Infection of primary human macrophages with hepatitis C virus \textit{in vitro}: induction of tumour necrosis factor-\textalpha{} and interleukin 8

Marek Radkowski,\textsuperscript{1,3} Agnieszka Bednarska,\textsuperscript{2} Andrzej Horban,\textsuperscript{2} Janusz Stanczak,\textsuperscript{2} Jeffrey Wilkinson,\textsuperscript{3} Debra M. Adair,\textsuperscript{3} Marek Nowicki,\textsuperscript{4} Jorge Rakela\textsuperscript{3} and Tomasz Laskus\textsuperscript{3}

\textsuperscript{1}Institute of Infectious Diseases, Warsaw Medical Academy, Poland
\textsuperscript{2}Municipal Hospital for Infectious Diseases, Warsaw, Poland
\textsuperscript{3}Department of Medicine, SC Johnson Bldg Sj3, Mayo Clinic Scottsdale, AZ 85259, USA
\textsuperscript{4}Maternal–Child Virology Research Laboratory, University of Southern California, Los Angeles, CA 90033, USA

Hepatitis C virus (HCV) has been reported to replicate in monocytes/macrophages in infected patients. However, it is unclear whether macrophages are susceptible to infection \textit{in vitro} and whether such an infection is consequential. Sera from 26 HCV-infected patients were incubated with primary human macrophages collected from healthy donors. Virus negative strand was detected by a \textit{Tth} enzyme-based strand-specific assay and virus sequences were analysed by single strand conformation polymorphism (SSCP) and sequencing. Concentrations of the cytokines tumour necrosis factor-\textalpha{} (TNF-\textalpha{}) and interleukin (IL)-1\textbeta{}, IL-6, IL-8, IL-10 and IL-12p70 were measured in culture supernatants and respective mRNAs were analysed in cell extracts by quantitative RT-PCR. For 15 sera, HCV RNA was detectable in 2- and 3-week cultures from at least one donor. Virus negative strand was detected in 29\% of macrophage samples in this group. In four cases, HCV RNA sequences amplified from macrophages differed from those amplified from sera suggesting evolution during infection. Concentrations of TNF-\textalpha{} and IL-8 were found to be significantly higher in supernatants from HCV-infected cultures. In conclusion, these preliminary data suggest that primary human macrophages are susceptible to HCV infection \textit{in vitro} and this infection is associated with the induction of cytokines TNF-\textalpha{} and IL-8.

\underline{INTRODUCTION}

Hepatitis C virus (HCV) was originally thought to be a strictly hepatotropic virus, but there is mounting evidence that it can also replicate in peripheral blood mononuclear cells (PBMC), particularly under conditions of immunodeficiency associated with human immunodeficiency virus type 1 (HIV-1) infection. The infected cells were reported to contain HCV RNA negative strand, which is a virus replicative intermediate, and virus genomic sequences were often found to be distinct from those found in serum and liver (Laskus \textit{et al}., 1998, 2000b; Lerat \textit{et al}., 1998; Navas \textit{et al}., 1998). The presence of HCV replication was also documented in haematopoietic cells inoculated into severe combined immunodeficiency (SCID) mice (Bronowicki \textit{et al}., 1998). Furthermore, it was also reported that human T and B cell lines are capable of supporting HCV infection \textit{in vitro} (Nakajima \textit{et al}., 1996; Shimizu \textit{et al}., 1993) and some virus strains were found to be lymphotropic both \textit{in vitro} and \textit{in vivo} in infected chimpanzees (Shimizu \textit{et al}., 1997).

Within the population of PBMC, the cells harbouring replicating virus have been identified as belonging to monocytes/macrophages, as well as T-cell and B-cell lineages (Bain \textit{et al}., 2001; Laskus \textit{et al}., 2000a; Sansonno \textit{et al}., 1996). Whether the infection of macrophages is consequential is currently unclear; it was reported that chronic infection by HCV is associated with an allostimulatory defect and impaired maturation of monocyte-derived dendritic cells (Auffermann-Gretzinger \textit{et al}., 2001; Bain \textit{et al}., 2001; Kanto \textit{et al}., 1999). However, it is impossible to differentiate between changes caused by virus replication itself and those due to factors released from other cells of the immune system and/or from infected liver. Furthermore, although the presence of replication of HCV in macrophages in patients with chronic infection is highly likely, it is unclear whether these cells are susceptible to infection \textit{in vitro}. Moreover, it is unclear whether macrophage infection affects the infected cell. The current study suggests that primary human macrophages can be infected by HCV \textit{in vitro} and that the ensuing infection leads to induction of
tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interleukin 8 (IL-8) in the infected cells.

