Hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Choo et al., 1989; Saito et al., 1990). Infection with HCV is frequently associated with B-cell-related diseases, such as mixed cryoglobulinaemia and non-Hodgkin’s lymphoma (Hausfater et al., 2000). A number of studies have suggested that HCV can replicate in lymphocytes. However, in vitro culture systems to investigate replication of HCV in lymphocytic cells are severely limited. Robust HCV culture systems have been established using the HCV JFH-1 strain and Huh-7 cells. To gain more insights into the tissue tropism of HCV, we investigated the infection, replication, internal ribosome entry site (IRES)-dependent translation and polyprotein processing of the HCV JFH-1 strain in nine lymphocytic cell lines. HCV JFH-1 failed to infect lymphocytes and replicate, but exhibited efficient polyprotein processing and IRES-dependent translation in lymphocytes as well as in Huh-7 cells. Our results suggest that lymphocytic cells can support HCV JFH-1 translation and polyprotein processing, but may lack some host factors essential for HCV JFH-1 infection and replication.

Virological characterization of the hepatitis C virus JFH-1 strain in lymphocytic cell lines

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While hepatocytes are the major site of hepatitis C virus (HCV) infection, a number of studies have suggested that HCV can replicate in lymphocytes. However, in vitro culture systems to investigate replication of HCV in lymphocytic cells are severely limited. Robust HCV culture systems have been established using the HCV JFH-1 strain and Huh-7 cells. To gain more insights into the tissue tropism of HCV, we investigated the infection, replication, internal ribosome entry site (IRES)-dependent translation and polyprotein processing of the HCV JFH-1 strain in nine lymphocytic cell lines. HCV JFH-1 failed to infect lymphocytes and replicate, but exhibited efficient polyprotein processing and IRES-dependent translation in lymphocytes as well as in Huh-7 cells. Our results suggest that lymphocytic cells can support HCV JFH-1 translation and polyprotein processing, but may lack some host factors essential for HCV JFH-1 infection and replication.

We first sought to determine whether HCV JFH-1 can infect lymphocytic cell lines. We chose nine lymphocytic cell lines derived from Burkitt's lymphoma, the EBV-immortalized human B cell line, lymphoblasts and acute T-cell leukaemia. C1R, IB4, Namalwa, P3HR1 and Raji cells were Epstein–Barr virus (EBV)-positive (Table 1). Infectious HCV was generated from HCV JFH-1 RNA in Huh-7 cells (Shirakura et al., 2007; Wakita et al., 2005) and the calculation of the 50% tissue culture infectious dose (TCID50) was based on methods described previously (Lindenbach et al., 2005). These cell lines (1 × 10^5 cells per well of a six-well plate) were incubated with 2 ml inoculum (5 × 10^3 or 5 × 10^4 TCID50 ml^-1) for 3 h, washed three times with PBS, and cultured in fresh medium. The culture medium was changed every 2 days. Cells were harvested at 0 (3 h post-infection [p.i.]), 4 and 8 day p.i. HCV core antigen within cells was quantified by immunoassay (Ortho HCV-core ELISA kit; Ortho-Clinical Diagnostics). As shown in Fig. 1(a), increasing the HCV titre of the inoculum resulted in a 7.2-fold increase in the levels of HCV core protein in Huh-7 cells (Shirakura et al., 2007; Wakita et al., 2005) and the calculation of the 50% tissue culture infectious dose (TCID50) was based on methods described previously (Lindenbach et al., 2005). These cell lines (1 × 10^5 cells per well of a six-well plate) were incubated with 2 ml inoculum (5 × 10^3 or 5 × 10^4 TCID50 ml^-1) for 3 h, washed three times with PBS, and cultured in fresh medium. The culture medium was changed every 2 days. Cells were harvested at 0 (3 h post-infection [p.i.]), 4 and 8 day p.i. HCV core antigen within cells was quantified by immunoassay (Ortho HCV-core ELISA kit; Ortho-Clinical Diagnostics).
P3HR1 and Raji cells, suggesting that HCV can bind to these cell lines (Fig. 1a). In contrast, the levels of HCV core protein in IB4, Jurkat and Ramos cells at 3 h p.i. were below the detection limits and there were no significant differences in the levels of the core protein in Bjab cells and Namalwa cells, suggesting that HCV binding to these cells was very inefficient (Fig. 1a). Moreover, the levels of HCV core protein increased in Huh-7 cells but, in the case of all lymphocytic cell lines, including Raji cells, the core titre did not increase at day 4 and 8 p.i., suggesting that HCV JFH-1 does not infect and/or replicate efficiently in these lymphocytic cell lines (Fig. 1b).

