De novo infection and propagation of wild-type Hepatitis C virus in human T lymphocytes in vitro

Sonya A. MacParland, Tram N. Q. Pham, Shashi A. Gujar and Tomasz I. Michalak

Molecular Virology and Hepatology Research, Division of Basic Medical Sciences and Discipline of Laboratory Medicine, Faculty of Medicine, Health Sciences Centre, Memorial University, St John's, NL A1B 3V6, Canada

While exploring previous findings that ex vivo treatment of lymphoid cells from Hepatitis C virus (HCV)-infected individuals with T cell-stimulating mitogens augments detection of the residing virus, an in vitro HCV replication system was established, in which mitogen-induced T cell-enriched cultures served as HCV targets and the derived T cells multiplied virus during repeated serial passage. HCV replication was ascertained by detecting HCV RNA positive and negative strands, HCV NS5a and E2 proteins, release of HCV virions and nucleocapsids (confirmed by immunoelectron microscopy) and de novo infection of mitogen-induced T cells prepared from healthy donors. Further, affinity-purified normal human T lymphocytes were also susceptible to HCV infection in vitro and HCV replication was detected in pure T cells isolated from a patient with chronic hepatitis C. These results document that T cells can support propagation of HCV both in vivo and in vitro. The infection system established offers a valuable tool for in vitro studies on the entire cycle of HCV replication, virus cytopathogenicity and evaluation of antiviral agents against wild-type HCV in the natural host-cell milieu.

INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded RNA virus with a genome of 9600 bp, which encodes a single polypeptide that is subsequently cleaved co- and post-translationally to at least ten structural and non-structural proteins (Bartenschlager & Lohmann, 2000). HCV is thought to propagate via synthesis of the so-called 'negative strand'.

Although considered to be primarily hepatotropic, accumulated evidence indicates that HCV also replicates in the lymphatic system (Laskus et al., 1998; Lerat et al., 1996; Shimizu et al., 1997). Its replicative intermediate has been demonstrated in peripheral blood mononuclear cells (PBMCs) from patients with progressing chronic hepatitis C (CHC) (Laskus et al., 1998; Okuda et al., 1999; Willems et al., 1994) and individuals with apparently complete resolution of the disease (Pham et al., 2004, 2005; Radkowski et al., 2005a, b). HCV replication has been documented in T lymphocytes (Zignego et al., 1992), B cells (Morsica et al., 1999; Zignego et al., 1992; Baré et al., 2005), monocytes (Laskus et al., 2000; Radkowski et al., 2004) and dendritic cells (Goutagny et al., 2003) in CHC patients. In addition, recent studies from this laboratory have shown that ex vivo mitogen treatment of PBMCs from patients with CHC or those with occult HCV infection augmented HCV replication in this compartment (Pham et al., 2004, 2005; reviewed by Pham & Michalak, 2006).

Further support for the notion that lymphotropism is a natural propensity of HCV has stemmed from works with transformed or immortalized lymphoid-cell cultures. For instance, HCV propagation has been demonstrated in Epstein–Barr virus-transformed B cells isolated from PBMCs of patients with CHC (Sung et al., 2003). The presence of HCV RNA and proteins has also been reported in in vitro-infected human T-cell lines, such as MOLT-4 co-infected with human T-lymphotropic virus (Shimizu et al., 1992). However, many of these investigations relied on the target cells being co-infected with other viral pathogens and, overall, were difficult to reproduce. The lack of an adequate understanding of the mechanisms of HCV infection and a possible role of co-infecting viruses in promoting lymphoid-cell susceptibility to HCV raise a concern that the initiation of HCV infection and propagation of the virus in these systems might have been altered.