**METHODS**

**Biological samples.** Sera used for macrophage infections were drawn from 26 HCV-infected patients who presented for clinical care at participating centres. The inclusion criteria were: HCV RNA positivity and hepatitis G virus (HGV) RNA negativity in serum and no anti-HCV or anti-HIV antiviral therapy prior to the study. Neither HCV virus load nor infecting genotype was known at that time. Seven patients, all of them intravenous drug users, were co-infected with HIV-1. Sera from 12 HCV-negative and HIV-negative subjects were used in control experiments.

Monocytes/macrophages were collected from 10 healthy donors. Specifically, they were all anti-HCV and anti-HIV negative in serum and HCV RNA, hepatitis B virus (HBV) DNA and HGV RNA negative both in serum and in unfractioned PBMC. To obtain monocytes/macrophages, PBMCs were isolated from 50 ml of blood by centrifugation over a density gradient (Ficoll-Hypaque, Pharmacia). Cells were removed from the upper band located at the plasma and resolving medium interface and washed three times with Mg\(^{2+}\) and Ca\(^{2+}\)-free PBS (pH 7.4). The typical yield of PBMCs was 2–4 \(\times\) 10⁵; these cells were determined to be free of granulocytes and platelets. To separate monocytes/macrophages from other cells, PBMCs were resuspended in 10 ml RPMI 1640 medium containing 10 % foetal bovine serum (FBS) and incubated in plastic six-well cell culture dishes (Costar), 1·5 ml of suspension per well. After incubation at 37 °C for 1 h, non-adhering cells were removed by vigorous washing four times with Mg\(^{2+}\)- and Ca\(^{2+}\)-free PBS while adhering cells (approximately 2 \(\times\) 10⁵ cells per well) were maintained in RPMI 1640 (Gibco/BRL) with 10 % FBS. The typical purity of the cells was >95 % based on morphological features in light microscopy and flow cytometry staining analysis of detached cells using monoclonal anti-CD68 and anti-CD14 antibodies. After 24 h, the macrophages were incubated for 4 h with HCV-positive serum diluted 1:10 in RPMI 1640 with occasional agitation. Subsequently, the cells were washed 3 \(\times\) 10 min with PBS and maintained in RPMI with 10 % FBS. The culture medium was changed every 3–4 days.

Each of the HCV-positive sera was incubated with three independent macrophage cultures from two different donors (six cultures total for each serum). RNA was extracted from macrophages after 1, 2 or 3 weeks of culture and stored at −80 °C until analysis. RNA was extracted from cells and 100 \(\mu\)l of serum by means of a modified guanidinium thiocyanate/phenol/chloroform technique using a 10-fold serial dilutions of the RNA template. The titres were calculated by assuming that the end-point dilution contains 10⁵ genomic equiv. when tested with the \(T_hh\)-based assay. Specificity controls, which consisted of serial dilutions of synthetic template, were included in each series of experiments.

**Standard RT-PCR.** MMLV RT-based amplification of the 5′ UTR has been described in detail previously (Laskus et al., 1998). This assay was capable of detecting approximately 10 genomic equiv. of the correct synthetic template but was not strand specific. Similarly to \(T_hh\)-based assay, the addition of cellular RNA would slightly lower the sensitivity by up to one log. The established detection limit was approximately 10−100 genomic equiv. per 1 \(\mu\)l of total RNA. In serum the approximate detection limit was 100 genomic equiv. per 1 ml. The non-structural (NS) 5B region was amplified by RT-PCR as described previously (Laskus et al., 2001).

Appropriate measures, described elsewhere (Laskus et al., 1997, 1998), were employed to prevent and detect contamination. Nested protocols, which are prone to carry over contamination, were not used for detection purposes. All RT-PCR runs included positive controls, consisting of end-point dilutions of respective RNA strands, and negative controls, which included macrophages from uninfected subjects and normal sera.

**Analysis of HCV quasispecies.** The analysis was conducted on the stable 5′ UTR and relatively stable NS5B region because a small number of expected virus variants within quasispecies allows for reliable comparison, and we have previously found that variations in the 5′ UTR may correlate with extrahepatic replication (Laskus et al., 1998, 2000a). In addition, comparison of the highly variable E2 region may be unreliable due to selective adsorption by human cells of virus quasispecies variants differing in the E2 region (Laskus et al., 2000b). For the purpose of sequence comparison, nested protocols were used to maximize the yield of PCR product. Amplification of the 5′ UTR and NS5B regions was conducted with RT-PCR assay as previously described (Laskus et al., 1998, 2001).

