Mitogen-induced upregulation of hepatitis C virus expression in human lymphoid cells

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Considering growing evidence indicating that hepatitis C virus (HCV) replicates in lymphoid cells, establishment of a reliable and sensitive method for detection of HCV in these cells may provide means for monitoring the infection and the efficacy of sterilizing antiviral therapy. In this study, conditions for ex vivo augmentation and detection of the HCV genome in peripheral blood mononuclear cells (PBMCs) from patients with chronic hepatitis C (CHC) or after a sustained virological response (SVR) to antiviral treatment were assessed. Following stimulation with combinations of mitogens and/or cytokines, PBMCs and, in certain cases, affinity-purified T and B cells were examined for HCV positive- and negative-strand RNA by using RT-PCR followed by nucleic acid hybridization, while the presence of viral NS3 protein was determined by flow cytometry. HCV RNA augmentation was assessed by quantification of Southern and dot-blot hybridization signals. The results showed that treatment of peripheral lymphoid cells with mitogens stimulating T- and B-cell proliferation and with cytokines supporting their growth significantly increased HCV RNA detection in patients with both CHC and SVR. This enhancement was up to 100-fold for the HCV genome and fivefold for the NS3 protein compared with untreated cells. In conclusion, HCV RNA can be readily detected in circulating lymphoid cells in progressing hepatitis C and following SVR after ex vivo cell stimulation. As such, this method offers a new investigative tool to study HCV lymphotropism and to monitor virus presence during the course of HCV infection.

INTRODUCTION

Hepatitis C virus (HCV) infection is a worldwide problem. Up to 85% of infected individuals are believed to succumb to chronic hepatitis C (CHC) (Cohen, 1999). HCV is an enveloped virus that contains a positive-stranded RNA of approximately 9400 nt encoding a single polyprotein, which is cleaved into 10 structural and non-structural proteins (Brechot, 1996; Houghton et al., 1994). HCV replicates by making a complementary negative-sense RNA strand, also known as the replicative strand.

The liver is the main site of HCV replication. However, accumulated data indicate that the virus also replicates in the lymphatic system. This is reflected by a higher frequency of type II mixed cryoglobulinaemia (El-Serag et al., 2002) and non-Hodgkin’s lymphoma (Musto, 2002) in HCV-infected persons. The notion of lymphotropism being an intrinsic property of HCV has gained strong support from recent studies demonstrating the HCV genome and its replicative intermediate in peripheral blood mononuclear cells (PBMCs) from individuals with CHC and after spontaneous or therapeutically induced resolution of hepatitis C (Castillo et al., 2004; Laskus et al., 2000; Pham et al., 2004; Radkowski et al., 2000). In a previous study (Pham et al., 2004), we provided evidence for long-term persistence of HCV RNA and its replicative strand in PBMCs and monocyte-derived dendritic cells after apparent complete recovery from hepatitis C. HCV RNA was also detected in PBMCs and liver tissue from patients with occult HCV infection accompanied by long-standing abnormal liver function tests (Castillo et al., 2004). In addition, different types of lymphoid cells, including T (Shimizu et al., 1992, 1998) and B lymphocytes (Morsica et al., 1999), as well as monocytes (Radkowski et al., 2004), have been shown to support HCV propagation and the virus derived in vitro can be infectious (Kato et al., 1995; Shimizu et al., 1998; Sung et al., 2003).

Considering that lymphotropism characterizes many viruses capable of long-term persistence (Grosjean et al.,
1997; Oldstone, 1996), the establishment of a method for reliable and sensitive detection of HCV in lymphoid cells may shed new light on the natural history of HCV infection and provide a tool for monitoring its replication. In earlier studies from this laboratory on occult infections with woodchuck hepatitis virus (WHV) (Coffin & Michalak, 1999; Michalak, 2000; Michalak et al., 1999, 2004) and HCV (Pham et al., 2004), it was shown that treatment with certain non-specific mitogens led to greater expression of viral genomes in lymphoid cells, allowing identification of the residing virus in apparently negative cells.

