Hepatitis C virus infection of primary tupaia hepatocytes leads to selection of quasispecies variants, induction of interferon-stimulated genes and NF-κB nuclear translocation

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Systems for *in vitro* culture of *Hepatitis C virus* (HCV) are essential tools to analyse virus–cell interactions and to investigate relevant pathophysiological aspects of HCV infection. Although the HCV replicon methodology has increased our understanding of HCV biology, this system does not reproduce the natural infection. Recently, tupaia (*Tupaia belangeri chinensis*) hepatocytes have been utilized for *in vitro* culture of HCV. In the present work, primary tupaia hepatocytes infected *in vitro* with HCV were used to analyse the evolution of HCV quasispecies in infected cells and the ability of the virus to influence antiviral and proinflammatory responses in cells sustaining virus replication. The results confirmed the potential of tupaia hepatocytes as a model for HCV infection, although this system is limited by rapid loss of differentiated cell phenotype in culture. These findings revealed an extraordinary plasticity of HCV quasispecies, which underwent rapid evolution to tupaia-tropic variants as early as 24 h after infection. It was also shown that HCV could activate interferon-sensitive genes, albeit modestly in comparison with other viruses such as *Semliki Forest virus*. Importantly, HCV activated NF-κB in primary hepatocytes and upregulated NF-κB-responsive genes including the chemokines MCP-1 and CXCL2 (MIP-2). This effect may play a role in induction of the hepatic inflammatory reaction *in vivo*. In summary, HCV quasispecies adapt rapidly to the specific biology of the host and HCV stimulates a blunted interferon response while inducing a proinflammatory phenotype in the infected cell.

INTRODUCTION

Hepatitis C virus (HCV) is a positive-strand RNA virus belonging to the genus *Hepacivirus* within the family *Flaviviridae*. The majority of HCV-infected individuals develop chronic hepatitis, which may progress to liver cirrhosis and hepatocellular carcinoma. Six major genotypes (1–6) and multiple minor subtypes have been described (Robertson *et al.*, 1998). Moreover, due to an error-prone RNA polymerase that causes a high mutation rate, HCV circulates as a heterogeneous population (Forns *et al.*, 2000) of closely related variants termed quasispecies (Manzin *et al.*, 1998). These variants are subjected to host immune

pressure, which does not affect all viral proteins uniformly. Genetic variability is segregated within particular segments of the HCV genome, resulting in a number of highly variable regions. The most variable region is hypervariable region 1 (HVR1) of the E2 glycoprotein (Weiner *et al.*, 1991).

The biological significance of the viral diversity is not clear, but differences in pathogenesis, cell tropism, viral persistence and response to therapy have been reported (Farci & Purcell, 2000; Lopez-Labrador *et al.*, 1999; Pessoa *et al.*, 1999). Combined therapy with pegylated interferon plus ribavirin induces a sustained viral elimination in more than 80 % of patients infected with genotypes 2 and 3, but in less than 50 % of those infected with genotypes 1 and 4. Due to the high prevalence of genotype 1, there is an urgent need to develop new anti-HCV drugs and therapeutic strategies.

Animal models of HCV infection and systems for *in vitro* HCV culture are essential tools for an understanding of HCV biology and virus–host interactions and to identify new therapeutic targets. The chimpanzee is the only

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non-human host for HCV infection, but the use of these animals is restricted due to problematic availability, high maintenance costs, ethical considerations and regulations banning the use of this species for many experimental studies. Recently, subgenomic and genomic HCV replicons have been increasingly used to study the cellular effects of HCV replication (Bukh *et al.*, 2002; Lohmann *et al.*, 1999). However, although these systems have allowed a better understanding of HCV biology, they do not serve as true models of HCV infection (Su *et al.*, 2002). Systems based on infection of primary cells in culture have been described. Susceptibility of human haematopoietic cells (dendritic cells, peripheral blood mononuclear cells and macrophages) has been reported (Cribier *et al.*, 1995; Navas *et al.*, 2002; Radkowski *et al.*, 2004), but these systems suffer from poor reproducibility and very low levels of HCV replication. Other groups have used primary hepatocytes from humans or chimpanzees (Fournier *et al.*, 1998; Lanford *et al.*, 1994; Rumin *et al.*, 1996), but the limited availability of these cells makes these systems impractical for routine use. Very recently, a virus production system that allows secretion of viral particles has been developed (Wakita *et al.*, 2005). The system is based on an HCV 2a clone and Huh-7-derived cell lines and holds considerable promise for the study of virus–host interactions and for testing antiviral compounds and vaccines. However, an important limitation of this culture system is that it only contains RNA from one of the six HCV genotypes.