HCV quasispecies were compared by the single-strand conformation polymorphism (SSCP) assay as described previously (Laskus et al., 1998), with minor modifications. In brief, PCR products were purified with a DNA-binding resin system (Wizard PCR, Promega) and resuspended in 50 \(\mu\)l of water. Next, 2–2 \(\mu\)l of the purified product was diluted in 15 \(\mu\l\)l of low ionic strength solution (10 % saccharose, 0·5 % bromophenol blue, 0·5 % xylene cyanol), denatured by heating at 97 °C for 3 min, immediately cooled on ice and subjected to non-denaturing 8 % PAGE in 1 \(\times\) Tris/borate/EDTA buffer with 400 V applied for 5–6 h at a constant temperature of 25 °C. The bands were visualized with silver staining (Silver Stain, Promega). This assay enables detection of minor variants representing 3 % of the whole population (Laskus et al., 1998).
All analysed products were sequenced directly using Perkin Elmer ABI 377 automatic sequencer. HCV genotypes were determined by direct sequencing of the NS5B region (Simmonds et al., 1993). HCV RNA was quantified using branched DNA assay (Quantiplex HCV-RNA 2.0, Roche).

**Cytokine assays.** Concentrations of cytokines TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12p70 were measured in cell culture supernatants using a commercially available Cytometric Bead Array (BD). In this assay soluble analytes are captured on particles and then measured using a fluorescence-based detection system and flow cytometry analysis (Cook et al., 2001; Oliver et al., 1998).

The mRNAs of the above cytokines were quantified using the multiplex PCR (MPCR) kit marketed by Maxim Biotech, Inc. When the initial analysis suggested the presence of quantitative mRNA differences, analysis was repeated using Dual Quantitative PCR (Maxim Biotech, Inc.), which coamplifies only the housekeeping gene and the gene of interest. Amplified products were fractioned on an agarose gel, stained with ethidium bromide and quantified using the Bio-Rad UV camera and analysis software (Quantity One version 4.1.1, Bio-Rad). For comparison, gene expressions were normalized against the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

The relative percentage of target specific gene expression was calculated as follows: 

\[
\frac{X}{Y} \times 100 \% \text{ normalized target specific gene expression,}
\]

where \(X\) = target specific gene expression level and \(Y\) = housekeeping gene expression level.

For confirmation of the above assay, which is largely semi-quantitative, transcripts of TNF-α and IL-8 were quantified by real-time RT-PCR with SYBR Green I detection. The assays were performed with a LightCycler (Roche) with single-round amplification. Reaction mixtures included 2 μl of Fast Start DNA Master SYBR Green I (Roche), 4 mM MgCl₂, 0.5 μM concentration of each primer and 2 μl of the template to a total volume of 20 μl. After initial incubation at 95 °C for 10 min, 40 cycles of amplification were performed each cycle consisting of 30 s at 95 °C, 5 s at 55 °C, and 30 s at 72 °C. Specificity of the signal was checked by melting curve analysis and target specific gene expression was normalized against histone mRNA (H3F3A). The following primers were used: H3F3A (sense 5′-CCACTGAAACT-TCTGATTGC-3′; antisense 5′-GGCTGCTAGCTGGATGTCTT-3′), IL-8 (sense 5′-GACGGCGATGCGGCTGATG-3′; antisense 5′-CCATCCCTGGTTCTCCTTGC-3′), TNF-α (sense 5′-GGCTCAAGCCGTTG-GCTCCATTTC-3′; antisense 5′-AGAGCCGGAATGCGCTGATG-3′).

NCBI accession numbers for H3F3A, IL-8 and TNF-α genes, which were the basis for designing of the primers, were NC_002107, BC013615, and AY214167, respectively.

Statistical analysis was performed using the SPSS version 9.0 package. Means were compared by the non-parametric Mann–Whitney U test and paired data were compared by Wilcoxon signed rank sum test. Proportions were analysed by Fisher’s exact test.

**RESULTS**

**HCV RNA in macrophage cultures**

Each of the 26 HCV-positive sera was incubated with macrophages from two different healthy donors and each culture was done in triplicate resulting in 156 cultures overall. Cultures were terminated and RNA was extracted after 1, 2 and 3 weeks. For serum samples #1–11 either no HCV RNA was present in macrophages or it was detected in the 1-week culture only (Table 1). These samples were considered to be uninfected and the occasional presence of HCV RNA at seventh day of culture was probably due to the small amount of serum virus remaining after incubation. HCV RNA negative strand was not detected in any of the macrophage samples in this group.