Considerable efforts to establish HCV replication in hepatocyte cultures have recently succeeded, with a system in which transfection of Huh7 hepatoma cells with a full-length HCV replicon led to secretion of infectious viral particles (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). However, this system is robust only for genotype 2a replicons.
The present study was undertaken in an effort to establish an in vitro infection system in which: (i) plasma or lymphoid cell-derived wild-type HCV can be used as an inoculum; (ii) virus-transformed or -immortalized cells will not be used as infection targets; and (ii) infectious HCV will be propagated. Over the course of this work, we designed conditions allowing infection of human T-cell-enriched cultures with wild-type HCV and its productive replication in the derived T cells. The system created should be of value for studies on the complete cycle of HCV replication, factors determining host susceptibility to HCV and cytopathic consequences of wild-type HCV infection. It could also be utilized for testing the efficacy of anti-HCV agents in the natural host-cell milieu.

**METHODS**

**Plasma-derived HCV inocula.** Plasma from patient N07/M with serologically and histologically documented CHC was used as the main source of wild-type HCV. This inoculum, designated HCV N07/M, carried HCV genotype 1a at 1 × 10^6 virus genome equivalents (vge) ml⁻¹, as determined by real-time RT-PCR (Pham et al., 2004). To establish whether the inoculum would be able to infect lymphoid cells, PBMCs isolated from N07/M were examined and found to be positive for both HCV RNA strands by RT-PCR/nucleic acid hybridization (RT-PCR/NAH) assays (Pham et al., 2004). The estimated HCV load was approximately 10^6 vge per 10^6 cells. Plasma samples from five CHC patients were also used as sources of wild-type HCV: N08/M plasma contained HCV genotype 1b at 5 × 10^6 vge ml⁻¹; C07/F, genotype 1a at 1·6 × 10^6 vge ml⁻¹; C32/M, genotype 1a at 8·5 × 10^6 vge ml⁻¹; C34/M, genotype 1a at 2·6 × 10^6 vge ml⁻¹; and N23/M, genotype 1b at 1·9 × 10^6 vge ml⁻¹. HCV replication in PBMCs was evident in these patients (not shown). The study was approved by the local Human Investigation Committee and samples were collected after informed consent had been obtained.

**Preparation of lymphoid cells.** Lymphoid cells serving as HCV infection targets were isolated from a healthy donor (A/M) with no clinical history or molecular indication of HCV exposure, as confirmed by RT-PCR/NAH assay (sensitivity of ≤ 10 vge ml⁻¹) (Pham et al., 2004), and who was seronegative for anti-HCV (enzyme immunoassay; Abbott Diagnostics). For some experiments, lymphoid cells from two other HCV RNA- and anti-HCV-negative individuals, B/M and C/F, were utilized. For direct infection or serial passage of HCV, PBMCs were isolated from 40 ml blood. Monocyte-depleted cells were resuspended in culture medium at 1 × 10^6 cells ml⁻¹ (Pham et al., 2004). For some experiments, T lymphocytes were affinity-purified from PBMCs of donor A/M. In parallel, T cells from a CHC patient (N09/F), who carried genotype 1a at 1 × 10^6 mitogen-induced A/M T cells by using a previously published protocol (Zhong et al., 2005). T cells preincubated with an isotype-control mAb and inoculated with the same HCV served as controls. The cells were cultured for 14 days before analysis.

**Direct infection with plasma-derived HCV.** Monocyte-depleted lymphoid cells were exposed to 5 µg phytohaemagglutinin (PHA; ICN Biomedicals) ml⁻¹ for 48 h prior to inoculation with HCV (Pham et al., 2004). For direct infection, PHA-treated cells (1 × 10^6 cells ml⁻¹) were exposed to 250 µl heat-inactivated plasma containing approximately 2·5 × 10^4 vge. In parallel, the cells were exposed to 250 µl plasma from donor B/M for mock infection. Inocula were removed after 24 h, then the cells were washed and cultured with 20 U recombinant human interleukin-2 (IL-2; Roche Diagnostics) ml⁻¹ for 72 h (phase A). At 4 days post-infection (p.i.), supernatant was collected and the cells were cultured with 5 µg PHA ml⁻¹ and 20 U IL-2 ml⁻¹ (designated PHA/IL-2) for the next 72 h (phase B). At 7 days p.i., supernatant was harvested and the cells were again cultured for 3 days in medium with IL-2 (phase C). The culture supernatant collected at 10 days p.i. was centrifuged at 400 g for 30 min and used as the source of HCV for the serial-passage experiment. In a supplementary experiment, affinity-purified T cells from donor A/M were infected with N07/M or N08/M inoculum and cultured for 10 days p.i. as described above.

