In vitro infection of peripheral blood mononuclear cells by hepatitis C virus

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To study the in vitro susceptibility of peripheral blood mononuclear cells (PBMC) to hepatitis C virus (HCV), we incubated cells from healthy donors with HCV-positive sera. Using RT-PCR and in situ hybridization, the genomic viral RNA was detected in PBMC and in their supernatants until 25 days post-incubation. The PBMC of the different donors were not all permissive to HCV, but results were more constantly positive when cells from four donors were pooled. Quantification of the genomic viral RNA by the branched-DNA assay showed a decrease in the HCV RNA concentration during the first week of culture followed by a peak during the second or third week, and also an increase in the total amount of viral RNA in the inoculated cells. Although HCV RNA could be detected in the supernatants by RT-PCR, the concentration was very low. Using a sense-specific RT-PCR method, the HCV negative-strand was also detected in the cells but not in the supernatants. In two experiments PBMC were successfully infected using HCV-positive culture supernatants, therefore suggesting that infectious particles can be produced in this system. Our findings demonstrate that PBMC are permissive for HCV replication in vitro but the replication level is very low. The HCV RNA concentration measured in PBMC of 10 chronically infected patients was not significantly higher than the maximal concentration obtained in PBMC infected in vitro.

Introduction

Hepatitis C virus (HCV) is the major cause of post-transfusional non-A and non-B hepatitis (Alter et al., 1989; Kuo et al., 1989). It has a positive-strand RNA genome of about 10 kb in length (Choo et al., 1991), which has significant sequence homology with pestiviruses and flaviviruses (Miller & Purcell, 1990). The viral particles are spherical with spike-like projections, and have morphological features similar to those of flaviviruses (Kaito et al., 1994). HCV is now grouped in the family Flaviviridae. Peripheral blood mononuclear cells (PBMC) of patients infected with HCV have been shown to be targets for the virus (Bouffard et al., 1992; Zignego et al., 1992; Ferri et al., 1993). Recently, the negative-strand of HCV, which is the putative replicative intermediate, has been detected in PBMC isolated from HCV-infected patients by both RT–PCR (Wang et al., 1992; Willems et al., 1994; Henin et al., 1994) and in situ hybridization (Moldvay et al., 1994). PBMC are therefore suspected to be a possible site of extra-hepatic replication of HCV. The pathogenesis of HCV infection is not well understood because there have been only a few attempts to study HCV replication in vitro. Evidence of in vitro replication has been shown in three human T cell lines: MOLT-4 cells (Shimizu et al., 1992), HPB-Ma cells (Shimizu et al., 1993) and H9 cells (Nissen et al., 1994). In two other studies, replication of HCV was observed in fetal hepatocytes (Iovacci et al., 1993) and in a human bone-marrow-derived B cell line (Bertolini et al., 1993). Nevertheless, there is no satisfactory model of in vitro HCV replication. Using an HCV-positive serum incubated with PBMC from a healthy donor, Muller et al. (1993) demonstrated the incorporation of [3H]uridine into HCV RNA. In order to evaluate HCV replication in PBMC in vitro, we incubated cells from healthy donors with HCV-positive sera. The presence of both positive- and negative-strands of HCV RNA was detected by RT–PCR, and by in situ hybridization. In order to compare our results with those observed in vivo, HCV RNA was quantified by the use of the branched-DNA assay in PBMC infected in vitro and in PBMC obtained from chronically infected patients.

Methods

HCV-positive sera and PBMC. Two patients from the Hôpitaux Universitaires of Strasbourg who were HCV-positive on ELISA (3rd generation Abbott) and who had circulating HCV RNA in their serum
as demonstrated by RT-PCR gave their consent for a serum sample to be taken for use in virus cultivation in vitro. These different sera served as HCV inocula, and were named K and P. PBMC were obtained from 10 patients chronically infected by HCV and with a quantifiable HCV virema.