Some reports have shown that the tree shrew or tupaia (*Tupaia belangeri*) is susceptible to infection *in vivo* with human hepatitis viruses such as hepatitis A, B and delta virus (Li *et al.*, 1995; Walter *et al.*, 1996; Yan *et al.*, 1996; Zhan *et al.*, 1981). Tupaias are small squirrel-like animals ($\sim$150 g) endemic to South-East Asia that are closely related to primates and classified in their own order (Scandentia) (Martin, 1990). Studies from our group have shown that one subspecies of these animals (*Tupaia belangeri chinensis*) is partially susceptible to HCV infection after immunosuppression and subsequent intravenous inoculation of infectious HCV sera (Xie *et al.*, 1998). Moreover, recent papers have demonstrated that tupaia primary hepatocytes can be infected *in vitro* with HCV (Barth *et al.*, 2005; Zhao *et al.*, 2002). In the present study, we assessed the potential of cultured tupaia hepatocytes as a system for culturing HCV *in vitro* and used this model to analyse the evolution of HCV quasispecies and the ability of the virus to influence the antiviral and proinflammatory response in the infected cell.

METHODS

Animals. Adult tupaias (*T. belangeri chinensis*) were kept in the animal facilities of the University of Navarra (Spain) in steel cages at a temperature of $26 \pm 2\,^\circ C$, with a relative humidity of 50–70 % and a 12/12 h light/dark cycle. Handling and care of the tupaias were evaluated and approved by the Animal Ethical Committee of the University of Navarra.

Isolation and culture of primary hepatocytes. Tupaias were anaesthetized by intramuscular injection with a mixture of ketamine, xylazine and atropine (16 mg, 0·75 mg and 3 µg per 100 g body weight, respectively). Hepatocytes were isolated through a two-step collagenase liver perfusion procedure employing 50–70 mg Hepatocyte Qualified Collagenase (Gibco-BRL) for 10–15 min. The viability of isolated cells was assessed by the trypan blue exclusion test. Dead cells were removed on a 40 % Percoll (Amersham Pharmacia) gradient and hepatocytes were seeded at confluence ($1\cdot2$–$1\cdot5 \times 10^6$ cells per well) on collagen-covered, six-well plates (Becton Dickinson) allowing overnight attachment of the cells. After seeding, cells were maintained in 2 ml Williams' E medium with GlutaMAX-I (Gibco-BRL) supplemented with 100 U penicillin and streptomycin ml^{-1} (Gibco-BRL), 5 mg bovine insulin (Sigma) l^{-1}, 2 % DMSO (Sigma), $3\cdot5 \times 10^5$ M hydrocortisone hemisuccinate (Sigma) and 2 % AB normal human serum (BioWhittaker) (Rumin *et al.*, 1999). Cells were incubated at $37\,^\circ C$ in a humidified atmosphere of 5 % CO_2 and the medium was changed every 2–3 days. As a negative control, primary rat hepatocytes were isolated and cultured using a similar procedure to that described for tupaia hepatocytes.

***In vitro* infection of primary hepatocytes.** Hepatocytes were infected 1 day after plating by overnight incubation of cells with 50 µl of different human infectious sera in 1 ml medium without AB normal human serum per well. The inoculum was then removed and the cells were washed five to six times with PBS (Gibco-BRL) and finally refed with medium. HCV mock-infected cells were incubated with 50 µl human non-infectious serum. Hepatocytes from three rats were infected under exactly the same conditions as tupaia hepatocytes. Supernatants and hepatocytes were collected at various time points during the culture.

All infectious serum samples used belonged to patients that were non-responders to interferon therapy, presented a high titre of HCV RNA (10^7–10^9 IU ml^{-1}) genotype 1 and were negative for hepatitis B virus and human immunodeficiency virus markers.

In one experiment, hepatocytes were infected with *Semliki Forest virus* (SFV) (Liljestrom & Garoff, 1991) at an m.o.i. of 45. Cells were incubated with virus for 6 h, washed twice with PBS and finally refed with medium.

Harvest of samples and RNA extraction. For collection of hepatocytes, cells from one well were washed twice with PBS and then scraped, pelleted and stored at $-80\,^\circ C$ in 500 µl using the Ultraspec RNA Isolation system (Biotecx Laboratories). Supernatants were also collected and stored at $-80\,^\circ C$.

RNA was purified from 500 µl supernatant or from cells contained in one well or from 100 µl infectious inocula using the Ultraspec RNA Isolation system according to the manufacturer's instructions. RNA was resuspended in 20 µl DEPC-treated water, quantified by measurement of absorbance and stored at $-80\,^\circ C$. In some cases, a double RNA extraction was performed.

cDNA synthesis. For reverse transcription, a quarter of the RNA extracted was first denatured for 1 min at $90\,^\circ C$. cDNA synthesis was then performed for 1 h at $37\,^\circ C$ in a reaction mixture containing 60 U Moloney murine leukaemia virus reverse transcriptase (MMLV RT; Gibco-BRL), RT buffer, 5 mM dithiothreitol, 2 mM dNTP mix (Roche Molecular Biochemicals), 100 ng random primers (Roche Molecular Biochemicals) and 12 U RNase inhibitor (Promega) in a final volume of 10 µl.