With regard to occult HCV infection, HCV positive- and negative-strand RNAs were detected in the majority (81 and 75%, respectively) of individuals with resolved hepatitis C when their PBMCs were cultured with phytohaemagglutinin (PHA) and interleukin-2 (IL2) (Pham et al., 2004). In the above study, the use of a highly sensitive RT-PCR nucleic acid hybridization (RT-PCR/NAH) assay [sensitivity of $\leq 10$ virus genome equivalents (vge) ml$^{-1}$] undoubtedly contributed to enhanced detection of HCV RNA in PBMCs. The same assay revealed the virus genome in the sera of 88% of the same individuals who were repeatedly HCV RNA non-reactive by a standard clinical assay (sensitivity of $10^2$–$10^3$ vge ml$^{-1}$). However, when taking the results from PBMCs and sera together, all individuals were HCV RNA positive (Pham et al., 2004), raising the possibility that detection of the HCV genome in lymphoid cells could be further improved.

Based on the above reasoning, the aim of the current study was to design conditions for the most effective augmentation of HCV detection in lymphoid cells. We explored synergistic effects of mitogens stimulating T and B cells and the cytokines supporting their survival. The data reported here show that the detection of HCV positive- and negative-strand RNA, as well as HCV NS3 protein, in PBMCs can be markedly enhanced after ex vivo mitogen treatment. This approach offers a new tool to examine HCV lymphotropism and to monitor HCV infection.

### METHODS

Six patients (three males and three females, aged 34–48 years) with progressing CHC and three individuals (one male and two females, aged 33–48 years) with documented resolution of CHC after standard antiviral therapy were randomly selected for this study (Table 1). CHC was diagnosed based on clinical and biochemical evidence of liver disease, repeated detection of HCV RNA in serum using the Roche Amplicor HCV Monitor v2.0 assay (sensitivity $10^2$ vge ml$^{-1}$ or 500 IU ml$^{-1}$; Roche Molecular Diagnostics) and by liver histology. These six patients did not receive any antiviral therapy prior to the investigation. The other three resolved CHC following a 48-week treatment with interferon-α 2b and ribavirin (Table 1). All had a sustained virological response (SVR), i.e. serum HCV RNA was undetectable by a standard assay for at least 6 months after therapy and the liver function tests were repeatedly normal. These individuals were followed for 11–36 months prior to the study. However, all carried HCV RNA at the time of this investigation (Table 1) when tested by an in-house RT-PCR/NAH assay

### Table 1. Clinical characteristics and detection of HCV RNA in sera and in circulating lymphoid cells from patients with CHC and individuals with resolved CHC due to antiviral therapy

<table>
<thead>
<tr>
<th>Diagnosis/case</th>
<th>Age/sex</th>
<th>Route of infection</th>
<th>Antiviral treatment (duration in weeks)</th>
<th>HCV genotype</th>
<th>HCV RNA Serum* [vge ml$^{-1}$]</th>
<th>HCV RNA PBMCs† [vge (μg RNA)$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>44/M</td>
<td>Transfusion</td>
<td>None</td>
<td>1b</td>
<td>$1 \times 10^5$</td>
<td>$1 \times 10^5$*</td>
</tr>
<tr>
<td>2</td>
<td>45/F</td>
<td>Transfusion</td>
<td>None</td>
<td>1b</td>
<td>$4 \times 10^6$</td>
<td>$4 \times 10^2$</td>
</tr>
<tr>
<td>3</td>
<td>40/F</td>
<td>Intravenous drug user</td>
<td>None</td>
<td>2a/c</td>
<td>$1 \times 10^5$</td>
<td>$\sim 1 \times 10^4$</td>
</tr>
<tr>
<td>4</td>
<td>48/M</td>
<td>Transfusion</td>
<td>None</td>
<td>1a</td>
<td>$9 \times 10^5$</td>
<td>$\sim 1 \times 10^4$</td>
</tr>
<tr>
<td>5</td>
<td>47/M</td>
<td>NK</td>
<td>None</td>
<td>NK</td>
<td>$8 \times 10^6$</td>
<td>$\sim 1 \times 10^4$</td>
</tr>
<tr>
<td>6</td>
<td>34/F</td>
<td>Intravenous drug user</td>
<td>None</td>
<td>1a</td>
<td>$2 \times 10^6$</td>
<td>$\sim 1 \times 10^4$</td>
</tr>
<tr>
<td>Resolved hepatitis C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>40/F</td>
<td>Inhalation</td>
<td>IFN/ribavirin (48)</td>
<td>1a</td>
<td>$8 \times 10^4$</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>48/M</td>
<td>Endemic</td>
<td>IFN/ribavirin (48)</td>
<td>1a</td>
<td>$8 \times 10^4$</td>
<td>$\sim 1 \times 10^4$</td>
</tr>
<tr>
<td>9</td>
<td>33/F</td>
<td>Inhalation</td>
<td>IFN/ribavirin (48)</td>
<td>1§</td>
<td>$1 \times 10^6$</td>
<td>ND</td>
</tr>
</tbody>
</table>

