

# Hepatitis C virus in human B lymphocytes transformed by Epstein-Barr virus *in vitro* by in situ reverse transcriptase-polymerase chain reaction

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**Subject headings** hepatitis C-like viruses; herpes virus 4, human; B-lymphocytes; cells cultured

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## Abstract

**AIM** To study persistence and replication of hepatitis C virus (HCV) in patients' peripheral blood mononuclear cells (PBMC) cultured *in vitro*.

**METHODS** Epstein-Barr virus (EBV) was used to transform the hepatitis C virus from a HCV positive patient to permanent lymphoblastoid cell lines (LCL). Positive and negative HCV RNA strands of the cultured cells and growth media were detected by reverse transcriptase-polymerase chain reaction (RT-PCR) each month. Core and NS5 proteins of HCV were further tested using immunohistochemical SP method and in situ RT-PCR.

**RESULTS** HCV RNA positive strands were consistently detected the cultured cells for one year. The negative-strand RNA in LCL cells and the positive-strand RNA in supernatants were observed intermittently. Immunohistochemical results mediated expression of HCV NS3 and C proteins in LCL cytoplasm mostly. The positive signal of PCR product was dark blue and mainly localized to the LCL cytoplasm. The RT-PCR signal was eliminated by overnight RNase digestion but not DNase digestion.

**CONCLUSION** HCV may exist and remain functional in a cultured cell line for a long period.

## INTRODUCTION

Hepatitis C virus has a positive-strand RNA genome of about 10kb in length, which is grouped in the family Flaviviridae<sup>[1,2]</sup>. The viral particles are spherical with spike-like projections, and have morphological features similar to those of the Flavivirus<sup>[3]</sup>. The liver is the main target for replication of HCV *in vivo*<sup>[4,5]</sup>, which occurs via minus-strand RNA as a replicative intermediate<sup>[7,8]</sup>. Recently, positive and negative strands have been detected in peripheral blood mononuclear cells (PBMC) isolated from HCV infected patients by both RT-PCR<sup>[6,7]</sup> and *in situ* hybridization<sup>[9]</sup>. PBMC are therefore suspected as a possible site of extra-hepatic replication of HCV. It has been recognized for almost 31 years that Epstein-Barr virus (EBV) is capable of transforming lymphocytes into immortal cell lines<sup>[10]</sup>. We detected HCV RNA of the cultured cells and growth media by reverse transcriptase-polymerase chain reaction (RT-PCR) each month. Antigens of HCV were further tested using the immunohistochemical and the streptavidin/peroxidase (SP) staining methods and *in situ* RT-PCR. Our results offer strong evidence for the persistence of HCV RNA in mononuclear blood cells.

## MATERIALS AND METHODS

### *Human peripheral blood mononuclear cells*

PBMCs were obtained from a female patient. PBMCs were whose serum tested positive for both anti-HCV antibodies and HCV RNA isolated from heparinized peripheral blood by using a diatrizoate-Ficoll (Eurobic) density gradient and washed three times in PBS before being resuspended in RPMI-1640 medium containing 20% fetal calf serum (FCS) (Gibco BRL).

### *Epstein-Barr virus preparation*

Viral stocks were prepared from the growth medium of a B95-8 cell line EBV-transformed marmoset lymphocyte cells. The viral stocks were centrifuged at 400 × g to sediment cells, frozen and thawed three times, and then passed through a 0.45 μm Millipore filter. They were determined to be free of mycoplasma and bacteria by culture.

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### Cell culture<sup>[11,12]</sup>

Before exposure to EBV, isolated PBMC populations were maintained for 13-24 hours at  $2 \times 10^6$  cells/mL in 96-well plates. This procedure removed many phagocytic cells. Medium was RPMI-1640 supplemented with 20% heat-inactivated (56°C, 30 min) fetal calf serum, penicillin (50U/mL), and streptomycin (50 µg/mL). The cells were cultured at 37°C in an atmosphere of 5% CO<sub>2</sub>. Phytohemagglutinin A-M (PHA-M) (Difco) was dissolved in RPMI-1640, sterilized by passage through a 0.45 µm Millipore filter, and added to the lymphocytes 24 hours before addition of EBV. Twenty-four hours after EBV transformation, the cultures were treated with Cyclosporin A (0.5 g/mL and 1.0 g/mL).