To assess the replication of JFH-1 in our lymphocytic cell lines, we utilized the HCV replicon system. To visualize the

**Table 1.** Summary of the virological characterization of HCV JFH-1 in lymphocytes

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>EBV</th>
<th>Concentration of G418 for HCVcc infection (µg ml⁻¹)</th>
<th>HCV-RNA replication</th>
<th>Translation*</th>
<th>Polyprotein processing</th>
<th>HCV-RES</th>
<th>EMCV-RES</th>
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<tr>
<td>Bjab</td>
<td>Burkitt's lymphoma</td>
<td>+</td>
<td>600-800</td>
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<td>++</td>
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<td>++</td>
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<td>++</td>
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<td>++</td>
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<tr>
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<td>1000</td>
<td>++</td>
<td>++</td>
<td></td>
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<td>++</td>
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<tr>
<td>IB4</td>
<td>Burkitt's lymphoma</td>
<td>+</td>
<td>1000</td>
<td>++</td>
<td>++</td>
<td></td>
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<td>++</td>
</tr>
<tr>
<td>Jurkat</td>
<td>Burkitt's lymphoma</td>
<td>+</td>
<td>1000</td>
<td>++</td>
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<tr>
<td>Namalwa</td>
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<td>++</td>
<td>++</td>
<td></td>
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</tr>
<tr>
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<td>+</td>
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<td>Burkitt's lymphoma</td>
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<td>++</td>
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<tr>
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<td>400</td>
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<td>+</td>
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<tr>
<td>Huh7</td>
<td>Hepatoma</td>
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<td>100</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

* | ++, 0.25 fold IRES activity of Huh-7; +++, 0.75-1.5-fold; ++++, >1.5-fold.

**Fig. 1.** HCV infection assay. (a) HCV core protein levels 3 h after infection. A total of 1×10⁵ cells were infected with 2 ml of the inoculum (5×10⁳ [white bars] or 5×10⁴ [grey bars] TCID₅₀ ml⁻¹) for 3 h at 37 °C and harvested at 3 h p.i. HCV core protein in cell lysate was quantified by ELISA. The average values with standard deviations from triplicate samples are shown. The cut-off value of the immunoassay is indicated by an arrow and a dotted line. The difference between low m.o.i. (white bars) and high m.o.i. (grey bars) was significant (*, P<0.05; **, P<0.01, Student's t-test).

(b) Time-course of HCV core protein levels after infection. In total, 1×10⁵ cells were infected with 2 ml of the inoculum (5×10⁴ [a] or 5×10⁴ [b] TCID₅₀ ml⁻¹) for 3 h and harvested at 0, 4 and 8 days p.i. HCV core protein in cell lysate was quantified by ELISA. Average values ± SD from triplicate samples are shown.
replicating cells, a reporter replicon plasmid was constructed as follows. The gene encoding green fluorescence protein (GFP) was fused to the neomycin resistance gene using an overlap PCR amplification technique and the fusion product was inserted into pSGR-JFH1. The resultant plasmid was pSGR-GFPneo-JFH1. This plasmid was linearized with XbaI and used as a template for in vitro transcription using an AmpliScribe T7 High Yield Transcription kit (Epicentre Biotechnologies). RNA was transfected with high transfection efficiency and low cytotoxicity using the Nucleofector system (Amamax Biosystems) (Coughlin et al., 2004; Miyahara et al., 2005; Van De Parre et al., 2005). The transfection efficiencies ranged from 60 to 80 % after optimization of transfection conditions (Table 1). GFP expression was monitored periodically during the selection of HCV-replicon cells by G418 (Table 1). The GFP-expressing cells were detected at day 3 post-transfection (p.t.) in Huh-7, P3HR1, Raji, C1R and Namalwa cells. The rate of GFP expression in Huh-7 cells was more than 50 %, the rate of GFP-expression in lymphocytic cell lines was less than 1 %, despite the high transfection efficiencies. After 3 weeks of G418 selection, SGR-GFPneo-JFH1 replicon cells were established in Huh-7 cells, but not in lymphocytic cells. These data suggest that JFH-1 subgenomic replicon RNA cannot replicate in the lymphocytic cell lines.