*HCV RNA load determined by real-time RT-PCR.
†HCV RNA detected in naive (mitogen-unstimulated) PBMCs.
§HCV RNA load estimated based on band density of the amplicon after direct or nested RT-PCF/NAH analysis using 10-fold serial dilutions of rHCV UTR–E2 as a quantitative standard.
§Subtype could not be determined.
IVDU, intravenous drug user; NK, not known; ND, not detectable.
Results of this study have been described previously (Pham et al., 2004). The study was approved by the local Human Investigation Committees and samples were collected after informed consent had been obtained.

In all cases, serum and PBMC samples were stored at −80°C or in liquid nitrogen, respectively. PBMCs were isolated from whole blood by Ficoll-Hypaque (Pharmacia Biotech) gradient centrifugation (Lew & Michalak, 2001; Pham et al., 2004). Isolation of T and B cells was done on freshly prepared PBMCs. B cells were purified by positive selection using MACS magnetic beads coated with anti-CD19 antibodies and T cells were isolated from the non-B fraction by negative selection with beads carrying antibodies to CD11b, CD16, CD19, CD36 and CD56 (Miltenyi Biotec). Flow cytometry with anti-CD19 or anti-CD3 monoclonal antibody (both from Chemicon International) confirmed that the purity of the isolated B cells was above 98% and that of T cells above 97%.

Previous investigations of occult infections with WHV (Coffin & Michalak, 1999; Michalak et al., 1999, 2004) and HCV (Pham et al., 2004) showed that stimulation of lymphoid cells with mitogens could significantly enhance trace virus replication allowing detection of virus in cells that were supposedly non-reactive. To determine the most effective conditions augmenting HCV RNA in PBMCs, we assessed the effect of mitogens stimulating T and B cells and cytokines supporting effective conditions augmenting HCV RNA in PBMCs, we assessed the expression of cytokines with mitogens, either alone or in combination with each other. The following were tested: PHA (ICN Biomedicals), a T-cell proliferation inducing mitogen; pokeweed mitogen (PWM; ICN Biomedicals), a B- and T-cell proliferation-inducing lectin; IL2 (Roche Molecular Diagnostics), a T-cell-supportive cytokine; and IL4 (Roche Molecular Diagnostics), a B-cell-supportive cytokine (Table 2). Thus, 5 x 10^5 PBMCs or T or B lymphocytes were cultured for 72 h in supplemented RPMI 1640 (Pham et al., 2004) in the presence of mitogens and/or cytokines at the concentrations indicated in Table 2.

Detailed protocols for RNA extraction and detection of HCV positive- and negative-strand RNA by direct and, if required, nested PCR with primers specific for the HCV 5'-untranslated region (5'-UTR) have been described previously (Pham et al., 2004). Specificity of the RT-PCR products and validity of the controls were routinely confirmed by NAH using a recombinant HCV UTR–E2 (rHCV UTR–E2) probe (Pham et al., 2004). The sensitivity of the nested RT-PCR/NAH assay for positive-strand detection was ≤10 vge ml⁻¹ and for negative-strand detection was 100 vge ml⁻¹ with the latter non-specifically detecting ≥10^9 vge ml⁻¹ of the incorrect strand. Ten-fold serial dilutions of HCV positive- and negative-strand synthetic RNA (sRNA) fragments were used as quantitative standards and to confirm the specificity of detection (Pham et al., 2004). A sample containing water instead of test cDNA and a mock sample were routinely included as contamination controls, with cDNA from PBMCs from a healthy donor as a negative control.

To assess the relative increase in HCV RNA expression in lymphoid cells after mitogen/cytokine treatments, PCR products (20 μl) of test samples and known amounts of HCV positive-strand sRNA were 10-fold serially diluted and immobilized by microfiltration on to a nylon membrane (Hybond; Amersham Biosciences). Blots were then hybridized to the rHCV UTR–E2 probe (Pham et al., 2004).