In the case of the remaining 15 sera (sera #12–26), HCV RNA was detectable in macrophages cultured for 3 weeks from at least one donor and, for nine of these sera, macrophages from both donors tested positive. Virus negative strand was detected in 26 (29 %) of macrophage samples in this group (Table 1). These reactions were unlikely to represent false-positive results because non-specific detection of the incorrect strand might be expected when the latter is present at a concentration of at least 10⁶ genomic equiv. per reaction. However, the concentration of HCV RNA in samples containing virus negative strand was no more than 10⁴ genomic equiv. per reaction as determined by testing serial dilutions of extracted RNA.

To determine whether infection is reproducible, sera #14 and #15 were used to infect macrophages from two additional donors (Table 1). Virus positive strand was detectable in all cell cultures and virus negative strand was detectable in the majority of the samples suggesting that the implicated sera were infectious for macrophages from different donors. Furthermore, HCV RNA was detected in supernatant from every cell culture infected with sera #14 and #15 although the titre was low (5 x 10⁵–5 x 10⁶ genomic equiv. ml⁻¹). To determine the ratio of positive to negative strands, 10-fold serial dilutions of RNA extracted from macrophages incubated with serum #14 were tested for the presence of positive and negative HCV RNA by the Tth-based RT-PCR. The titre of virus negative strand was found to be one log lower than the titre of the positive strand (data not shown).

The two groups did not differ with respect to the distribution of HCV genotypes; type 1b was dominant as it was present in 12 out of 15 (80 %) and in 8 out of 11 (73 %) infective and non-infective sera, respectively. The second prevalent genotype was 3a and it was present in two sera from each group (Table 1). However, the infective sera had a higher mean virus load than the non-infective sera (7.3 x 10⁸ versus 1.9 x 10⁶ copies ml⁻¹) and this difference was statistically significant (P<0.02). An important role of virus titre is further suggested by sera dilution experiments. Both sera #14 and #15, while infectious at dilution 1:10, did not infect macrophages when diluted 1:1000. Presence of HIV coinfection could have facilitated HCV infection as the mean HCV RNA load was lower in HIV positive sera transmitting infection when compared to HIV-negative sera transmitting infection (2.2 x 10⁶ versus 9.8 x 10⁶ copies ml⁻¹). However, as the HIV-coinfected group was small, this difference did not reach statistical significance. HIV itself did not infect macrophages as all culture supernatants tested negative for the p24 antigen.
Table 1. Detection of HCV RNA in primary human macrophages after incubation with 26 HCV-positive sera

