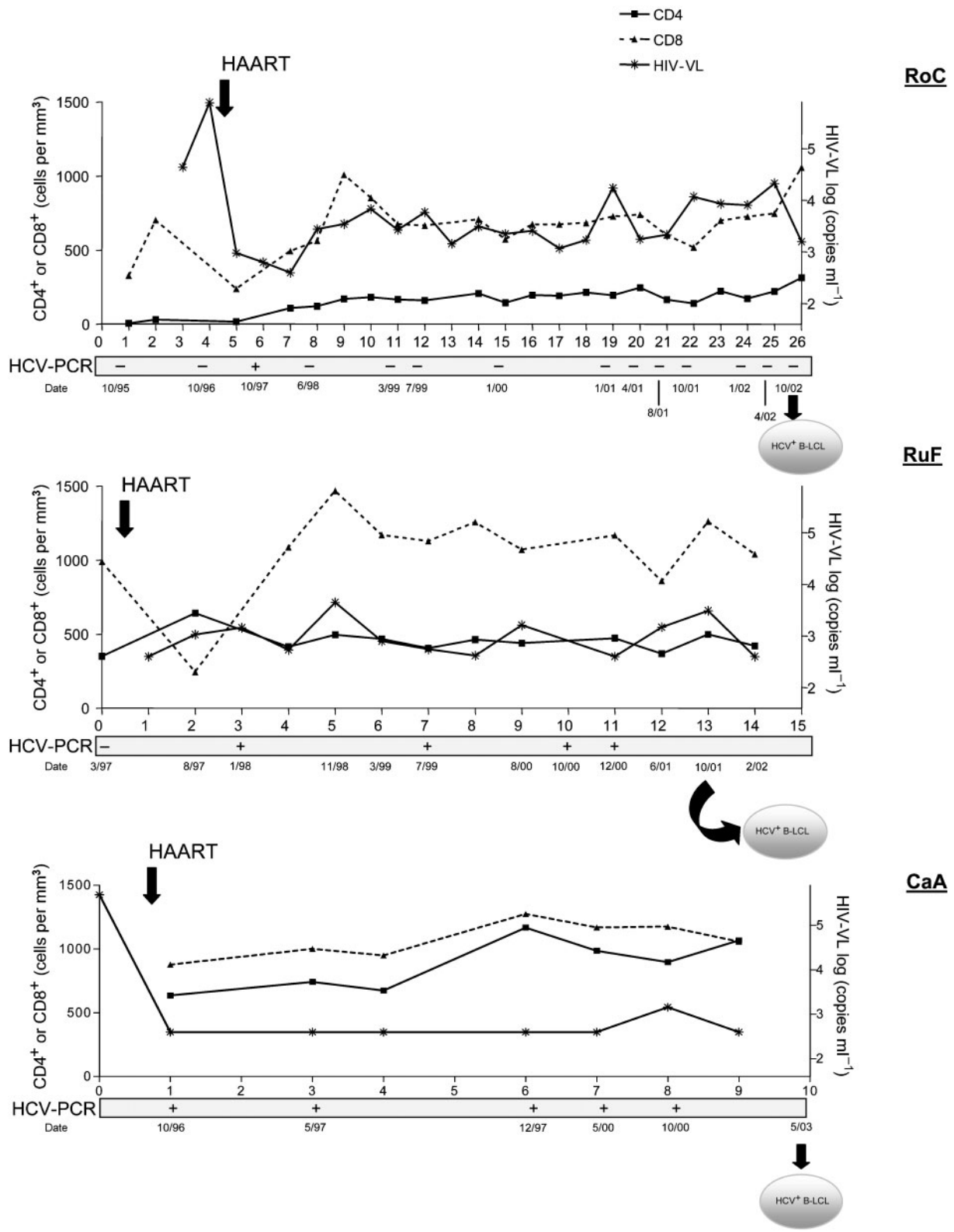
Fig. 4. HCV^{+} B-LCL were obtained from three HIV/HCV patients on HAART for 6–7 years. CD4^{+} and CD8^{+} T-cell counts (cells per mm³), HIV-VL [log (copies ml⁻¹)] and HCV genome detection (+ or −) are shown for each date of analysis.