**Serial passage of HCV in T-cell cultures.** PHA-stimulated target cells were incubated with clarified supernatant after phase C from the direct infection, normally containing approximately 10^6 vge HCV. After 24 h, the cells were collected, washed and stimulated with IL-2 or PHA/IL-2 (phases A, B and C) as described above. At day 10 p.i., supernatant was harvested and cells were cultured for 96 h with PHA/IL-2 (phase D). The culture ended at 14 days p.i. with collection of the supernatant and cells. A sample of the phase D supernatant was preserved for analysis, whereas the remainder was used to infect fresh PHA-induced lymphoid cells. This was repeated 14 consecutive times, spanning a total of 28 weeks. The phase D supernatants were examined for completeness of cell removal by phase-contrast microscopy. Randomly selected phase D supernatants were amplified for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by using primers and PCR conditions reported previously (Hodgson & Michalak, 2001) and no signals were detected. To determine whether HCV produced after the multiple passage in A/M T cells could infect cells from other healthy donors, PHA-pretreated lymphoid cells from B/M and C/F were incubated with pooled supernatant after phases A–C of passage 11 and cultured as described for the direct-infection experiment.

**Blocking of HCV infection in T cells with anti-CD81.** The experiment was carried out with anti-CD81 monoclonal antibody (mAb) JS81 (Pharmingen) at 1:20 and 1:100 dilutions in 50 µl containing 5 × 10^6 mitogen-induced A/M T cells by using a previously published protocol (Zhong et al., 2005). T cells preincubated with an isotype-control mAb and inoculated with the same HCV served as controls. The cells were cultured for 14 days before analysis.

**Treatment with alpha interferon (IFN-α).** To reaffirm that active HCV replication was established in T cells, the cells were treated in triplicate with 1000, 10 or 1 µm recombinant human IFN-α 2b (Research Diagnostics) ml⁻¹ at the time of HCV inoculation. The same IFN-α amounts were added to the culture each time that the medium was changed (phases A–D). The cells were cultured for 14 days as described above. IFN-α concentrations were non-toxic to lymphoid cells in an MTT assay (data not shown). Cells inoculated with HCV but not treated with IFN-α served as controls.