<table>
<thead>
<tr>
<th>No.</th>
<th>HCV RNA titre (equiv. ml(^{-1}))</th>
<th>HIV status</th>
<th>Genotype</th>
<th>Macrophage donor</th>
<th>HCV RNA in macrophages (weeks after infection)</th>
<th>Sequence changes during infection (region)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1.</td>
<td>6.79 × 10^5</td>
<td>Pos.</td>
<td>1b</td>
<td>A</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2.</td>
<td>1.67 × 10^6</td>
<td>Neg.</td>
<td>3a</td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>3.</td>
<td>7.04 × 10^6</td>
<td>Neg.</td>
<td>1b</td>
<td>A</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4.</td>
<td>2.21 × 10^5</td>
<td>Neg.</td>
<td>3a</td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>5.</td>
<td>8.68 × 10^6</td>
<td>Neg.</td>
<td>1b</td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>6.</td>
<td>&lt;2.00 × 10^5</td>
<td>Neg.</td>
<td>1b</td>
<td>A</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7.</td>
<td>4.07 × 10^5</td>
<td>Pos.</td>
<td>4d</td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>8.</td>
<td>8.55 × 10^5</td>
<td>Neg.</td>
<td>1b</td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>9.</td>
<td>4.90 × 10^5</td>
<td>Neg.</td>
<td>1b</td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>10.</td>
<td>7.65 × 10^5</td>
<td>Neg.</td>
<td>1b</td>
<td>A</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>11.</td>
<td>2.68 × 10^5</td>
<td>Neg.</td>
<td>1b</td>
<td>A</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>12.</td>
<td>7.0 × 10^6</td>
<td>Neg.</td>
<td>1b</td>
<td>A</td>
<td>+*</td>
<td>+*</td>
</tr>
<tr>
<td>13.</td>
<td>3.7 × 10^6</td>
<td>Neg.</td>
<td>1b</td>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14.</td>
<td>9.5 × 10^6</td>
<td>Neg.</td>
<td>1a</td>
<td>A</td>
<td>+*</td>
<td>+*</td>
</tr>
<tr>
<td>15.</td>
<td>5.5 × 10^7</td>
<td>Neg.</td>
<td>1b</td>
<td>A</td>
<td>+*</td>
<td>+*</td>
</tr>
<tr>
<td>16.</td>
<td>9.0 × 10^6</td>
<td>Neg.</td>
<td>1b</td>
<td>A</td>
<td>+*</td>
<td>+*</td>
</tr>
<tr>
<td>17.</td>
<td>2.62 × 10^6</td>
<td>Pos.</td>
<td>3a</td>
<td>A</td>
<td>+</td>
<td>+*</td>
</tr>
<tr>
<td>18.</td>
<td>1.60 × 10^6</td>
<td>Neg.</td>
<td>1b</td>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>19.</td>
<td>1.80 × 10^6</td>
<td>Neg.</td>
<td>1b</td>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>20.</td>
<td>3.12 × 10^6</td>
<td>Pos.</td>
<td>1b</td>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>21.</td>
<td>2.16 × 10^6</td>
<td>Neg.</td>
<td>1b</td>
<td>A</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>22.</td>
<td>1.06 × 10^6</td>
<td>Pos.</td>
<td>3a</td>
<td>A</td>
<td>+</td>
<td>+*</td>
</tr>
<tr>
<td>23.</td>
<td>&lt;2.0 × 10^5</td>
<td>Neg.</td>
<td>1b</td>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

M. Radkowski and others
Analysis of HCV RNA in macrophage cultures by SSCP and sequencing

In the next step virus sequences amplified from cultured macrophages were compared by SSCP with sequences amplified from respective serum samples. In the vast majority of cases, SSCP band patterns between serum and cultured macrophages were indistinguishable, suggesting the presence of identical virus variant. However in four cases, virus sequences present in the macrophage cultures were different than those amplified from the infective serum either in the 5′ UTR or NS5B region. These differences were not present in 1-week culture and were found only in macrophages grown for the duration of 2–3 weeks. In cases where virus negative strand could be amplified, its SSCP band pattern was congruent with the positive strand, suggesting that it indeed represented a virus replicative intermediary. Fig. 1 shows SSCP analysis in the example case of serum #14 where the band patterns for serum- and macrophage-derived virus sequences for both 5′ UTR and NS5B regions were identical, and all four cases with dissimilar band patterns in either of these regions.

Table 1. (cont.)

<table>
<thead>
<tr>
<th>Infecting serum</th>
<th>HCV RNA in macrophages (weeks after infection)</th>
<th>Sequence changes during infection (region)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>HCV RNA titre (equiv. ml⁻¹)</td>
<td>HIV status</td>
</tr>
<tr>
<td>24.</td>
<td>7.48 × 10⁵</td>
<td>Pos.</td>
</tr>
<tr>
<td>25.</td>
<td>3.60 × 10⁶</td>
<td>Pos.</td>
</tr>
<tr>
<td>26.</td>
<td>7.90 × 10⁶</td>
<td>Neg.</td>
</tr>
</tbody>
</table>

*Negative strand HCV RNA detected by strand-specific RT-PCR.
NA, Not applicable.

Fig. 1. Analysis by SSCP of HCV sequences amplified from macrophages exposed to HCV-positive sera and cultured for 1, 2 and 3 weeks. In the case of serum #14 the macrophage-derived strain was identical to the one amplified from infecting serum. HCV RNA negative strands were detected in each culture infected with this serum. In the case of sera #17 and #23, virus sequences amplified from cultured macrophages differed from respective serum-derived sequences in the 5′ UTR, while in the case of sera #20 and #24, virus sequences differed in the NS5B region. The band pattern was identical to the serum pattern in 1-week culture, but became dissimilar in longer term cultures suggesting evolution of infecting virus strain. However, NS5B region could not be amplified from 3-week donor B macrophage culture infected with serum #20. The presence of identical and dissimilar virus sequences in the analysed samples was verified by direct sequencing. –, Negative strand; w, week of culture; A, macrophage donor A; B, macrophage donor B.