**Cell preparation.** Heparinized blood was obtained from volunteer healthy donors who were not infected with human immunodeficiency virus, hepatitis B virus or HCV. Cells from 10 different donors (D1 to D10) were first used. PBMC were isolated using a diatrizoate-Ficoll (Eurobio) density gradient, and washed three times in PBS before being resuspended in RPMI medium containing 10 % fetal calf serum (FCS) (Gibco BRL). The cells were then stimulated for 48 h with phytohemagglutinin A (PHA) (Sigma) at a concentration of 5 µg/ml. Cells from 20 other healthy donors were isolated and PHA-stimulated in the same way before being stored separately in liquid nitrogen in RPMI medium containing 20% FCS and 10% DMSO.

**Cell culture and incubation of cells with HCV-positive sera.** After 48 h incubation with PHA the fresh PBMC from donors D1 to D10 were centrifuged and adjusted to 10^7 cells/ml in fresh RPMI medium containing 10% FCS and 10% IL-2 (Boehringer). The cells were aliquoted into 24-well plates, each well containing 1 x 10^5 cells in 200 µl of RPMI medium, and incubated with 10 µl of HCV-positive serum for 4 h at 37 °C under gentle agitation. Cells from the same donor were also incubated with an HCV-negative serum and used as a control for cell growth and as a negative control for PCR. After incubation, all cells were removed from the plate and washed five times with PBS and then maintained in culture flasks (Falcon) with RPMI medium containing 10% FCS and 10% IL-2 at 2 x 10^6 cells/ml. The culture medium was changed completely every 1 or 2 days for the first week of culture and twice weekly for the next 2 to 3 weeks. After centrifugation, the cells were counted, cell and supernatant samples were stored at - 20 °C, and the remaining cells were resuspended in fresh RPMI medium containing 10% FCS and 10% IL-2 at 2 x 10^6 cells/ml.

**PBMC pools.** The PBMC obtained from the other donors were thawed at 37 °C, washed twice in PBS, and cells from five different donors were mixed together to give four different pools of PBMC. The pools of cells were adjusted to 10^7 cells/ml in RPMI medium containing 10% FCS and 10% IL-2 and were incubated with sera and cultured in the same way as described for fresh cells.

**Isolation of PBMC from HCV-positive patients.** Total PBMC were also isolated from 10 volunteer HCV-infected patients in the same way as PBMC from healthy donors. In order to eliminate contamination of the cells by HCV RNA present in plasma, the cells were pelleted, resuspended in 1 ml of PBS and treated with trypsin (final concentration 0.05%) and EDTA (final concentration 0.02%) (Gibco BRL) before being incubated for 20 min at 37 °C, as described by Willems et al. (1994). One hundred microlitres of RNase A (5 mg/ml) (Boehringer) was then added and the cell suspension was incubated at 37 °C for 15 min. Trypsin activity was then blocked by addition of FCS and the cells were washed twice in PBS. The cell pellets were stored at - 80 °C.

**RNA purification.** Total RNA was extracted from 100 µl of culture supernatant or from 10^7 cells resuspended in 100 µl of DEPC-treated water in a single-step method as described by Chomczinsky et al. (1987).

**Reverse transcription and nested PCR.** The synthesis of cDNA and the two PCR rounds were performed using oligonucleotide primers from the highly conserved 5' untranslated region of the genome: P1 (sense, -GCGACATCCACCATAAGG-; nucleotides 10-28) and P4 (antisense, -ACTGCAGACATCAAATACT-; nucleotides 303-285) for the first PCR round and P2 (sense, -CTGTGAGCAACTACGTCT-; nucleotides 36-55) and P3 (antisense, -CGTGTACTCAACGG-TTCC-; nucleotides 161-143) for the second PCR round. Ten µl of the RNA solution was denatured at 70 °C for 10 min and incubated at 37 °C for 1 h with 200 U of murine Moloney leukaemia virus reverse transcriptase and 50 pmol of the outer antisense oligonucleotide primer (P4). Synthesis of cDNA was stopped by heating the samples at 95 °C for 10 min. Amplification of the cDNA was performed by using 15 µl of the cDNA solution and 50 pmol of one of the outer primers (P1). Thirty cycles of DNA amplification were carried out, followed by an extension step for 10 min at 72 °C. Each cycle of PCR consisted of 95 °C for 60 s, 50 °C for 60 s and 72 °C for 120 s. The second PCR was carried out in the same way with 10 µl of the first PCR mixture and 50 pmol of each inner primer (P2 and P3). The amplified DNA was visualized by 2% agarose gel electrophoresis and ethidium bromide staining. The size of the second product generated by the PCR was 126 bp.