To facilitate quantification of replication, we performed luciferase assays using subgenomic replicon RNA (SGR-JFH1/Luc) carrying firefly luciferase as a reporter. SGR-JFH1/Luc RNA was in vitro-transcribed using the linearized pSGR-JFH1/Luc (Kato et al., 2005a) as template DNA. Cells were harvested at 4, 24, 48 and 72 h p.t. and luciferase activities were assayed with Luciferase assay reagent (Promega). Assays were performed at least in triplicate. There were significant differences in luciferase activities at 4 h p.t. among the cell lines, probably because there were differences in transfection efficiencies and the doubling time of the cell lines. Thus, the replication activity was expressed relative to the reporter activity determined at each 4 h p.t. for each cell line, which was set to 1 (Fig. 2a). HCV subgenomic replicon RNA efficiently replicated in Huh-7 cells (Fig. 2a). Replication-deficient subgenomic replicon RNA encoding a GDD to GND mutation in NS5B served as a negative control in Huh-7 cells. The luciferase activities of replication-deficient subgenomic replicon RNA in lymphocytic cell lines also decreased rapidly (data not shown). As shown in Fig. 2(a), the luciferase activities of HCV subgenomic replicon RNA in lymphocytic cell lines decreased rapidly, suggesting that HCV subgenomic replicon RNA did not replicate efficiently in lymphocytic cell lines. Thus, these two different replicon assays demonstrated that the HCV JFH-1 subgenomic replicon failed to replicate in our lymphocytic cell lines.

To determine which steps of the HCV life cycle are impaired, we further examined translation and polyprotein processing. At first, we assessed HCV IRES-dependent translational efficiencies in the lymphocytic cell lines. Cells were co-transfected with the subgenomic replicon RNA (SGR-JFH1/Luc) and a capped RNA encoding Renilla luciferase (cap-luc). Cap-luc RNA was in vitro-transcribed using a T7 mMessage mMachine kit (Ambion). The HCV IRES activities in IB4, Namalwa and P3HR1 cells were as high as in Huh-7 cells. The HCV IRES activities in Jurkat and Raji cells were about 50 % of those in Huh-7 cells, and the HCV IRES activities in Bjab, BL41 and Ramos cells were less than 25 % of those in Huh-7 cells. On the other hand, the HCV IRES activity in C1R cells was about twofold higher than in Huh-7 cells (Fig. 2b). Replication-deficient subgenomic replicon RNA encoding a GDD to GND mutation in NS5B showed a luciferase activity level similar to that of the wild-type, suggesting that the luciferase activity at 4 h after transfection reflected translational levels but not replication levels (data not shown). Our data indicate high HCV IRES activities in all cell lines, except in Bjab, BL41 and Ramos.

The HCV polyprotein is translated in subgenomic replicon cells in an encephalomyocarditis virus (EMCV) IRES-dependent manner. To rule out the possibility that the EMCV IRES-dependent translation is impaired in lymphocytic cell lines, we assessed the EMCV IRES-dependent translational efficiencies. We assayed EMCV IRES activity using EMCV IRES-driven luciferase RNA (EMC-luc) and Cap-luc RNA. The EMCV IRES activity was five- to tenfold higher in C1R, Namalwa, IB4 and P3HR1 than in Huh-7 cells (Fig. 2c). From these results, HCV IRES and EMCV IRES exhibited sufficient translational activity in C1R, Namalwa, P3HR1 and Raji cells, suggesting that IRES-dependent translation was not impaired in these lymphocytic cell lines.