In some instances, our in-house real-time RT-PCR assay using the LightCycler Fast Start Master Hybridization Probes (Roche) specific for HCV 5'-UTR (Pham et al., 2004) was used to quantify HCV RNA levels in sera and PBMCs of the individuals investigated. Ten-fold serial dilutions of rHCV UTR–E2 were used for enumeration of viral load.

In cases where the cell number was sufficient and HCV RNA was detectable after stimulation with Combo 5, detection of HCV NS3 protein by flow cytometry was attempted. For this purpose, 5 x 10^5 PBMCs cultured with or without Combo 5 were permeabilized with 100 μl 0.1% saponin in PBS, pH 7.4, containing 1% BSA, 1 mM CaCl₂, 1 mM MgSO₄, 0.05% NaN₃ and 10 mM HEPES for 1 h at room temperature. The cells were then exposed to mouse anti-HCV NS3 IgG, monoclonal antibody (ViroGen) or to a relevant immunoglobulin isotype control for 1 h at 4°C. Following washing with permeabilization buffer, cells were incubated with FITC-conjugated goat anti-mouse IgG and IgM antibody (Jackson Immunoresearch Laboratories), washed, fixed in 1% paraformaldehyde and analysed using a FACS Calibur cytometer (BD Biosciences Pharmingen). The results were interpreted with the help of the CellQuest Pro software (BD Biosciences).

### RESULTS

The main aim of this study was to establish conditions allowing maximal augmentation of HCV RNA expression in circulating lymphoid cells from individuals with either symptomatic infection or after recovery from CHC. Hence, PBMC samples from both groups were subjected to treatment with mitogens, either alone or in combination with each other or with cytokines, and extracted RNA was analysed by RT-PCR/NAH.

### Table 2. Mitogen and cytokine treatment of lymphoid cells

<table>
<thead>
<tr>
<th>Lymphoid cells</th>
<th>Treatment</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMCs</td>
<td>IL2/IL4</td>
<td>IL2, 20 U ml⁻¹; IL4, 1 ng ml⁻¹</td>
</tr>
<tr>
<td></td>
<td>PHA/IL2</td>
<td>PHA, 5 μg ml⁻¹; IL2, 20 U ml⁻¹</td>
</tr>
<tr>
<td></td>
<td>PWM</td>
<td>PWM, 5 μg ml⁻¹</td>
</tr>
<tr>
<td></td>
<td>PHA/PWM</td>
<td>PHA, 5 μg ml⁻¹; PWM, 5 μg ml⁻¹</td>
</tr>
<tr>
<td>Combo 1-25</td>
<td></td>
<td>PHA, 1-25 μg ml⁻¹; PWM, 1-25 μg ml⁻¹; IL2, 20 U ml⁻¹; IL4, 1 ng ml⁻¹</td>
</tr>
<tr>
<td>Combo 2-5</td>
<td></td>
<td>PHA, 2-5 μg ml⁻¹; PWM, 2-5 μg ml⁻¹; IL2, 20 U ml⁻¹; IL4, 1 ng ml⁻¹</td>
</tr>
<tr>
<td>Combo 5</td>
<td></td>
<td>PHA, 5 μg ml⁻¹; PWM, 5 μg ml⁻¹; IL2, 20 U ml⁻¹; IL4, 1 ng ml⁻¹</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>PHA/IL2</td>
<td>PHA, 5 μg ml⁻¹; IL2, 20 U ml⁻¹</td>
</tr>
<tr>
<td>B lymphocytes</td>
<td>PWM/IL4</td>
<td>PWM, 5 μg ml⁻¹; IL4, 1 ng ml⁻¹</td>
</tr>
</tbody>
</table>
Fig. 1 illustrates enhanced detection of the HCV genome in PBMCs from a patient with CHC (Case 1), which were virus-RNA reactive prior to treatment. Stimulation of the cells with PWM alone, PHA/IL2 or Combo 5 gave an increase in the HCV RNA level ranging from 1-4-fold (for PWM) to 3-5-fold (for Combo 5) compared with untreated PBMCs, as determined by densitometric analysis of Southern blot hybridization signals. Interestingly, in this case, treatment with PHA/IL2 led to slightly better enhancement of HCV RNA upregulation. The possibility that both T and B cells were infected and contributed to the augmented expression of virus genome was supported by the result from PBMCs treated with Combo 5 (Table 2), which was the strongest enhancer of virus expression in this case (Fig. 1). Identical treatment of healthy PBMCs did not lead to the appearance of a hybridization signal. PCR contamination controls were negative, further confirming the specificity of the augmentation detected (Fig. 1).