Detection of genomic HCV RNA by nested RT-PCR. Supernatants and hepatocytes were analysed to detect genomic HCV RNA by nested PCR from the 5′ non-coding region (5′NCR). Outer primers used were JL1 (sense): 5′-CCTGTGAGGAACTACTGTCT-3′

and JL2 (antisense): 5′-CTATCAGGCAGTACCACAAG-3′. Inner primers were JL3 (sense): 5′-ACTGTCTTCACGCAGAAAGC-3′ and JL4 (antisense): 5′-GACCCAACACTACTCGGCTA-3′. PCR conditions for both rounds consisted of an initial denaturation of 95 °C for 1 min, followed by 35 cycles of 95 °C for 15 s, 52 °C for 12 s and 72 °C for 20 s, and a final extension of 72 °C for 1 min. Reactions were carried out with 3 µl cDNA or first-round PCR product, 1 U Expand High Fidelity *Taq* DNA polymerase (Roche Diagnostics), 1·5 mM MgCl$_2$, 0·8 mM dNTP mix and 0·5 µM inner or outer primers in a final volume of 20 µl. All genomic HCV detection assays included negative controls consisting of mock-infected tupaia cells and water. RNA isolated from HCV-positive serum was used as a positive control. The sensitivity of the assay was 10^2 RNA copies.

Detection of negative-strand HCV by strand-specific recombinant (r)*Tth* RT-PCR assay. Specific detection of negative-strand HCV RNA was performed using the r*Tth* RT-PCR method described by Lanford *et al.* (1995) with some modifications. Briefly, a 10 µl RT reaction containing 0·5–1 µg RNA was performed at 70 °C with 0·75 µM 5′NCR sense primer (JL3) and 5 U r*Tth* DNA polymerase (Applied Biosystems). After addition of chelating buffer, still at 70 °C, the PCR mixture (2 mM MgCl$_2$ and 0·15 µM JL2 antisense primer) was added. PCR conditions consisted of an initial denaturation of 94 °C for 1 min, followed by 35 cycles of 94 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s, and a final extension of 72 °C for 7 min. A second PCR round was required to detect visible bands. For the nested PCR, 3 µl first-round PCR product was reamplified using the primers JL3 and JL4 as described above.

To assess the sensitivity and specificity of the method, synthetic positive and negative strands of HCV RNA encompassing the 5′NCR were transcribed *in vitro* to be used as standards in our assay. HCV RNA transcripts were synthesized from a PCR II TOPO vector (TOPO TA Cloning kit; Invitrogen) containing the insert JL1–JL2 of HCV 5′NCR sequence using either T7 (Roche Molecular Biochemicals) or SP6 (Amersham Pharmacia) RNA polymerase. RNA transcripts were treated twice with DNase I (Gibco-BRL). The absence of residual DNA template was ascertained by performing nested PCR without the RT step of both transcripts (from 10^7 to 10^{10} RNA copies). Dilutions of synthetic RNA were made in control liver RNA to mimic conditions in hepatocytes.

Quasispecies analysis. For sequence analysis of HCV HVR1, RNA purified from infectious inocula and tupaia hepatocytes was amplified by reverse transcription and the nested PCR procedure with primers derived from the E2 region. Outer primers used were HV1 (sense) and HV2 (antisense) (Lanford *et al.*, 1995) and the inner degenerate primers were HV3 (sense): 5′-TGGTGGGGAACTGGGCYAAGGT-3′, and HV6 (antisense): 5′-ARGGCAGTCCTGTTGATGTGCCA-3′. PCR conditions for both rounds consisted of an initial denaturation of 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 1 min, and a final elongation step of 72 °C for 7 min. Specific reverse transcription was performed for 1 h at 42 °C in a reaction mixture containing 1·5 µM HV2. For the first round, PCR was carried out with 3 µl cDNA, 1 U Expand High Fidelity *Taq* DNA polymerase (Roche Diagnostics), 1·5 mM MgCl$_2$, 0·8 mM dNTP mix, 0·5 µM HV1 and 0·2 µM HV2 in a final volume of 20 µl. For the second round, 3 µl first-round PCR product and 0·75 µM inner primers were used. A high-fidelity polymerase was used in order to exclude artefacts due to nucleotide misincorporations.