**Ultracentrifugation and buoyant density gradients.** To determine HCV RNA in T-cell culture supernatants, samples (5 ml) were centrifuged at 400 g for 30 min and then at 150 000 g for 2 h at 4°C in an SW50.1 rotor (Beckman Instruments). RNA was extracted from the pellets and analysed by RT-PCR/NAH. To examine ultrastructural features of the released viral particles, samples (5 ml) of passage 5, 8 and 11 supernatants were clarified, ultracentrifuged and analysed by electron microscopy. As controls, the supernatant from A/M cells not infected with HCV, but cultured under identical conditions, was prepared. To analyse properties of HCV RNA reactive particles further, 15 ml pooled supernatant obtained after phases A–C from passage 8 was clarified and ultracentrifuged as described above. The resulting pellet was resuspended in 800 µl 10 mM Tris/HCl buffer (pH 7·2) with 0·15 M NaCl and 10 mM EDTA and overlaid onto a 12 ml discontinuous gradient of 1·1–1·6 g caesium chloride (CsCl) ml⁻¹ prepared in the same buffer. In parallel, plasma samples (800 µl) from patient C26/F containing 1·1 × 10^6 vge ml⁻¹ and from a healthy donor were fractionated. After centrifugation at 200 000 g for 48 h at 10°C in a Beckman SW41 rotor, fractions (750 µl) were collected from the top of each gradient and analysed for HCV RNA by and electron microscopy.
**RNA extraction and RT-PCR/NAH.** Total RNA was extracted by using TRIzol (Invitrogen) from $1 \times 10^7$ cells (which usually yielded approx. 15 µg RNA) or from 100 µl of the pellet suspension obtained by ultracentrifugation of culture supernatants. RNA was also isolated from samples (650 µl) of the CsCl fractions by using TRIzol LS (Invitrogen). RNA (1–4 µg) was transcribed with Moloney murine leukemia virus reverse transcriptase for HCV RNA positive-strand detection or with recombinant Tth DNA polymerase for virus RNA negative-strand detection (Pham et al., 2004). PCR was carried out by using primers and conditions reported previously (Pham et al., 2004). A water sample and a mock-treated test RNA were always included as contamination controls. cDNA from PHA-treated A/M cells exposed to B/M plasma (mock infection) and cultured as the infected cells served as a negative control, whilst 10-fold dilutions of recombinant HCV UTR-E2 fragment (rHCV UTR-E2) acted as quantitative standards. Specificity of the detection and validity of controls were confirmed routinely by Southern blot hybridization (Pham et al., 2004). Detection of HCV synthetic RNA (sRNA) positive and negative strands was not affected by the presence of cellular RNA from healthy PHA-stimulated lymphoid cells. HCV RNA in plasma was quantified by real-time RT-PCR using LightCycler Fast Start Master hybridization probes (Roche Diagnostics) (Pham et al., 2004).

**Detection of HCV NS5a and E2 proteins.** For Western blotting, proteins of T cells infected with HCV and recovered after passages 3, 11 and 14 were separated on 10 % SDS-polyacrylamide gels and blotted as reported previously (Michalak et al., 2000). Blots were probed with anti-HCV NS5a mAb (Biodesign) or anti-HCV E2 (ALP98) mAb (provided by Dr A. Patel, Institute of Virology, University of Glasgow, UK). HCV replicon cell line AB12-A2FL, containing full-length HCV genotype 1b (provided by Dr C. Richardson, Ontario Cancer Institute, University of Toronto, Canada), was used as a positive control. A/M T cells not exposed to HCV, but cultured under the same conditions, were a negative control. Reactions were visualized by a horseradish peroxidase-conjugated secondary antibody and chemiluminescence. To assess intracellular expression of HCV E2 protein in in vitro-infected T cells, confocal immunofluorescence microscopy was carried out by using AB12-A2FL Huh7 cells, naïve Huh7 cells and HCV-naïve T cells as controls. Cells were grown overnight on polylysine-coated 16-well glass slides (Nalge Nunc International), fixed with 4 % paraformaldehyde and permeabilized with 0.5 % Triton X-100. HCV E2 protein and tubulin were identified with anti-HCV E2 (AP33) mAb and rat anti-tubulin (Chemicon), respectively. Cy2-labelled donkey anti-mouse or Cy5-labelled donkey anti-rat.
antibodies (both from Jackson ImmunoResearch) were used in the second layer. Cells were examined in a FluoView FV300 confocal system (Olympus).

Transmission and immune electron microscopy. For routine microscopy, aliquots of the pellets recovered after ultracentrifugation or fractions from CsCl gradients and respective controls were applied onto Formvar–carbon-coated 200-mesh microscropic grids. The grids were washed and stained negatively with 1% phosphotungstic acid (PTA). To precipitate HCV cores, aliquots of the pellets were incubated with 20 μg anti-HCV core IgG2a mAb (Virogen) or with an isotype-control mAb for 1 h at ambient temperature. After alternating stimulation of both virus-naïve and HCV-exposed cells with PHA and/or IL-2 for 14 days, 96% of them were CD3-positive (T cells), 16% were CD19-positive (B cells, macrophages and/or dendritic cells), 24% were CD40-reactive (B cells, macrophages and/or dendritic cells), 16% were CD19-positive (B cells) and <1% were CD14-positive (monocytes) (data not shown). After alternating stimulation of both virus-naïve and HCV-exposed cells with PHA and/or IL-2 for 14 days, 96–6% of the cells were CD3-positive (Fig. 1). Interestingly, whilst the ratio of CD4- to CD8-positive cells was 2:1 for uninfected T cells, that for the infected cells was 1:3.4. Fewer than 1% CD19-reactive cells were detected in HCV-infected cultures (data not shown). These data showed that T cells were essentially the only cell type supporting HCV propagation at phase D of the passage from which supernatant was used to transmit infection.