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The presence of identical and dissimilar sequences in macrophage cultures and respective sera used for infection was confirmed by direct sequencing. In case #17, the macrophage-derived virus differed in the 5' UTR from the serum-derived virus by a single nucleotide substitution. However, these differences were identical for each of the 2- and 3-week macrophage cultures from both donors (Fig. 2). In case #23, the serum-derived virus differed from the macrophage sequence by two and three nucleotide substitutions at 2 and 3 weeks, respectively (Fig. 2). The NS5B region sequence recovered from infecting serum #20 differed by one nucleotide substitution from the sequence recovered from donor A macrophages, and by three nucleotide substitutions from the sequences recovered from donor B macrophages (Fig. 3). All these changes were silent. In case #24, the macrophage-derived sequence differed from the serum sequence by two nucleotide substitutions, which were predicted to change the amino acid sequence at positions 2728 and 2731, respectively (Fig. 3).

In two out of four cases with sequence changes, macrophages from both donors were infected. In case #17 virus sequences in macrophages from both donors were identical, while in case #20 they were different. However, as individual cultures were independent of each other and macrophages were derived from two distinct donors, various outcomes are not entirely unexpected.

**Secretion and expression of cytokines by infected macrophages**

To determine whether infection of macrophages in vitro entails any biological effect, we measured the concentrations of TNF-α, IL-1ß, IL-6, IL-8, IL-10, and IL-12p70 in cell culture supernatants at 2 weeks. The timing was not chosen arbitrarily: in 1-week cultures we could not determine whether the infection was successful or not, as evidenced by the common presence of HCV RNA in cases where no later cultures tested positive (Table 1), while in the 3-week cultures the cells were already undergoing normal death. Altogether, 63 2-week culture supernatants were available for analysis. The concentrations of IL-1ß, IL-6, IL-12p70 were below the cut-off value of the assay in 90–95% of the analysed samples and these cytokines did not undergo any further analysis. However, as shown in Fig. 4(A), the concentrations of TNF-α and IL-8 were significantly higher in cases with putative HCV infection compared to uninfected cases. Similarly, IL-10 concentration was also higher in supernatants from infected macrophages, but this difference did not reach statistical significance ($P=0.07$).

To confirm the above findings, a different set of experiments was devised. Sera from 16 HCV-infected patients containing high-titre virus load ($>5 \times 10^6$ virus equiv. ml$^{-1}$) and from 12 uninfected controls were incubated with macrophages from healthy donors as described above. In parallel, a second set of identical experiments was conducted using the same sera heated to 60 °C for 4 h in a water bath to render the virus non-infectious (Nowak et al., 1993). Therefore, in these experiments each serum was the control for itself, lowering the likelihood that the previously observed differences in cytokines production were related to some serum factors and not necessarily to HCV infection itself. After 7 days, HCV RNA was uniformly detected in the macrophages incubated with non-inactivated serum samples and in the majority of heat-inactivated samples as well. The latter results are compatible with observation that pasteurization, while destroying the infectivity of HCV, does not affect its detectability by RT-PCR (Hilfenhaus et al., 1997). Thus, HCV RNA detected in macrophages incubated with inactivated sera probably represented inactivated virus adsorbed on, or trapped within, the cells. The cytokine concentrations were determined in 1-week culture supernatants, and TNF-α and IL-8 were found to be significantly higher in non-heated sera macrophage cultures than in heat-inactivated sera cultures. This difference was even more pronounced when analysis was limited to 10 cases in which HCV RNA negative strand was detectable (Fig. 4B). However, as illustrated in Fig. 4(C), this effect was not observed for control HCV-negative sera, suggesting that the observed differences between HCV-positive non-inactivated and inactivated sera were not related to the heating process itself.

To determine whether there are differences in cytokine transcription levels, mRNAs for TNF-α, IL-1ß, IL-6, IL-8, IL-10 and IL-12 were quantified using the multiplex PCR (MPCR). Except for TNF-α and IL-8, mRNAs for other cytokines were amplified only occasionally and were therefore not analysed further. For quantification purposes, TNF-α and IL-8 mRNA was RT-PCR amplified using Dual Quantitative PCR, which amplifies only the housekeeping gene and the gene of interest. The mRNA for the cellular enzyme GAPDH was monitored as an internal control to allow comparison of transcript levels between macrophages incubated with infective and heat-inactivated sera. Under the conditions used, both TNF-α and IL-8 gene expression was in a linear range as determined on serial dilutions of the template. Sufficient quality amplification allowing for analysis was available in all 16 HCV-positive cases and in 9 out of 12 HCV-negative controls. As seen in Fig. 5, the quantity of amplified mRNA was lower in heat-inactivated serum cultures than in paired non-inactivated serum macrophage cultures. These differences were even more pronounced when transcripts were analysed by real-time RT-PCR. Again, parallel analysis using HCV-negative sera did not reveal significant differences between activated and inactivated samples, suggesting that the observed differences were not related to the process of heating per se (Fig. 5).