Based on the finding that PBMCs treated with Combo 5 showed the greatest increase in HCV RNA detection compared with cells treated with PHA/IL2 or PWM alone (Fig. 1), the effect of different concentrations of PHA and PWM in a Combo cocktail was tested. As depicted in Fig. 2, treatment of lymphoid cells from Case 2 with Combo 2-5 (Table 2) increased HCV RNA detection by 41-fold compared with untreated cells, in which HCV genome was barely detectable. Significant enhancement (26-fold) of HCV RNA was also detected in PBMCs stimulated with Combo 5. In the same experiment, culture of PBMCs with IL2/IL4 did not augment HCV RNA, whereas PHA/PWM (Table 2) gave almost a 32-fold increase in HCV RNA expression. These findings confirmed that the upregulation was mitogen, not cytokine, driven. Dot-blot hybridization analysis improved detection of the amplified HCV sequences, usually by 10-fold compared with Southern blot hybridization, and gave similar relative increases in HCV RNA detection to those determined by densitometric analysis of Southern blots (Fig. 2).

Since PBMCs from Case 2 treated with Combo 2-5 somehow yielded a stronger HCV RNA signal than cells cultured with Combo 5 (Fig. 2), the effect of Combo 1-25 (Table 2) was tested. As shown in Fig. 3(a), exposure of lymphoid cells from Case 3 to Combo 1-25 did not exert any effect on HCV RNA expression. The same result was obtained when PBMCs from another patient with CHC were cultured with...
Combo 1-25 (data not shown). These results demonstrated that HCV RNA augmentation depended upon the dose of the mitogens. Furthermore, treatment of PBMCs from the same patient (Case 3) with Combo 5 enhanced only slightly the HCV RNA level compared with Combo 2-5, and stimulation with PHA/IL2 was similarly effective (Fig. 3a). Dot-blot hybridization of the PCR products showed that Combo 5, Combo 2-5 and PHA/IL2 all produced an approximately 90-fold increase in HCV RNA expression. Nevertheless, considering the data obtained as a whole, Combo 5 was chosen as the most effective enhancer of HCV genome expression in PBMCs derived from different patients and used as a standard treatment in subsequent experiments.

To confirm whether the observed increases in HCV positive-strand RNA detection were an attribute of virus replication, the presence of the HCV negative-strand RNA was examined. As shown in Fig. 3(b), PBMCs treated with PHA/IL2, Combo 2-5 or Combo 5 showed an increase in the replicative intermediate of 20-, 52- or 55-fold, respectively. In sharp contrast, relative to untreated PBMCs, those cultured with Combo 1-25 did not show any upregulation in the level of detectable HCV negative-strand RNA. Overall, the data from this evaluation were compatible with those for the positive strand in the same PBMCs (Fig. 3a).

To determine whether augmentation of viral RNA was connected to enhanced synthesis of virus proteins, lymphoid cells from patients with CHC (Cases 4 and 5) were treated with Combo 5 and analysed for HCV RNA by RT-PCR/NAH and for the presence of NS3 protein by flow cytometry. As illustrated in Fig. 4(a), Combo 5 led to an approximately fourfold enhancement in the level of HCV RNA. Similarly, the production of NS3 protein was more evident in Combo 5-treated cells than in untreated cells, with an increase in the NS3 protein-positive cell population ranging from 3-9 to 5-3 % (Fig. 4b). In mitogen-untreated cells, up to 1 % of the cells were NS3 reactive. The fact that cells from a healthy individual gave neither a specific signal by RT-PCR/NAH nor a shift after staining with anti-NS3 antibody validated the results obtained. Incubation of Combo 5-stimulated cells with an unrelated antibody of the same isotype (IgG1) was included to account for background binding.

In an attempt to recognize the contribution of HCV RNA present in T and B cells to the total load detected in PBMCs, T and B lymphocytes were isolated from a CHC patient (Case 6), treated with PHA/IL2 and PWM/IL4, respectively, and analysed for HCV RNA by RT-PCR/NAH. Combo 5-stimulated PBMCs and, where possible, untreated cells were also included. As depicted in Fig. 5(a), mitogen treatment enhanced HCV positive-strand RNA detection by 2-2-fold.