**Detection of the HCV negative-strand.** Because the detection of the negative-strand by conventional RT–PCR can be due to self-priming or to non-specific priming (MacGuiness et al., 1994; Willems et al., 1994; Lanford et al., 1994) we used a tagged RT–PCR method, adapted from the work by Lanford et al. (1994). The oligonucleotide primer for negative-strand for cDNA synthesis contained a tag sequence (in bold) unrelated to HCV at the 5' end and the outer sense primer P1 (5' TCATGTTGCGGAATAGCCGACTCCACCATAGAT 3'). The cDNA synthesis reaction was performed using 50 pmol of this tag-P1 primer and was performed as previously described. In order to eliminate the RT activity, the cDNA was heated for 1 h at 95 °C. Subsequently, the samples were treated with RNase A at a concentration of 50 µg/ml for 30 min at 37 °C, in order to digest all remaining RNA molecules and to avoid a possible reverse transcription and non-specific amplification of HCV RNA positive-strands during the PCR procedure. As a negative control, we also performed cDNA synthesis without primer using RNA extracted from sera containing high quantities of HCV. The cDNA obtained by this method was then amplified using 50 pmol of P4 as outer antisense primer and 50 pmol of the tag sequence alone (5' TCATGTTGCGGAATAGCCGACTCCACCATAGAT 3') as a sense primer. One-tenth of the first PCR product was amplified in a second PCR round, using primer P3 as inner antisense primer and the tag sequence as sense primer. The two PCR rounds were performed in the same way as described above. One-quarter of the second PCR product was analysed by agarose gel electrophoresis, followed by Southern transfer to nylon Hybond-N+ (Amersham) and hybridization with a digoxigenin-labelled probe internal to the PCR primers (5' GCAGAAAGCGTCTAACGATCCGTTTAGAT 3', nucleotides 59-98). The probe was labelled using a DIG oligonucleotide tailing kit (Boehringer) according to the manufacturer's instructions. The nylon membranes were prehybridized at 68 °C for 1 h in a buffer containing 0.2% SDS, 5 x SSC, 0.1% N-laurylsarcosine and 1% blocking reagent (Boehringer), and were then hybridized with the HCV-specific probe overnight at 68 °C, using the same buffer. The membranes were then washed twice at room temperature in 2 x SSC, 0.1% SDS (w/v) and twice at 68 °C with 0.1% SSC, 0.1% SDS (w/v). Detection of chemiluminescence was then done with the DIG luminescence detection kit (Boehringer) using CSPD as substrate for alkaline phosphatase. The membranes were exposed to Hyperfilm (Amersham) for 2 h at room temperature.

**Determination of HCV genotype**

Genotyping was done according to Okamoto et al. (1992) using specific primers for each genotype (I, II, III and IV), except that we performed a separate PCR with each type-specific primer, instead of mixing them all together.

**In situ hybridization.** (i) Preparation of slides. Cells (1 x 10^5–3 x 10^5) were attached to slides by cytocentrifugation. After air-drying the cells were fixed with 4% paraformaldehyde pH 7.3 (PFA) for 15 min,
washed in PBS and fixed again in ethanol-acetic acid (3:1, v/v) for 10 min. The slides were stored at −20°C.

(ii) Preparation of probes. Primers 1 and 4 were used as HCV negative-strand probe and HCV positive-strand probe respectively. A 19-mer feline immunodeficiency virus (FIV) oligonucleotide probe (5'-GCACACATCCCCCTGTAGC-3') from the gag region of the FIV genome was also used as a negative control. The probes were 3'-end labelled with 35S (35S)dATP, specific activity 1000 Ci/mmol; ICN) using a Boehringer DNA Y-end labelling kit according to the manufacturer's recommendations. Unincorporated nucleotides were removed by column filtration on a NACS PREPAC Mini-Column (Gibco BRL). After precipitation in ethanol and centrifugation, the probe was dissolved in distilled water.