Stock of B lymphocytes transformed by EBV and its growth medium. After about one month, B lymphocytes transformed by EBV and subcultured were observed to have achieved immortalization. The culture medium was exchanged semi-quantity every week. The exchanged LCL was centrifuged at  $400 \times g$  for 5 minutes to sediment the cells. The cell pellets were placed in RPMI medium containing 20% FCS and 10% DMSO. The cells and supernatants were separately stored in liquid nitrogen. Before PCR, the fresh or stored sedimentary cells were washed ten times in DEPC-treated PBS. The last wash was collected and saved. The cells were diluted at  $5 \times 10^7$  cells/mL.

### RNA purification

Total RNA was extracted from 100 µL of culture supernatant or from  $5 \times 10^7$  cells resuspended in 100 µL of DEPC-treated water by a single-step method as described by Chomczynsky<sup>[13]</sup>.

### Reverse transcription and nested PCR<sup>[14-17]</sup>

The synthesis of cDNA and the two rounds of PCR were performed using oligonucleotide primers from the highly conserved, untranslated 5'-region of the genome: P1 (Sense strand: 5'-CTGTGAGGAACTACTGTCTT-3', nucleotides 28-47), P2 (Antisense strand: 5'-AACACTACTCGGCTAGCAGT-3', nucleotides 229-248) for the first PCR round and P3 (Sense strand: 5'-TTCACGCAGAAAGCGTCTAG-3', nucleotides 46-65), P4 (Antisense strand: 5'-GTTGATCCAAGAAAGGACCC-3', nucleotides 171-190) for the second PCR round. Detection of the HCV positive strand: ten L of the RNA solution was denatured at 70°C for 10 min and incubated in 42°C for 40 min with 1U AMV and 50pmol the outer antisense oligonucleotide primer (P2). Synthesis of cDNA was stopped by heating the samples at 95°C for 10 min.

Amplification of the DNA was performed using 10 µL cDNA solution and 50pmol 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 60s, 55°C for 90s and 72°C for 120s. The second PCR was carried out in the same way with 5 µL of the first PCR mixture and 50pmol of each inner primer (P3 and P4). The amplified DNA was detected by 6% polyacrylamide gel electrophoresis and ethidium bromide staining. The size of the second DNA fragment generated by the PCR was 145 bp. Detection of the HCV negative-strand: 10 µL RNA solution was denatured at 70°C for 10 min and incubated in 42°C for 40 min with 1U AMV and 50pmol of the outer sense oligonucleotide primer (P1). The following PCR was as same as the detection of the HCV positive-strand.

### Immunocytochemistry

Immunocytochemistry was performed according to streptavidin/peroxidase (SP) staining method<sup>[18-21]</sup> using antibodies against HCV Core and NS5. The anti-HCV Core monoclonal antibody was kindly supplied by L Haoying (Academy of Military Medical Sciences, Beijing, China). The antibody was produced by immunizing a mouse with synthetic peptide based on the sequence of the HCV genome. Monoclonal antibodies to HCV NS3 proteins were produced against a recombinant HCV NS5 protein and were kindly supplied by Zhan Meiyun (Institute of Virology, Chinese Academy of Preventive Medicine) and Gao Jianen (Institute of Hepatology, People's Hospital, Beijing Medical University). Histostain TM-SP kit was a ZYMED product (U.S.A.).

### In situ RT-PCR<sup>[22-30]</sup>

LCL cells were carefully washed in sterile saline, resuspended in ice-cold 10% buffered formaldehyde solution and kept for 2 hours at 4°C. For each set of experiments,  $2 \times 10^6$  cells were aliquoted in 0.5 mL Eppendorf tubes and treated with a 1% sterile saline solution of Triton-X100 (Sigma, St Louis, MO) at 4°C for 10min with gentle shaking. Permeabilized LCL cells were then resuspended in 20 µL RT buffer (50 mmol/L Tris-HCl, 75 mmol/L KCl, 3 mmol/L MgCl<sub>2</sub>) containing 10 mmol/L dithiothreitol, 200U cloned Moloney's murine leukemia virus RT (Gibco-BRL, Gaithersburg, MD), 40U RNase inhibitor (RNA guard; Pharmacia), 1 mmol/L each dGTP, dATP, dTTP, dCTP, and 50pmol of the outer antisense oligonucleotide primer (P2) of HCV. The tubes were incubated at 37°C for 1 hour, 95°C for 10 min to