To determine whether HCV polyprotein is properly processed in lymphocytes, we examined the processing of HCV non-structural (NS) proteins. The construct pSGR-JFH1/Luc expresses the polyprotein NS3-NS4A-NS4B-NS5A-NS5B. The HCV NS3/4A protease is responsible for proteolytic processing at each cleavage site. We used the eukaryotic transient-expression system based on a recombinant vaccinia virus carrying bacteriophage T7 RNA polymerase (T7vac) (Fuerst et al., 1989). To express the SGR-JFH1/Luc encoding HCV NS proteins, 5 x 10⁶ cells were transfected with 5 μg pSGR-JFH1/Luc and infected with 2.5 x 10⁹ p.f.u. T7vac, harvested at 24 h p.i., and analysed by Western blotting. Completely processed NS3, NS3A and NS5B proteins were detected in Bjab, Raji, IB4 and Namalwa cells as well as in pSGR-JFH1/Luc-transfected Huh-7 cells and HCV-JFH1-infected Huh-7 cells (Fig. 2c). The unprocessed polyprotein was not detected by immunoblotting in these lymphocytic cell lines (data not shown). These results suggest that the HCV polyprotein is efficiently processed in these lymphocytic cells.

In this study, we demonstrated that HCV JFH-1 failed to infect and replicate in nine lymphocytic cell lines. In contrast, HCV IRES-dependent translation and polyprotein processing by NS3/NS4A protease functioned properly
in these cells. Moreover, subgenomic replicon RNA failed to replicate in these cell lines. Our data suggest that lymphocytic cell lines may lack some host factors required for infection and replication of HCV-JFH1.

Viral entry often requires sequential interactions between viral proteins and several cellular factors. Several molecules (CD81, Claudin-1, Scavenger receptor class B member IR, LDL-receptor and glycosaminoglycans) have been reported to be involved in HCV binding and entry (Barth et al., 2003; Evans et al., 2007; Pileri et al., 1998; Scarselli et al., 2002). Further investigation will be required to clarify HCV binding and entry into lymphocytic cell lines.

HCV IRES and EMCV IRES exhibited sufficient translational activities in C1R, IB4, P3HR1, Namalwa and Raji cells. All these cell lines are EBV-positive. EBV-encoded nuclear antigen (EBNA1) has been reported to support HCV replication (Sugawara et al., 1999). Two small EBV-encoded RNA species (EBERs) bind to the HCV IRES region (Wood et al., 2001). These findings raise the possibility that HCV IRES activities may be modified by the EBV genome.

HCV JFH-1 subgenomic replicon RNA could not replicate in all lymphocytes tested in this study. The HCV SB strain, however, has been reported to infect Raji, Daudi, Molt-4...
and Jurkat cells (Kondo et al., 2007; Sung et al., 2003). Still unknown is how hepatotropism and lymphotropism of HCV are determined. The GB virus B (GBV-B) is most closely related to HCV and the GBV-B infection of tamarins has been proposed as a good surrogate model for chronic hepatitis C (Bukh et al., 2001; Jacob et al., 2004; Lanford et al., 2003; Martin et al., 2003). A recent report has shown that GBV can disseminate to not only liver but also a variety of extrahepatic tissues such as haematolymphoid and genital tissues in tamarins (Ishii et al., 2007). Viral RNA cloned from plasma and liver from the tamarins showed no sequence heterogeneity, suggesting that host factors determine the pleiotropism (Ishii et al., 2007). It remains unclear how host factors and/or viral factors determine the tissue tropism of HCV. Further studies will be required to clarify the molecular mechanisms of HCV tissue tropism.

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