(iii) In situ hybridization. The slides were rehydrated through an ethanol series (100%–50%) and rinsed in PBS for 5 min. and then the cells were digested with 1 μg/ml proteinase K (Boehringer) for 30 min at 37°C. The slides were rinsed twice in PBS containing 0.2% glycine (10 min each), refixed in 4% PFA for 5 min at room temperature and then rinsed three times for 10 min in 2 × SSC buffer; 10 mM DTT was then added to the slides. Dehydration in an ethanol series (50%–100%) was followed by acetylation in a 0.1 M solution of triethanolamine in 0.25% acetic anhydride for 10 min. The probe was denatured for 5 min at 95°C and added to hybridization solution (50% formamide, 10% dextran sulphate, 2 × SSC, 2 × Denhardt’s solution, 0.1% Triton X-100, 0.01 M-DTT, 200 ng/μl herring sperm DNA). The final concentration of the probe was 8500 c.p.m./μl. The slides were denatured for 2 min at 95°C, 8 μl of the probe was added and a coverslip applied. Hybridization was performed at 42°C overnight. The coverslips were removed and slides were rinsed three times in 1 × SSC buffer and washed twice in 50% formamide-1 × SSC for 15 min at 42°C. After two washes at 42°C in 1 × SSC and another wash in 0.1 × SSC (10 min each), the slides were dehydrated through an ethanol series and dried under vacuum. LM-1 emulsion (RPN 40, Amersham) was added and specimens were exposed for 21 days and then counterstained with methionine blue. Uninfected PBMC from the same donor were used in the final wash of the cells.

Quantification of HCV RNA. We used the branched-DNA assay (Quantiplex HCV-RNA, Chiron) in order to quantify the HCV RNA in the sera and in the cultured cells and their supernatants. The serum samples were analysed according to the manufacturer’s instruction book: a 50 μl serum or plasma sample was lysed, hybridized and captured and the signal was amplified in a single well of a 96-well plate. The presence of HCV RNA positive-strand was determined by RT-PCR both in cells and in supernatants in the 10 experiments. Table 1 shows the results of two different experiments which are representative of the results. In three experiments (D8 to D10), no HCV RNA could be detected after day 3 post-incubation (p.i.) in cells or supernatants, as shown in Table 1(b) for D10. In cells from the seven other donors (D1 to D7) we observed intermittently positive RT-PCR from day 0 to day 28 p.i., as shown in Table 1(a) for D7. In these seven experiments, positive RT-PCR were constantly noted both in cells and in supernatants between day 10 and day 15. In five experiments, HCV RNA could be detected during the third and fourth week p.i. We also observed that HCV RNA was present in the supernatants less often than in the cells. Immediately after inoculation and five washes, the RT-PCR were negative on the buffer used in the final wash of the cells.

(iii) In situ hybridization. In situ hybridization was done in six different experiments. Using the HCV positive-strand probe, positively labelled cells could be

Table 1. Detection of HCV RNA positive-strand by RT-PCR in PBMC and in their supernatants after incubation with serum K

<table>
<thead>
<tr>
<th>Days p.i.</th>
<th>0</th>
<th>3</th>
<th>5</th>
<th>8</th>
<th>12</th>
<th>15</th>
<th>19</th>
<th>22</th>
<th>26</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) RT-PCR in cells</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>RT-PCR in supernatants</td>
<td>−</td>
<td>ND</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>(b) RT-PCR in cells</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<td>RT-PCR in supernatants</td>
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</table>

* (a) Cells from donor 7; (b) Cells from donor 10. ND, Not determined.