inactivate residual RT activity, and immediately chilled on ice. After the RT step, LCL cells were washed again with ice-cold sterile saline, spun down, and resuspended in 25  $\mu$ L of PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-Cl, 2.5 mmol/L MgCl<sub>2</sub>) containing 1 mmol/L each of dGTP, dATP, dTTP, dCTP, and 25 pmol of both antisense (P2) and sense (P1) primers. After preheating the tubes for 7 min at 82°C, 1U Taq DNA polymerase (Promega) was added to each sample. Pre-melting at 95°C for 5 min was followed by 20 cycles of 1 min each at 94°C (DNA denaturation), 55°C (primer annealing), and 72°C (primer extension). After the first PCR, LCL cells were washed again with ice-cold sterile saline, spun down, and resuspended in 25  $\mu$ L PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-Cl, 2.5 mmol/L MgCl<sub>2</sub>) containing 1 mmol/L each dGTP, dATP, dTTP, dCTP, and 25 pmol of both antisense (P3, its N-end labelled biotin) and sense (P4) primers. The second PCR processes were the same as the first. At the end of suspension PCR, LCL cells were carefully washed 3 times again with ice-cold sterile saline and 1 time in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.4), with gentle agitation at 37°C. Cells (between  $2 \times 10^3$  and  $4 \times 10^3$ ) were spread on a glass slide coated with polytetrafluoroethylene. The slides were air dried and then washed in  $2 \times$  saline sodium citrate (SSC), buffer (0.3M sodium chloride and 0.03M sodium citrate). Amplification products were incubated with alkaline phosphatase-conjugated streptavidin (AP-SA) for one hour at 37°C. After incubation, slides were thoroughly washed with phosphate-buffered saline. The color was developed with nitroblue tetrazolium /5-bromo-4-chloro-1-3 indolyphosphate (NBT/BCIP) as the chromagen, which yields a dark blue color at the site of biotin binding avidin. The counterstain, nuclear fast red, stains nuclei pale pink and does not stain the cytoplasm.

## RESULTS

### Hepatitis C virus persistence in cell lines

We observed two distinct morphologies as early as 24 hours post-infection with EBV, indicating successful transformation of the virus into the B cells: ① blastogenesis became evident resulting in enlargement of the lymphocytes or ② there was increasing development of cell aggregates of proliferative lymphoblast cells (Figure 1). After one month, the EBV transformed lymphocytes exhibited huge, spherical cell bodies, a few projections on the body surface, and large, varying nuclei (Figure 2).

### HCV of LCL and medium detected by nested RT-PCR

In order to observe the persistence of HCV in LCL, the appearance of viral plus-strands and minus-strands of the cell line were assayed in cell lysates and cultured media and the tenth wash of LCL each month during culturing continuously. The plus-strand HCV RNA in cell was successively detected during 12 months in the subcultured cells. The plus-strand HCV RNA in supernatants and the minus-strand HCV RNA in cells were intermittently positive RT-PCR (Table 1, Figures 3-5).

**Table 1** Detection of HCV-RNA positive-strand and negative-strand by RT-PCR in EBVTB and their growth medium after incubation

	Incubation months											
	1	2	3	4	5	6	7	8	9	10	11	12
Cells (p/n)	+/+	+/-	+/-	+/+	+/-	+/-	+/+	+/-	+/-	+/-	+/-	+/+
Growth medium (p/n)	+/-	-/-	-/-	-/+	-/+	-/-	-/-	-/-	+/-	-/-	-/+	-/-

p = plus-strand, m = minus strand; +:PCR positive; -: PCR negative.