**DISCUSSION**

In a previous study we reported on the presence of virus replicative forms in monocytes/macrophages from HCV-infected patients (Laskus et al., 2000a). The current study...
HCV-1

Serum 17
Donor A 1w
2w (−)
3w

Donor B 1w
2w
3w

Serum 23
Donor A 1w
2w
3w

--- C--- TG--- GT--- G--- A--- CA--- A--- --- TCA---
--- C--- TG--- GT--- G--- A--- CA--- A--- --- TCA---
--- C--- TG--- GT--- G--- A--- CA--- A--- --- TCA---
--- C--- TG--- GT--- G--- A--- CA--- A--- --- TCA---
--- C--- TG--- GT--- G--- A--- CA--- A--- --- TCA---
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Fig. 2. Nucleotide sequence alignment of the 5' UTR fragments of HCV recovered from sera #17 and #23 and macrophage cell cultures exposed to these sera. The 5' UTR sequences are compared with the prototype type 1a sequence published by Choo et al. (1991) shown on the top line. −, Sequence identity; _ gap introduced to preserve sequence integrity; (−), negative strand; w, week of culture.
**Fig. 3.** Nucleotide sequence alignment of NS5B fragments of HCV recovered from sera #20 and #24 and from cultured macrophages exposed to these sera. –, Sequence identity; w, week of culture.
HCV infection of macrophages

**Fig. 4.** A) TNF-α, IL-10 and IL-8 concentrations in supernatants of human macrophage cultures 2 weeks after exposure to HCV-positive sera. Twenty-nine cultures were considered infected as HCV RNA was detectable in cells, while 34 cultures were considered uninfected as the cells were HCV RNA negative. B) TNF-α, and IL-8 production by cultured human macrophages exposed to 16 untreated and heat-inactivated high-titre HCV-positive sera. A second set of analyses includes only 10 samples in which HCV RNA negative strand was detectable. C) TNF-α, and IL-8 production by cultured human macrophages exposed to untreated and heat-treated 12 HCV-negative control sera. All bars represent the means ± SEM. Readings below the cut-off were arbitrarily assigned the lower detection limit value of 2 pg ml$^{-1}$ for IL-6 and TNF-α, and 20 pg ml$^{-1}$ in the case of IL-8. Data in (A) were analysed by Mann–Whitney U test while paired data in (B) and (C) were compared by Wilcoxon signed rank test.
extends these findings by demonstrating that native human macrophages may be susceptible to HCV infection in vitro. We showed that after exposure to infectious sera in vitro, cultured macrophages can retain HCV RNA for 3 weeks, virus negative strand RNA can be detected, and the infecting strains may undergo changes, although it is unclear whether this is due to evolution or a successful growth of a minor variant already present in the infecting serum. While the mere presence of HCV RNA in phagocytic cells could come, at least theoretically, from virions entrapped inside these cells or adsorbed on their surface, detection of virus negative strand and the occasional changes in sequence, argue for the presence of genuine virus replication. Moreover, in a number of cases, the same serum infected macrophages from only one of the two donors, which is an additional argument against virus adsorption/entrapment as the explanation for long-term HCV RNA detectability in macrophage cultures. HCV RNA negative strand was detected only in a minority of cases, however, it is likely that the strand-specific assays are not sensitive enough to

Fig. 5. Expression of IL-8 and TNF-α mRNA in cultured human macrophages exposed to infectious and heat-inactivated HCV-positive sera and to untreated and heat-treated HCV-negative control sera. Cytokine mRNAs were amplified by quantitative RT-PCR (Dual Quantitative PCR; Maxim Biotech, Inc) and real-time PCR and gene expressions were normalized against the housekeeping gene GAPDH and H3F3A, respectively, and expressed as per cent of the housekeeping gene expression. The bars represent the means ± SEM.
detect low-level extrahepatic replication. Indeed, in several studies they were found to be at least one log less sensitive than standard RT-PCR (Lanford et al., 1994, 1997). Moreover, in cells supporting HCV replication, negative RNA strands are generally detected at a level one to two logs lower than the levels of positive strands (Lanford et al., 1994, 1997), and the titre of positive strand HCV RNA in cultured macrophages was low. Infection of monocytes/macrophages by HCV is not unexpected as these cells are known to be permissive to a wide range of viruses, including some other flaviviruses (Mogensen, 1979). Nevertheless, our findings require confirmation by detection of HCV proteins in macrophages and successful passaging of the infection into new cell cultures.