Incubation of fresh PBMC from a single donor with HCV-positive serum K

(i) Detection of HCV positive-strand. In the first series of experiments, fresh PBMC of donors D1 to D10 were incubated with the same inoculum (K). Cell growth was not affected by incubation with the HCV-positive serum. The presence of HCV RNA positive-strand was determined by RT-PCR both in cells and in supernatants in the 10 experiments. Table 1 shows the results of two different experiments which are representative of the results. In three experiments (D8 to D10), no HCV RNA could be detected after day 3 post-incubation (p.i.) in cells or supernatants, as shown in Table 1(b) for D10. In cells from the seven other donors (D1 to D7) we observed intermittently positive RT-PCR from day 0 to day 28 p.i., as shown in Table 1(a) for D7. In these seven experiments, positive RT-PCR were constantly noted both in cells and in supernatants between day 10 and day 15. In five experiments, HCV RNA could be detected during the third and fourth week p.i. We also observed that HCV RNA was present in the supernatants less often than in the cells. Immediately after inoculation and five washes, the RT-PCR were negative on the buffer used in the final wash of the cells.

(ii) Detection of HCV RNA negative-strand. The presence of the HCV negative-strand was determined in cells of four of the seven donors (D2, D3, D6 and D7) using the tag RT-PCR method. We observed a positive result between day 10 and day 15 in the PBMC of four donors, but the HCV negative-strand was detected less frequently than the HCV positive-strand. In two assays, the negative-strand could be found at day 19 and day 20 p.i. Fig. 1 shows the positive results observed at days 5, 15, 19, 22 and 26 in cells from D7, after Southern transfer and hybridization with an HCV-specific probe. The HCV negative-strand could not be detected in the supernatants in these four experiments.

(iii) In situ hybridization. In situ hybridization was done in six different experiments. Using the HCV positive-strand probe, positively labelled cells could be

Results

Characterization of HCV inoculum

The HCV RNA levels in pure sera K and P measured by branched-DNA assay were 180 × 10^5 genome eq./ml and 310 × 10^6 genome eq./ml, respectively. Both sera contained HCV type I according to the classification of Okamoto et al. (1992), which is type 1a according to Simmonds et al. (1993).
Fig. 1. Detection of HCV RNA negative-strand in PBMC from donor D7 and in controls. The amplified product was analysed by agarose gel electrophoresis and Southern hybridization with an HCV-specific probe labelled with digoxigenin. The size of the amplified product is 186 bp. Lanes d0 to d30, detection of the negative-strand in the cells from day 0 to day 30 p.i.; tc, PBMC incubated with an HCV-negative serum; th, tag RT-PCR performed without RNA, as a reagent control; s1 and s2, tag RT-PCR performed on two HCV-positive sera; s1' and s2', tag RT-PCR performed on the same sera without oligonucleotide primer during the RT reaction.

Fig. 2. *In situ* hybridization on HCV-infected PBMC of D2 (a, d) and D6 (b, c). (a) With an HCV positive-strand probe, positively labelled cells were observed at day 4 p.i. (b) An HCV negative-strand probe labelled several days at day 10 p.i. (c) Using the HCV positive-strand probe, positively labelled cells could be observed at day 10 p.i. (d) An FIV probe used as a negative control gave no signal in PBMC of D2, 4 days p.i.

observed at various times p.i. from day 3 to day 15 in five of these six experiments (Fig. 2a, c). We also observed positively labelled cells with the HCV negative-strand probe in three of these experiments at day 3 and day 10 p.i. (Fig. 2b). As observed with RT-PCR, *in situ* hybridization with the negative-strand probe was positive.
In vitro infection of PBMC by HCV

Fig. 3. Change of HCV RNA concentration with time in four parallel experiments using the same pool of PBMC inoculated either with serum K (a, b) or with serum P (c, d). The HCV RNA concentration was measured in $5 \times 10^6$ cells by the branched DNA assay, after RNA extraction.

### Table 2. Quantification of HCV RNA in pooled PBMC at the time of maximal HCV concentration

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<th>PBMC pool (no.):</th>
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<tr>
<td>Inoculum:</td>
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<td>P</td>
<td>K</td>
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<tr>
<td>HCV RNA</td>
<td>6.5x10^4</td>
<td>6x10^4</td>
<td>5.7x10^4</td>
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<td>$5 \times 10^6$ cells</td>
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less often than with the positive-strand probe. Between one and five positively labelled cells were identified per $10^5$. Uninfected PBMC from the same donors showed no labelling with either the positive or negative-strand probe. The FIV probe used as a negative control in all slides showed no hybridization (Fig. 2d). Based on morphological criteria, positively labelled cells were either lymphocytes or monocytes.