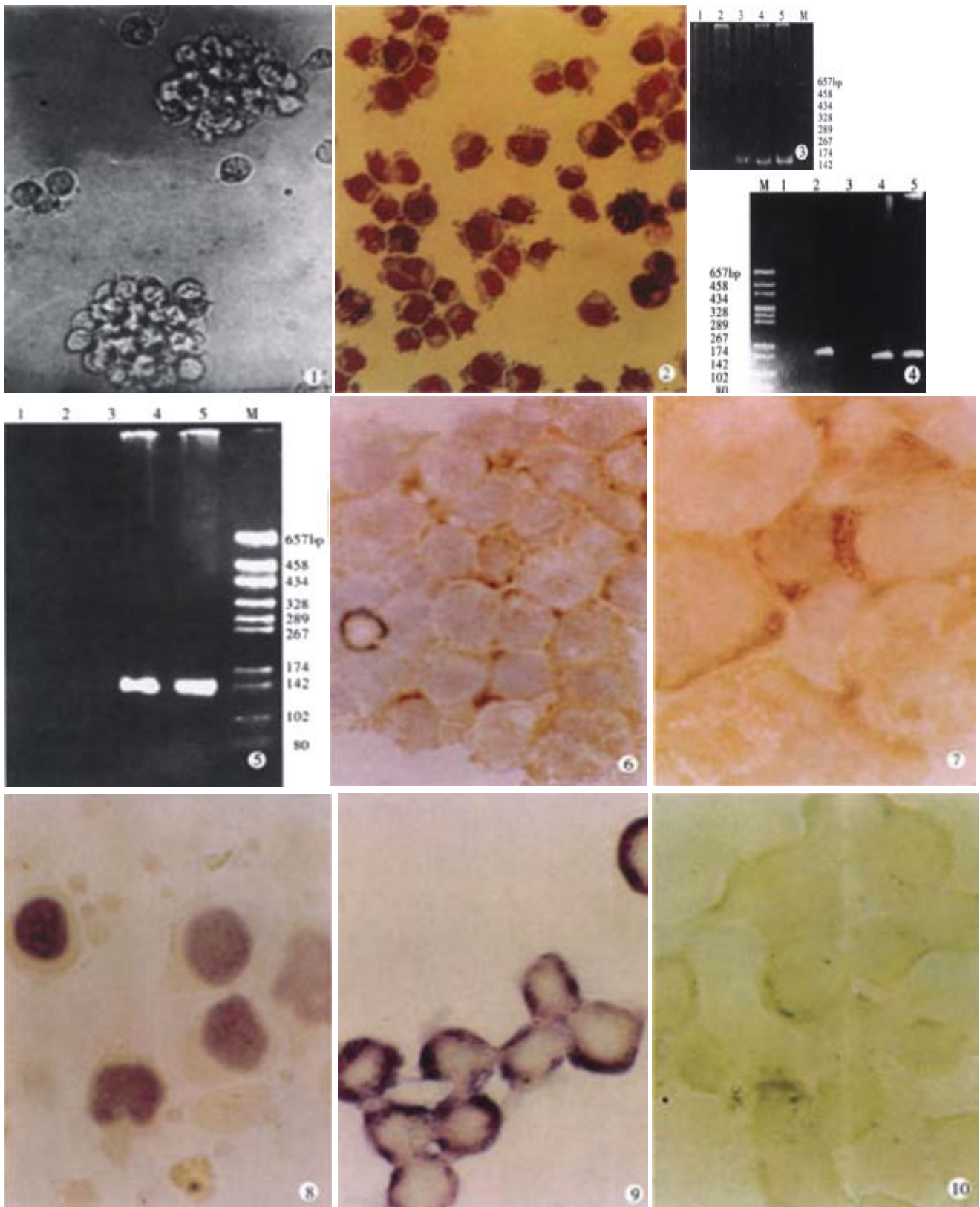
### HCV core and NS3 antigens of immunocytochemistry positive in LCL

Expression of the virus-encoded proteins in the cells was tested by immunocytochemistry using a monoclonal antibody to synthetic HCV core peptide and recombinant HCV NS3. The immunocytochemical staining for both antigens was detected in the cytoplasm. The staining granular was seen as a brown lump in a side cytoplasm (Figures 6-8).

To verify the specificity of the immunocytochemical staining, the following controls were employed: ① negative control, the PBMC of the normal donors; ② staining with a mouse monoclonal antibody to surface antigen of hepatitis B virus as a negative control antibody; ③ PBS taking the place of specific antibody as a replacement test; ④ the normal human PBMC transformed by EB virus as a negative control; and ⑤ omission of incubation with horseradish-peroxidase-labeled streptavidin to test for endogenous peroxidase activity in LCL. The immunocytochemical staining reaction was absent in the negative controls and endogenous peroxidase activity was not detected in the cells under the current condition of immunocytochemistry.

### HCV RNA signal of PCR product in situ RT-PCR

The positive signal of PCR product was dark blue and mainly localized to LCL cytoplasm (Figure 9). The specificity of the *in situ* cDNA product was checked in LCL cells. The RT-PCR signal was eliminated by overnight RNase digestion but not DNase digestion (Figure 10).



**Figure 1** There was increasing development of cell aggregates of proliferative lymphoblast cells.

**Figure 2** The immortal cell line of human peripheral blood mononuclear cells transformed by Epstein-Barr virus. The cell bodies are larger and spherical. There are the false foot-like hairs or thorns on their surface. The cell nuclei are large and varied. The cells multiply and grow as cell regiments or clusters.