HCV genome expression during de novo infection and serial passage in T-cell cultures

As shown in Fig. 2(a), HCV RNA positive strand was detected in both cells and culture supernatant after direct infection with HCV N07/M, whereas the mock-infected cells were negative. HCV load was approximately 10^5 vge per 10^7 cells. Comparable results were obtained when HCV-positive plasma from patients C32/M, C34/M and N23/M was used for direct infection of A/M cells (Fig. 2c, d). These results implied that infection of the T-cell cultures was not related

RESULTS

Selective expansion of T cells in culture

Anticipating that the intracellular environment supportive of HCV replication elicited by PHA (Pham et al., 2004) may also enhance lymphoid-cell susceptibility to virus, monocyte-depleted cells were PHA-treated prior to HCV exposure. After 48 h culture, the cells expanded by 20–45%. Approximately 64% of them were CD3-positive (T cells), with a CD4- to CD8-positive cell ratio of 2:4:1 (Fig. 1); 24% were CD40-reactive (B cells, macrophages and/or dendritic cells), 16% were CD19-positive (B cells) and <1% were CD14-positive (monocytes) (data not shown).
to the origin of the HCV inoculum. Heat inactivation of plasma did not influence HCV infectivity, although it improved cell survival occasionally.

Estimated levels of HCV RNA positive strand in T cells after passages 2, 4, 9 and 11 (Fig. 2a) ranged between $10^3$ and $10^6$ vge per $10^7$ cells, whilst the amount of HCV released during each serial passage was approximately $10^4$ vge ml$^{-1}$. Taken together, the total HCV produced during the entire passage experiment was estimated to exceed $10^7$ vge. Given that the HCV N07/M inoculum carried $2 \times 10^4$ vge and that $1.4 \times 10^4$ vge was recovered in culture medium after the inoculation, at least 1000-fold enrichment in the virus was achieved. Also, HCV RNA negative strand was found in T cells collected after direct infection and all passages analysed, but not in phase D supernatants (Fig. 2b). Semiquantitatively, there were approximately $10^4$ vge of the negative strand for $10^7$ cells.

To determine whether the virus obtained after multiple serial passages in A/M T cells could infect T cells of other healthy individuals, PHA-treated cells from donors B/M and C/F were exposed to pooled supernatants from passages 11 and 12. After direct infection, cells from donor C/F proliferated to the same extent as those from donors A/M and B/M, they remained HCV RNA-negative (Fig. 2c, d). This variation might be of biological significance and will be investigated in future.

**HCV protein display in in vitro-infected T lymphocytes**

To determine whether HCV infection was accompanied by synthesis of viral proteins, the presence of HCV NS5a and E2 proteins were examined by Western blotting. A protein band of approximately 56 kDa was detected in T cells after passages 3 and 11 when probed with anti-NS5a (or anti-E2 (ALP98) mAb. The 56 kDa NS5a and 70 kDa E2 protein bands are marked on the right and molecular mass markers on the left. A/M cells not exposed to HCV, but cultured as infected T cells, and naïve Huh7 cells were used as negative controls. In (c), T cells after direct infection with HCV N23/M inoculum were double-stained with anti-E2 (AP33) and anti-tubulin mAbs and analysed by confocal microscopy. Huh7 cells transfected with the HCV AB12-A2FL replicon served as a positive control.
T cells. A confocal microscopic analysis suggested that the E2 protein has intracytoplasmic and plasma membrane-associated localization in infected T cells (Fig. 3c).