**Incubation of pooled PBMC with HCV-positive sera and quantification of HCV RNA.**

In a second series of experiments we used pools of PBMC previously stored at $-170^\circ C$. Four different pools of PBMC (Nos 1, 2, 3 and 4) were incubated either with serum P or with serum K. In contrast to PBMC from a single donor, the detection of HCV RNA in the cells by RT-PCR was more constantly positive when pools of PBMC were incubated with HCV-positive sera. The HCV RNA was quantified in the cells at various times during the culture period after extraction of total RNA from $5 \times 10^6$ cells. The results of four of these experiments using pool 4 are detailed in Fig. 3. These data were obtained from experiments performed at the same time under the same conditions (inoculation, culture, RNA extraction, branched-DNA assay). Initially, there was a decrease in the concentration of HCV RNA during the first week p.i. and a peak at day 13 or day 20. A total of 12 experiments using four different pools of PBMC were done. In all experiments, we observed either an initial decrease in HCV RNA concentration or values below the cut-off during the first week of culture, followed by a peak of maximal concentration between day 10 and day 20 (Table 2). In some experiments, HCV RNA could also be quantified during the last week of the culture, between day 20 and day 26.

In the four experiments shown in Fig. 3, the initial HCV RNA concentration was measured in the cells after incubation with serum and five washes. The total amount of HCV RNA present after incubation and washes in experiments (a), (b), (c) and (d) represented 4%, 4.5%, 2.3% and 4% of the inoculum, respectively, and ranged between $21.6 \times 10^4$ and $37.2 \times 10^4$ genome eq. (see Methods for details of calculation). The total HCV RNA at the time of the peak was between $53.4 \times 10^4$ and $117.5 \times 10^4$ genome eq. in the same experiments, therefore showing production of viral genome during the culture period.

In experiments using pooled PBMC, the negative-strand HCV RNA could also be detected intermittently by tag RT-PCR. In two different experiments, positively labelled cells were also observed after *in situ* hybridization at various times during the culture period.
**HCV RNA concentration in cells from chronically infected patients.**

The HCV RNA concentration was determined in the serum and in $5 \times 10^6$ PBMC of 10 patients chronically infected by HCV. The highest concentration observed in the PBMC was $475 \times 10^4$ genome eq., although the viraemia in these patients was between $138 \times 10^4$ and $4200 \times 10^4$ genome eq./ml. When the viraemia was lower than $500 \times 10^4$ genome eq./ml, HCV RNA could not be quantified in the cells.

**Discussion**

There have been few reports of *in vitro* replication of HCV. In the present paper, we demonstrate infection of cultured PBMC using sera from HCV-infected patients, but the replication level obtained in this system was very low.

Due to the sensitivity of PCR, it is necessary to distinguish between the inoculum and newly replicated viral RNA, but our experiments provide evidence of HCV replication. (i) Positive RT–PCR were observed after day 8 in cells from seven out of ten separate donors, whereas in three donors the RT–PCR were negative after day 3. The presence of HCV RNA observed immediately after inoculation (day 0) and during the very first days of culture is therefore probably due to the inoculum. In contrast, the repeatedly positive RT–PCR for cells from seven separate donors and in pooled PBMC at days 10–15 are in favour of HCV replication; this hypothesis is reinforced by the frequent presence of HCV RNA in the cell supernatants at the same time. Moreover, when U937 cells and various other cell lines (KB, BHK and MRC5) were inoculated in the same way in separate experiments, RT–PCR for the cells were never positive after days 3–6 and were always negative for the supernatants (data not shown). Although RT–PCR is very sensitive, detection of HCV RNA in the cells after the second week of culture cannot be due to the persistence of inoculum in PBMC.