**Figure 3** Identification of HCV RNA with RT-PCR in serum and PBMC with the chronic hepatitis C patient.

Lane 1: negative control; Lane 2: the last wash; Lane 3: HCV RNA positive serum; Lane 4: PBMC from hepatitis C patient; Lane 5: positive control. Lane M: pGEM-72f(+)/HaeIII markers

**Figure 4** Identification of HCV RNA plus-strand with RT-PCR in the EBV transformed PBMC and the growth medium.

Lane 1: Negative control; Lane 2: LCL cells; Lane 3: the last wash of LCL; Lane 4: growth medium; Lane 5: positive control; Lane M: pGEM-72f(+)/HaeIII markers

**Figure 5** HCV RNA minus strand with RT-PCR in the EBV transformed PBMC and the growth medium.

Lane 1: Negative control; Lane 2: growth medium; Lane 3: In cells; Lane 4: Positive control; Lane M:pGEM-72f(+)/HaeIII markers

**Figure 6** Immunohistochemical SP method, DAB staining and hematoxylin counter staining of cell nuclei. NS3 antibody positive granules seen as a brown lump in the cytoplasm of LCL.  $\times 400$

**Figure 7** Immunohistochemical SP method, DAB staining and hematoxylin counter-staining cell nuclei. Core antibody positive granules are seen as a brown lump with in the cytoplasm of LCL.  $\times 400$

**Figure 8** Immunohistochemical negative control (anti-HBs replaced anti-HCV), a positive brown granule is not seen in the cytoplasm.  $\times 400$

**Figure 9** The blue-black positive signals of HCV RNA are exhibited in the LCL cell plasma but not in the nucleolus.  $\times 400$

**Figure 10** The blue-black signals are not exhibited in negative control cells *in situ* RT-PCR.  $\times 400$

## DISCUSSION

Our results indicate that PBMCs from hepatitis C patients may be easily transformed into immortal lymphocytes and subcultured over a long period of time. We did not observe any signs of humoral or cellular immunity against LCL cells in the culture media. The presence of HCV in cells was not achieved by incubation with HCV-positive sera. HCV plus-strand or minus-strand RNA was detected in the cells, and the plus-strand HCV was detected in the growth media. Our results offer strong evidence for *in vivo* HCV infection of PBMC, and indicate that HCV may persist in the cells transformed by EBV and cultured *in vitro*. The detection of viral particles and HCV gene expressing proteins in the cells by immunohistochemistry<sup>[18-21]</sup> and *in situ* PCR<sup>[23-29]</sup> confirmed further that such cells may support complete HCV multiplication. Investigation of the hepatitis C virus life cycle and the evaluation of novel anti-viral strategies are limited by the lack of an efficient cell culture systems. Although chimpanzees can be successfully infected with HCV sera from human patients, they do not serve as a good animal model due to their limited numbers and high cost. Many researchers have reported using the HCV positive sera incubated with Molf-4<sup>[31]</sup>, HPB-MA<sup>[31]</sup> and H9 cells<sup>[32]</sup> (the human T cell lines), fetal hepatocytes<sup>[33]</sup>, bone marrow-derived B cell line<sup>[34]</sup>, PBMC from a healthy donor<sup>[35]</sup>, and daudi cells (a human B cell line)<sup>[22]</sup> as a cell model of *in vitro* HCV replication. These experiments have provided abundant material towards the establishment of new satisfactory models. PBMCs have been suspected as a possible site of extra-hepatic replication of HCV and a bank of latent infection of HCV. It relates closely to relapse of chronic hepatitis C<sup>[36-41]</sup>.

Just for this reason, PBMCs from hepatitis C patients were transformed by EBV into immortal cell *in vitro* and subcultured. We hope to establish a new cell model which is more representable of the true state of *in vivo* HCV infection.

The development of human cytogenetics demonstrates scientific progress on new methodological approaches. Thus, the real beginning of clinical cytogenetics was marked by

introduction of the lymphocyte culture technique as an easily accessible source of mitotic cell. However, due to the limited life-span of cells, new blood sampling is necessary in cases of re-examination. This might be difficult or even impossible if patients are not available for different reasons. Our procedure is available for routine use and allows efficient transformation of peripheral B lymphocytes by EBV and thus the establishment of lymphoblastoid cell lines<sup>[42]</sup>. Compared to other methods of long-term cultivation, these cell lines have a number of advantages: ① the original material can easily be obtained from any hepatitis C patient. ② LCL is the ideal source for HCV molecular biology studies in humans as repeated HCV RNA preparations can be obtained without effort.

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