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**Fig. 3.** Enhanced detection of HCV positive- and negative-strand RNA in lymphoid cells following stimulation with different Combo cocktails and PHA/IL2. RNA extracted from PBMCs of a patient with CHC (Case 3), which were either untreated or cultured in the presence of the indicated mitogen/cytokine combinations, were analysed for HCV positive-strand (a) or negative-strand (b) RNA by RT-PCR/NAH. Dot-blot hybridization analysis of HCV positive-strand RNA amplicons was done as outlined in Fig. 2. Contamination and positive controls were as described in Fig. 1. Serial dilutions of HCV negative-strand sRNA amplified in parallel were used as semi-quantitative standards. Dilutions of HCV positive-strand sRNA were included to confirm the specificity of the negative-strand detection. Weak hybridization signals, although not visible on the blot images, gave low DU values as shown.
in PBMCs and 7.7-fold in T cells. Due to an insufficient number of B cells, HCV RNA was evaluated only after PWM/IL4 treatment and the cells were found to be reactive. The assessment of HCV negative-strand RNA in the same RNA preparations revealed that the level of the replicative intermediate was increased by 2.2-fold in PBMCs and 3.5-fold in T cells. B cells treated with PWM/IL4 were also reactive for HCV negative-strand RNA (Fig. 5b). Therefore, these results clearly showed that both T and B cells carried the virus and supported its active replication in a patient with progressing CHC.

Since Combo 5 demonstrated a greater ability to enhance HCV replication and HCV RNA detection than PHA/IL2, which had been used as a standard treatment in our previous study (Pham et al., 2004), we wanted to determine whether this new treatment regime could also augment detection of residual virus in PBMCs from patients with resolved hepatitis C. For this purpose, PBMCs from three convalescent individuals (Table 1) were treated with Combo 5 or PHA/IL2 and analysed for HCV positive-strand RNA and, where material permitted, for the negative strand or NS3 protein. As shown in Fig. 6, mitogen-stimulated cells exhibited up to a 25-fold increase in the expression of HCV positive-strand RNA in these individuals, with the Combo 5-induced enhancement greater than that of PHA/IL2 by approximately two to fivefold. Importantly, upregulation of positive-strand expression was accompanied, wherever tested, by that of the negative strand or NS3 protein (Fig. 6). This indicated that the treatment conditions established for PBMCs from patients with CHC also increased HCV replication in lymphoid cells from
individuals who were apparently free of the virus according to current clinical and virological criteria.

**DISCUSSION**

Based on data implying that T and B lymphocytes can support HCV replication (Kato et al., 1995; Morsica et al., 1999; Shimizu et al., 1992; Sung et al., 2003) and previous experience with detection of virus in lymphoid cells in occult infections caused by hepatotropic viruses (Coffin & Michalak, 1999; Michalak et al., 1994, 1999, 2004; Pham et al., 2004), we examined the effect of mitogens activating T and B cells on HCV genome expression and replication, and on the production of HCV NS3 protein. We showed that PHA and PWM, which activate lymphocytes by binding to their sugar moiety-specific receptors, enhanced HCV RNA detection. Moreover, the upregulation of HCV positive- and negative-strand RNA was greater when cells were cultured with both mitogens, compared with those treated with either one alone (Figs 1 and 3), suggesting that the lectins acted synergistically. In some cases, the almost 100-fold increase in the detection of the positive strand after Combo 5 stimulation was accompanied by up to a 55-fold augmentation in the level of the virus replicative intermediate in the same cells (Fig. 3). These data clearly demonstrated that upregulation was due to enhanced virus replication and they argue against the possibility that the treatment led to nonspecific virus adsorption or uptake by lymphoid cells. Although IL2 and IL4 had no influence per se on the heightened HCV RNA detection (Fig. 2), they were included in the Combo 5 cocktail since their presence is known to support T- and B-cell survival (Brams et al., 1993; Marrack et al., 1998). It was also evident that virus replication and expression were augmented to a variable degree in PBMCs from different individuals (see Figs 2 and 3) and that the magnitude of the response was usually lower in cases where the cells were HCV RNA reactive prior to the treatment. Furthermore, the fact that both T and B lymphocytes can support HCV replication was ascertained by the detection of HCV positive- and negative-strand RNA in T and B cells that were affinity-purified from a patient with CHC and treated ex vivo with appropriate mitogen and cytokine cocktails.