**Inhibition of HCV infection by anti-CD81 and treatment with IFN-α**

Preincubation of T cells with two concentrations of anti-CD81 mAb, but not with an isotype control, blocked HCV RNA expression (Fig. 4). To provide further evidence that active HCV replication was supported by T cells and to recognize applicability of our system for testing susceptibility of wild-type HCV to antivirals, the effect of IFN-α on the outcome of de novo infection in T cells was examined. The results showed a dose-dependent antiviral effect of IFN-α (Fig. 5). HCV replication was abrogated in cells treated with 1000 U ml⁻¹, but not inhibited uniformly by lower doses.

**HCV infection in affinity-purified T cells**

For further confirmation of the natural susceptibility of T cells to HCV infection, affinity-purified T cells from healthy donor A/M were infected with HCV N07/M or HCV N08/M. In parallel, pure T cells from patient N09/F with CHC were cultured exactly as those from donor A/M. At day 10 p.i., HCV RNA positive strand was detected at approximately 10⁵ vge per 10⁷ T cells infected with N07/M and N08/M inocula (Fig. 6a). Interestingly, T cells purified from patient N09/F carried HCV RNA positive strand at a level similar to that in T cells infected with HCV N07/M (Fig. 6a). The level of HCV RNA negative strand was approximately 10⁴ vge per 10⁷ cells infected with N07/M and N08/M inocula, and in T cells from patient N09/F (Fig. 6b).

**Buoyant density of HCV RNA reactive particles released by in vitro-infected T cells**

To recognize properties of HCV particles produced by T cells, culture supernatant obtained after passage 8 was subjected to isopycnic centrifugation. The fractions collected were examined for HCV RNA by RT-PCR/NAH and virus particles by electron microscopy. As shown in Fig. 7, the main peak of HCV RNA reactivity was identified at buoyant densities 1.16–1.19 g ml⁻¹ (fractions 5–7; peak 1). There was also a peak at 1.26–1.32 g ml⁻¹ (fractions 9–11; peak 2) and some minor increase in HCV RNA positivity at other densities. The greatest HCV copy numbers estimated were 1.4 × 10⁶ vge ml⁻¹ for fraction 6 (density, 1.18 g ml⁻¹) of peak 1 and 0.75 × 10⁵ vge ml⁻¹ for fraction 9 (density, 1.26 g ml⁻¹) of peak 2. Analysis of the fractions...
The vast majority of HCV particles detected by electron microscopy after direct ultracentrifugation of T-cell culture supernatants were non-enveloped cores (see Fig. 7). Virion-like particles were heterogeneous in diameter, ranging between 51 and 74 nm (mean, 67.4 nm) and in envelope thickness between 6 and 15.6 nm (mean, 11.4 nm). Fig. 8(b) illustrates examples of HCV virion-like particles after CsCl fractionation of the passage 8 supernatant [Fig. 8b(i–iii)] and C26/F plasma [Fig. 8b(iv–vi)]. Diameters of these virions were 62–85 nm (mean, 77.6 nm) and the envelope thickness ranged from 9 to 27 nm (mean, 20.6 nm).

To ascertain further that HCV virions were released by the in vitro-infected T cells, pellets recovered after ultracentrifugation of the T-cell culture supernatants or C32/M plasma were stained by immunogold with anti-HCV E2 mAb. Single enveloped virions dressed with gold particles were detected both in control C32/M plasma [Fig. 8c(i–ii)] and in the pooled T-cell culture supernatants recovered after passages 10 and 13 [Fig. 8c(iii–iv)]. Aggregates of gold-coated virions were also detected [Fig. 8c(v–vi)]. There were a few gold particles not associated with virions in the same supernatant after incubation with an isotype-control mAb [Fig. 8c(vii)].