Fig. 5. HCV detection in spontaneous B-LCLs SNs from HIV/HCV patients and from HCV-infected N-PBMC. (a) HCV genome production curve (1–11 days) in one of the B-LCLs (CaA) without culture medium replacement. The level of HCV production from an initial amount of 2×10^5 cells ml^{-1} suspended in 9 ml RPMI reached 4612 IU ml^{-1}. (b) Immunofluorescence pattern of HCV core protein deposits in B-LCL cells from patient RoC. Staining procedures as described in Methods. Original magnification $\times 1000$. (c) HCV genome detection in N-PBMC ($n = 2$) infected with HCV$^+$ B-LCL SN from RoC (performed by duplicate). PCR assays were carried out during culture in SN samples obtained from day 5 until day 40 of culture.

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-LCLs 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 HIV 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.

ACKNOWLEDGEMENTS

REFERENCES

Baré, P., Massud, I., Belmonte, L., Corti, M., Villafane, M., Perez Bianco, R., de Tezanos-pinto, M., de Bracco, M. M. & Ruibal Ares, B. (2003). HCV recovery from peripheral blood mononuclear cell culture supernatants derived from HCV-HIV co-infected hemophilic patients with undetectable HCV viraemia. *Haemophilia* 9, 598–604.

Beld, M., Penning, M., Lukashov, V., McMorrow, M., Roos, M., Pakker, N., van den Hoek, A. & Goudsmit, J. (1998). Evidence that both HIV and HIV-induced immunodeficiency enhance HCV replication among HCV seroconverters. *Virology* 244, 504–512.

Belmonte, L., Baré, P., Picchio, G. R., Pérez Bianco, R., de Tezanos Pinto, M., Corti, M., Villafañe, M. F., de Bracco, M. M. & Ruibal Ares, B. H. (2002). Decreased recovery of replication-competent HIV-1 from peripheral blood mononuclear cell-derived monocyte/macrophages of HIV-positive patients after 3 years on highly active antiretroviral therapy. *AIDS* 16, 1289–1292.

Boisvert, J., He, X. S., Cheung, R., Keeffe, E. B., Wright, T. & Greenberg, H. B. (2001). Quantitative analysis of hepatitis C virus in peripheral blood and liver: replication detected only in liver. *J Infect Dis* 184, 827–835.

Bonacini, M., Govindarajan, S., Blatt, L. M., Schmid, P., Conrad, A. & Lindsay, K. L. (1999). Patients co-infected with human immunodeficiency virus and hepatitis C virus demonstrate higher levels of hepatic HCV RNA. *J Viral Hepat* 6, 203–208.

Caussin-Schwemling, C., Schmitt, C. & Stoll-Keller, F. (2001). Study of infection of human blood derived monocyte/macrophages with hepatitis C virus in vitro. *J Med Virol* 65, 14–22.

Daar, E. S., Lynn, H., Donfield, S. & 7 other authors (2001). Relation between HIV-1 and hepatitis C viral load in patients with hemophilia. *J Acquir Immune Defic Syndr* 26, 466–472.

Davidson, F., Simmonds, P., Ferguson, J. C. & 19 other authors (1995). Survey of major genotypes and subtypes of hepatitis C virus using RFLP of sequences amplified from the 5′ non-coding region. *J Gen Virol* 76, 1197–1204.

Eyster, M. E., Fried, M., Di Bisceglie, A. M. & Goedert, J. J. (1994). Increasing hepatitis C virus RNA levels in hemophiliacs: relationship to human immunodeficiency virus infection and liver disease. *Blood* 84, 1020–1023.

Felsenstein, J. (1993). PHYLIP – Phylogenetic Interference Package Version 3.5. Department of Genetics, University of Washington, Seattle USA.

Giannini, C., Caini, P., Giannelli, F., Fontana, F., Kremsdorf, D., Brechot, C. & Zignego, A. L. (2002). Hepatitis C virus core protein expression in human B-cell lines does not significantly modify main proliferative and apoptosis pathways. *J Gen Virol* 83, 1665–1671.

Hu, Y., Shahidi, A., Park, S., Guilfoyle, D. & Hirshfield, I. (2003). Detection of extrahepatic hepatitis C virus replication by a novel, highly sensitive, single-tube nested polymerase chain reaction. *Am J Clin Pathol* 119, 95–100.