Due to limitations in the clinical material available, evaluation of both HCV positive- and negative-strand RNA and NS3 protein were only rarely possible in the same PBMC sample. Nevertheless, it should be emphasized that the treatment conditions established in this study, i.e. culture of PBMCs with Combo 5 for 72 h, consistently resulted in greater detection of at least two of the three viral parameters mentioned above, indicating that our findings were not random observations.

The ability of PHA and PWM to regulate the expression and replication of the viral genome positively is not unique to HCV, as similar observations have been made for other viral pathogens, including measles virus (MV) (Hyypia et al., 1985; Lucas et al., 1978), human herpesvirus 6 (Frenkel et al., 1990), cytomegalovirus (Braun & Reiser, 1986), human immunodeficiency virus type 1 (HIV-1) (Gowda et al., 1989) and feline immunodeficiency virus (FIV) (Joshi et al., 2004). In the case of MV and FIV, it has been shown that activation of latently infected lymphocytes by PHA or
Concanavalin A (ConA) gave a 5–15-fold increase in the number of virus-producing cells relative to that seen in unstimulated cells (Joshi et al., 2004; Lucas et al., 1978). Furthermore, infectious MV was released only by infected lymphoid cells treated with PHA, and the treated cells were able to establish a productive infection with features comparable to those of the lytic infection. It was postulated that lymphocytes not challenged by mitogen could persistently harbour the virus without being recognized as targets by immune effector mechanisms (Lucas et al., 1978). The fact that mitogen stimulation can enhance productive virus replication suggests that, for example, a coincidental activation of lymphocytes may play a role in the reactivation of latent infection or augment pathogenicity of the ongoing infection. In the case of HCV, virus reactivation in PBMCs has been reported following liver transplantation (Radkowski et al., 1998) or as a result of immunosuppression due to chemotherapy (Melisko et al., 2004) or HIV infection (Laskus et al., 1998).

Although molecular mechanisms underlying the ability of certain mitogen/cytokine combinations to upregulate HCV replication are not clear and will require separate studies, it is unlikely that the augmented HCV RNA expression was an attribute of cell proliferation, since we did not see a correlation between the rate of lymphoid-cell proliferation and the degree to which HCV genome detection was enhanced (data not shown). This discrepancy could be somehow similar to that recently described for different CD4⁺ T-lymphocyte subpopulations latently infected with FIV (Joshi et al., 2004). Specifically, it was found that although CD4⁺CD25⁺ T cells could proliferate efficiently in response to either IL2 or ConA, virus reactivation was only seen in cells treated with ConA. However, the reverse was true for CD4⁺CD25⁺ T cells, in which virus replication, but not cell proliferation, was seen following stimulation with ConA.

The enhanced detection of HCV positive- and negative-strand RNA in PBMCs after apparent complete resolution of
HCV infection observed in this study confirms our earlier findings (Pham et al., 2004), which demonstrated HCV persistence in circulating lymphoid cells during long-term follow-up of individuals with spontaneous resolution of acute hepatitis C or SVR following antiviral treatment of CHC. Furthermore, the current data clearly showed that treatment with mitogens inducing both T and B lymphocytes (i.e. Combo 5) resulted in a substantially greater detection of the virus genome in lymphoid cells in both study groups over that observed after PHA/IL2 treatment. Taking into consideration the fact that the amounts of HCV RNA and viral proteins are very low in hepatic tissue, even in active CHC (Bartenschlager & Lohmann, 2000), examination of PBMCs after their ex vivo treatment using conditions established in this study might be advantageous for identifying HCV occult infection. As such, it could also be used for monitoring HCV replication during the entire course of infection, including the pre-acute and acute phases, and potentially applied to the assessment of the efficacy of sterilizing antiviral therapy. Further investigations are required to validate fully the applicability of such evaluations for clinical purposes.

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REFERENCES


Castillo, I., Pardo, M., Bartolome, J. & 9 other authors (2004). Occult hepatitis C virus infection in patients in whom the etiology of persistently abnormal results of liver-function tests is unknown. J Infect Dis 189, 7–14.