While infection of monocytes/macrophages in HCV-positive patients is likely, its consequences for cell function are unclear. It was reported that monocyte-derived dendritic cells from patients with chronic HCV infection demonstrate an allostimulatory defect (Bain et al., 2001; Kanto et al., 1999). However, a direct relationship between virus replication and dendritic cell function could not be established in these studies. In the current study, in which primary human macrophages were infected in vitro and therefore the effects of factors unrelated to direct virus–macrophage interaction were likely to be eliminated, we found that exposure of primary macrophages to HCV-positive sera resulted in enhanced production of TNF-α and IL-8 and their respective mRNAs. Induction of these proinflammatory cytokines could be regarded as additional evidence for the susceptibility of human macrophages to HCV infection in vitro and suggests that such an infection is consequential to the cell.

TNF-α is a major component of the immune system involved in the control of virus infection through direct antiviral activity, usually in association with interferon-γ, and the induction of apoptosis (Herbeen & O’Brien, 2000). It is made mainly by monocytes and macrophages and its production was reported to increase when isolated human macrophages were infected with such RNA viruses as respiratory syncytial virus (Becker et al., 1991), or influenza A virus (Lehmann et al., 1996). Thus, production of TNF-α seems to be a natural response of macrophages to virus infection.

IL-8 was originally described as a neutrophil chemotactic factor, but subsequent studies demonstrated its various effects on T-cells and monocytes (Mukaida, 2000). There is growing evidence that infection with various viruses, or transfection with viral gene products, can induce IL-8 in different cells. For example, human cytomegalovirus (HCMV) infection can induce IL-8 production by concurrent activation of two distinct transcription factors, nuclear factor (NF)-κB and activator protein (AP)-1 (Murayama et al., 1997); similar mechanisms of IL-8 gene transactivation were described for human T-cell leukaemia virus type 1 Tax protein and hepatitis B virus X protein (Mahe et al., 1991; Mori et al., 1998). Interestingly, increased serum levels of IL-8 were recently found in chronic hepatitis C patients (Polyak et al., 2001b) and the same group of researchers demonstrated that expression of HCV non-structural 5A (NS5A) protein in human cells induced IL-8 mRNA and protein probably through a mechanism similar to that described previously for HCMV (Polyak et al., 2001a). Because monocytes are one of the main producers of IL-8 in vivo, they could be an important source of increased IL-8 serum levels in chronic hepatitis C patients. Notably, IL-8 itself may directly promote virus replication. It was reported that IL-8 enhances HCMV and HIV-1 replication, probably by acting through its receptors CXCR1, and CXCR1 and CXCR2, respectively (Lane et al., 2001; Murayama et al., 1994). The effect on virus replication could also be indirect, as IL-8 was reported to reduce the antiviral activities of IFN-α. This inhibitory action on IFN-α antiviral activity was associated with reduced 2’,5’-A oligoadenylate synthetase activity, a pathway well correlated with the antiviral action of IFN-α (Khabar et al., 1997). The latter mechanism has been postulated to promote HCV persistence and resistance to IFN therapy (Polyak et al., 2001b).

It has been long known that HCV RNA levels are higher in HIV-coinfected subjects and this phenomenon has been largely attributed to the HIV-induced immunosuppression with subsequent impairment of the containment effects of the immune system. However, several studies suggest a more direct interaction: HCV RNA levels were reported to be more closely associated with HIV RNA levels than with CD4+ T cell count (Thomas et al., 2001), and HIV seroconversion in HCV-infected patients is often associated with a burst of enhanced HCV replication (Beld et al., 1998). Interestingly, it was also reported that HCV infection is associated with higher HIV RNA levels and more rapid HIV disease progression (Daar et al., 2001; Greub et al., 2000). As both HIV and HCV induce IL-8 production, which in turn could enhance their replication, it could be speculated that IL-8 could play some role in HCV/HIV interactions.

In summary, our data suggest that human macrophages may be susceptible to HCV infection in vitro, although the level of ensuing replication seems to be small. Macrophage infection was associated with induction of TNF-α and IL-8 cytokines and their respective mRNAs.

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