**Ultrastructural features of HCV particles produced by T cells**

The vast majority of HCV particles detected by electron microscopy after direct ultracentrifugation of T-cell culture supernatants were non-enveloped cores [Fig. 8a(i–iii)]. Their diameters ranged between 25 and 50 nm (mean, 36.5 nm). These particles could be precipitated with anti-HCV core mAb [Fig. 8a(iv–vii)], but not with an isotype-control mAb (not shown). Fractions collected after a CsCl gradient showed the presence of both virion-like structures and non-enveloped cores (see Fig. 7). Virion-like particles were heterogeneous in diameter, ranging between 51 and 74 nm (mean, 67.4 nm) and in envelope thickness between 6 and 15.6 nm (mean, 11.4 nm). Fig. 8(b) illustrates examples of HCV virion-like particles after CsCl fractionation of the passage 8 supernatant [Fig. 8b(i–iii)] and C26/F plasma [Fig. 8b(iv–vi)]. Diameters of these virions were 62–85 nm (mean, 77.6 nm) and the envelope thickness ranged from 9 to 27 nm (mean, 20.6 nm).

To ascertain further that HCV virions were released by the in vitro-infected T cells, pellets recovered after ultracentrifugation of the T-cell culture supernatants or C32/M plasma were stained by immunogold with anti-HCV E2 mAb. Single enveloped virions dressed with gold particles were detected both in control C32/M plasma [Fig. 8c(i–ii)] and in the pooled T-cell culture supernatants recovered after passages 10 and 13 [Fig. 8c(iii–iv)]. Aggregates of gold-coated virions were also detected [Fig. 8c(v–vi)]. There were a few gold particles not associated with virions in the same supernatant after incubation with an isotype-control mAb [Fig. 8c(vii)].

**DISCUSSION**

In this study, lymphoid-cell cultures, established by ex vivo treatment of PBMCs from healthy individuals with a T cell-inducing mitogen, were susceptible to wild-type HCV and capable of supporting its complete cycle of replication. Productive HCV replication was documented by detection of HCV RNA positive and negative strands, of NS5a and E2 proteins and by secretion of complete virions, which could infect T cells from healthy donors de novo. The results also showed that circulating T cells in clinically evident HCV infection are an extrahepatic site of virus replication.

In the experiments preceding this study, we attempted to infect lymphoid cells with HCV without pre-treating them with PHA and found that they were recognized poorly by the virus (data not shown). However, exposure of the same cells to PHA led to HCV infection. In this context, stimulation of lymphoid cells with PHA alone or PHA and IL-2 has been shown to increase cell susceptibility to infections with other viruses, including Bovine leukemia virus (Chatterjee et al., 1985), Measles virus (Hyypiä et al., 1985) and herpes simplex virus (Braun & Kirchner, 1986; van der Meulen et al., 2002).

Data from the present and previous works (Pham et al., 2004, 2005) indicate that activation of lymphoid cells, which characterizes functioning, dividing and immature progenitor lymphoid cells, predisposes cells to HCV recognition and promotes an intracellular microenvironment supportive of virus replication. This may explain why HCV has been more readily identifiable in lymphoid-cell cultures or in PBMCs from patients co-infected with other viral pathogens (Beld et al., 1998; Laskus et al., 2004) and why mature, mitogen-untreated lymphoid cells are poorly permissive to HCV infection in vitro. Our data raise a possibility that lymphoid organs, which embrace the most active proliferative expansion of lymphoid cells in adulthood, are sites of
HCV propagation and long-term persistence of virus. The notion of lymphotropism is supported by data demonstrating HCV RNA in bone marrow, lymph nodes or spleens from patients with CHC or co-infected with human immunodeficiency virus type 1 (Laskus et al., 1998; Radkowski et al., 2000).