(ii) The HCV negative-strand was present intermittently in the cells as shown by RT–PCR and this finding was corroborated by the *in situ* hybridization results. In the experiment illustrated in Fig. 1, negative-strand RNA was present at various times during the culture period, therefore indicating newly replicated HCV RNA. In a similar experiment, the quantity of HCV RNA determined by the branched-DNA assay increased at day 15 (data not shown), which was when the negative-strand was detected. We often observed the presence of negative-strand RNA at two different times in the experiments, usually at the end of the first week and during the third week. Such intermittent detection of the negative-strand was also reported in an HPB Ma cell line inoculated *in vitro* with HCV (Shimizu et al. 1993).

(iii) In numerous experiments, quantification of HCV showed an initial decrease in HCV RNA concentration followed by an increase during the second or third week of culture. The HCV RNA concentration determined at the time of the peak was equal to or slightly greater than the concentration measured immediately after inoculation and washing. Since the cell multiplication rate between day 0 and the time of the HCV peak was 10 to 12 in the experiments detailed in Fig. 3, the HCV concentrations measured during the second and third week cannot be due to the inoculum. The increasing amount of total HCV RNA in the cultures from day 0 to day 13 or day 20 is evidence of HCV replication in these cells. Our findings are consistent with a productive *in vitro* infection of PBMC by HCV, and are in agreement with the results of Muller et al. (1993). However, these authors did not study replication of the virus after the first week of culture. The *in vitro* replication of HCV in PBMC correlates well with experiments which have shown that some T cell lines such as MOLT-4 (Shimizu et al., 1992), HPB Ma (Shimizu et al., 1993) and H9 (Nissen et al., 1994) are permissive to HCV.

HCV RNA was not often detectable in the culture supernatants. Nevertheless, in many experiments supernatants were RT–PCR negative during the first week, and became positive during the second and third weeks. These findings suggest that HCV RNA is released into the supernatants at the time of maximal replication. Using HCV-positive supernatants of PBMC inoculated *in vitro*, we inoculated PBMC from healthy donors. Seven different assays were performed, but positive RT–PCR in the cells and supernatants were obtained in only two of these experiments (data not shown). In attempts to effect a second passage we did not observe any significant HCV replication. This also suggests that the virus remains primarily cell-associated. However, this does not exclude the possibility that the system is capable of the complete replication cycle (Lanford et al., 1994).

Using fresh cells from a single donor we obtained variable results which probably reflect a variable susceptibility to HCV in the different donors. When cells were pooled, the results of RT–PCR were *more constant* positive, but the efficacy of replication did not improve. Although we observed an increase in the total amount of HCV during the culture period, our quantitative data clearly show that the replication rate was low in PBMC inoculated *in vitro*. Shimizu et al. (1992) showed that when MOLT-4 cells were infected by a murine retrovirus, the replication of HCV was more efficient, suggesting that cofactors could play an important role in HCV replication in PBMC. It is likely...
that in vitro systems using hepatocytes are more permissive to HCV. Yoo et al. (1995) established a human hepatoma cell line which is persistently infected with HCV, and Lanford et al. (1994) demonstrated a high level of replication in chimpanzee hepatocytes.

HCV replication was weak in our in vitro model, but the HCV RNA concentrations in PBMC of 10 HCV-positive patients were not significantly higher than those observed by us in vitro. The cellular HCV RNA concentration was low even in patients who had high HCV viraemias. These data suggest that PBMC play only a minor part in the production of HCV RNA present in the plasma. We did not determine which PBMC cell type is preferentially susceptible to HCV. Others have suggested that B lymphocytes are preferentially susceptible to HCV infection (Muller et al., 1993; Bertolini et al., 1994), but T lymphocytes (Zignego et al., 1992; Moldvay et al., 1994) and monocytes (Bouffard et al., 1992) may also be infected by HCV.

In conclusion, we have shown by different methods that PBMC can be productively infected in vitro by sera of patients chronically infected with HCV. Our results suggest that PBMC could be infected in vivo by circulating particles, without necessarily contacting infected hepatocytes. Nevertheless, both in vivo and in vitro studies show that the virus is present in only small amounts in PBMC.

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