Kim, A. Y., Lauer, G. M., Ouchi, K. & 12 other authors (2005). The magnitude and breadth of hepatitis C virus-specific CD8+ T cells depend on absolute CD4+ T cell count in HIV-1 coinfected individuals. *Blood* 105, 1170–1178.

Laskus, T., Radkowski, M., Piasek, A., Nowicki, M., Horban, A., Cianciara, J. & Rakela, J. (2000). Hepatitis C virus in lymphoid cells of patients coinfected with human immunodeficiency virus type 1: evidence of active replication in monocytes/macrophages and lymphocytes. *J Infect Dis* 181, 442–448.

Laskus, T., Radkowski, M., Jablonska, J., Kibler, K., Wilkinson, J., Adair, D. & Rakela, J. (2004). Human immunodeficiency virus facilitates infection/replication of hepatitis C virus in native human macrophages. *Blood* 103, 3854–3859.

Lérat, H., Rumin, S., Habersetzer, F., Berby, F., Trabaud, M. A., Trépo, C. & Inchauspe, G. (1998). In vivo tropism of hepatitis C virus genomic sequences in hematopoietic cells: influence of viral load, viral genotype, and cell phenotype. *Blood* 91, 3841–3849.

Moldvay, J., Deny, P., Pol, S., Bréchot, C. & Lamas, E. (1994). Detection of hepatitis C RNA in peripheral blood mononuclear cells of infected patients by in situ hybridization. *Blood* 83, 269–273.

Nikiforow, S., Bottomly, K. & Miller, G. (2001). CD4+ T-cell effectors inhibit Epstein-Barr virus-induced B-cell proliferation. *J Virol* 75, 3740–3752.

Pham, T. N., MacParland, S. A., Mulrooney, P. M., Cooksley, H., Naoumov, N. V. & Michalak, T. I. (2004). Hepatitis C virus persistence after spontaneous or treatment-induced resolution of hepatitis C. *J Virol* 78, 5867–5874.

Radkowski, M., Gallegos-Orozco, J. F., Jablonska, J. & 7 other authors (2005). Persistence of hepatitis C virus in patients successfully treated for chronic hepatitis C. *Hepatology* 41, 106–114.

Rodríguez-Iñigo, E., Casqueiro, M., Navas, S., Bartolomé, J., Pardo, M. & Carreno, V. (2000). Fluorescent ''in situ'' hybridization of

hepatitis C virus RNA in peripheral blood mononuclear cells from patients with chronic hepatitis C. *J Med Virol* **60**, 269–274.

Ruibal-Ares, B., Riera, N. E. & Bracco, M. M. E. (1997). Macrophages, multinucleated giant cells, and apoptosis in HIV$^+$ patients and normal blood donors. *Clin Immunol Immunopathol* **82**, 102–116.

Ruibal-Ares, B., Belmonte, L., Baré, P., Bayo-Hanza, C., Mendez, G., Bianco, R. P., de Tezanos Pinto, M. & de Bracco, M. M. E. (2001a). Monocyte differentiation and HIV replication after prolonged culture of peripheral blood mononuclear cells from HIV-infected individuals. *Cell Immunol* **210**, 11–20.

Ruibal-Ares, B., Belmonte, L., Baré, P., Scolnik, M., Palacios, M. F., Bayo-Hanza, C., Galmarini, C. M., Mendez, G. & de Bracco, M. M. (2001b). Immortalized Epstein-Barr virus-positive B-cell lines obtained by prolonged culture of peripheral blood mononuclear cells from human immunodeficiency virus type-1 positive patients. *J Hum Virol* **4**, 200–213.

Sung, V. M., Shimodaira, S., Doughty, A. L., Picchio, G. R., Can, H., Yen, T. S., Lindsay, K. L., Levine, A. M. & Lai, M. M. (2003). Establishment of B-cell lymphoma cell lines persistently infected with hepatitis C virus in vivo and in vitro: the apoptotic effects of virus infection. *J Virol* **77**, 2134–2146.

Thomas, D. L., Astemborski, J., Vlahov, D. & 7 other authors (2000). Determinants of the quantity of hepatitis C virus RNA. *J Infect Dis* **181**, 844–851.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Torres, B., Martin, J. L., Caballero, A., Villalobos, M. & Olea, N. (2000). HCV in serum, peripheral blood mononuclear cells and lymphocyte subpopulations in C-hepatitis patients. *Hepatol Res* **18**, 141–151.