In our system, passage of HCV by infection of T-cell cultures freshly prepared each time was associated with consistent detection of both HCV RNA positive and negative strands in the cells and of the positive strand in culture supernatants from all passages tested. The potency of the virus to infect the cells did not decrease during serial passage and the estimated amount of the virus produced appeared to be proportional to T-cell proliferation. On average, $10^5$ vge were detected per $10^7$ infected T cells at the end of each passage. Interestingly, a comparable rate of approximately $10^5$ vge per $10^7$ cells was detected for T cells affinity-purified from patient N09/F with CHC.

HCV in de novo-infected T cells was susceptible to IFN-α treatment, with complete inhibition of virus replication.
observed at 1000 U ml\(^{-1}\). This result resembles that reported for primary human hepatocytes infected in vitro with wild-type virus (Castet et al., 2002). In addition, as was shown for the JFH-1 replicon in HuH7 cells (Zhong et al., 2005), anti-CD81 mAb was able to inhibit HCV infection in our system.

To ascertain further that in vitro-infected T cells supported HCV replication and that the virus was assembled, ultrastructural characteristics of viral particles released by the cells were examined after either direct ultracentrifugation or fractionation in CsCl gradients. It was found that non-enveloped core particles were predominantly detected in the supernatants after direct centrifugation, whereas HCV virions were mainly seen in those fractionated throughout the density gradient, particularly in the fractions enriched with HCV RNA. These findings suggested that concentrating the virus without applying density equilibrium led to dissociation of viral particles and thus implied erroneously that HCV cores, but not complete virions, were mainly produced by infected T cells. In fact, depending on which method of HCV concentration is employed, separation of the viral envelope from the nucleocapsid has been observed (Fujita et al., 2001). Nevertheless, the HCV nucleocapsids detected in our T-cell supernatants displayed the expected size and were immunoprecipitated with anti-HCV core mAb, as observed by others (Kaito et al., 1994; Maillard et al., 2001). Similarly, HCV virion-like particles detected after isopycnic banding showed heterogeneous properties, as reported previously (Fujita et al., 2001; Hikijaka et al., 1993; Kanto et al., 1994; Pumeechochchai et al., 2002). Specificity of these particles was confirmed by immuno-gold staining with anti-E2 mAb, and both single, complete virions and immuno-aggregates of virions were detected. These results documented conclusively that viral particles with physical and structural properties of complete HCV virions were released by in vitro-infected T cells.

Overall, our results from a multiparametric analysis demonstrated that mitogen-induced normal human T cells can support the complete cycle of HCV replication and produce infectious virions. This suggests that they are equipped not only with appropriate molecules capable of recognition and uptake of HCV, but also with the machinery to multiply the virus. This system should be of value for studies on recognition of cytopathic mechanisms of HCV infection, investigation of host factors determining susceptibility and the efficiency of virus replication, and for testing antiviral agents against wild-type HCV propagating in the natural host-cell milieu.

ACKNOWLEDGEMENTS

The authors thank Dr F. R. Bursey from the Gastroenterology Unit, General Hospital, St John’s, Newfoundland, Canada, and Drs C. S. Coffin and S. S. Lee from the Liver Unit, University of Calgary, Calgary, Alberta, Canada, for providing clinical samples. We also thank Dr A. Patel from the Institute of Virology, University of Glasgow, Glasgow, UK, for providing mAbs ALP98 and AP33 against the HCV E2 protein and Dr C. Richardson from the Ontario Cancer Institute, Toronto, Canada, for providing the HCV AB12-A2FL replicon. The study was supported by grants EOP-41538 and MOP-77544 awarded to T. I. M. from the Canadian Institutes of Health Research, Ottawa, Canada. T. N. Q. P. is supported by a postdoctoral fellowship from the National Canadian Research Training Program in Hepatitis C. T. I. M. is the Canada Research Chair (Tier 1) in Viral Hepatitis/Immunology, sponsored by the Canada Research Chair Program and the Canadian Institutes of Health Research, Ottawa, Canada.

REFERENCES