HVR1 second-round PCR products were cloned using the TOPO TA Cloning kit (Invitrogen) according to the manufacturer's protocol. At least 10 clones from each transformation were purified with the TempliPhi Amplification kit (Amersham). Plasmids obtained were sequenced by fluorescence-based *Taq* dideoxy terminator cycle sequencing using the BigDye Terminator Cycle Sequencing Ready

Reaction kit (ABI PRISM; Applied Biosystems) and M13 universal primers in an automated sequencer (ABI PRISM 310 Genetic Analyser; Applied Biosystems).

Sequences were compared with GenBank using BLAST to confirm their identity as HCV HVR1 and deposited in GenBank under accession nos AY700854–AY700935.

Sequencing of tupaia mRNAs. One microgram of RNA from tupaia hepatocytes was treated with 1 U DNase I (Gibco-BRL) to avoid genomic contamination and reverse transcription was performed using MMLV RT enzyme as described above. One-tenth of the cDNA obtained was subjected to PCR amplification in a final reaction volume of 30 µl containing 1·5 U BioTaq DNA polymerase (Bioline), 1·5 mM MgCl$_2$, 0·3 µM each of sense and antisense primer and 0·5 mM dNTPs (Roche Molecular Biochemicals). General PCR conditions were as follows: initial denaturation at 94 °C for 1 min, 35–40 cycles of 94 °C for 20 s, 50–60 °C for 15 s and 72 °C for 15–30 s, and a final extension of 72 °C for 7 min. All PCRs were performed in a GeneAmp PCR System 2400 (Applied Biosystems). Primers for amplification of tupaia genes were designed on the basis of conserved regions in human, mouse and other mammals with known orthologous genes. If non-specific bands were present in agarose gels, sequencing of the correct band was performed to redesign new, more specific primers (see Supplementary Table available in JGV Online). The design of the majority of primers was done to guarantee that no genomic DNA could be amplified.

The identity of all amplified mRNA was verified by automatic sequencing in an ABI PRISM 310 Genetic Analyser (Applied Biosystems) as described above but using specific primers, followed by a query against the GenBank database using BLAST. Sequences of the tupaia genes Myxovirus resistance protein A (MxA), 2′5′-oligoadenylate synthetase (2′5′-OAS), interferon-induced protein with tetratricopeptide repeats 1 (p56), interferon regulatory factor 1 and 7 (IRF-1 and IRF-7), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), albumin, cytochrome P450 3A4 (CYP3A4), vimentin, monocyte chemoattractant protein-1 (MCP-1) and chemokine (CXC motif) ligand 2 (CXCL2/MIP-2) were deposited in GenBank under accession nos. AY699810–AY699818 and AY701519–AY701520.

Quantification of mRNAs in tupaia hepatocytes by real-time PCR. Levels of mRNA in the culture were quantified by real-time PCR using the LightCycler System (Roche Molecular Biochemicals). Amplification was performed in a 10 µl total volume containing 7 µl of the PCR mixture [1× LightCycler FastStart Reaction mix with SYBR Green I (Roche Diagnostics), 6 mM MgCl$_2$, 0·75 µM each primer) and 3 µl of a 1/10 dilution of cDNA template. PCR conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, 55–65 °C for 5 s and 72 °C for 12 s. Exact annealing temperatures are shown in Supplementary Table (available in JGV Online) for each gene. All amplification reactions were performed in triplicate. After the amplification was complete, a final melting curve was recorded to monitor the dissociation of specific PCR product. To improve SYBR Green I quantification, a high-temperature fluorescence measurement was performed (72–94 °C depending on the gene; see Supplementary Table available in JGV Online). As an internal control for each sample, PCR amplification of GAPDH was performed. The relative expression of each transcript was expressed using the formula: $2^{cp\text{GAPDH}-cp\text{gene}}$, where cp is the point at which the fluorescence rises appreciably above the background fluorescence.

Electrophoretic mobility shift assay (EMSA). NF-κB-binding activity was determined by EMSA with a commercial oligonucleotide containing the κB consensus site (Promega). EMSA was performed as previously described (Boya *et al.*, 2001). For competition experiments, 7 pmol unlabelled specific κB or non-specific AP-1 oligonucleotides were incubated with nuclear extracts and the labelled

probe. For supershift experiments, human-specific antibodies against p50 (20 µg) and p65 (10 µg) and the human non-specific antibody c-Jun (0·25 µg) (Santa Cruz Biotechnology) were added to the binding reaction. Samples were electrophoresed and band intensities were analysed using the Molecular Analyst/PC software (Bio-Rad).

Statistical analysis. Statistical analyses were performed using the non-parametric Kruskal–Wallis test and Mann–Whitney U-test. All P values were two-tailed and considered significant for values of $P < 0.05$. Descriptive data for continuous variables are reported as medians and interquartile range. Statistical analysis was carried out using SPSS 9.0 for Windows.

RESULTS

HCV infection of primary tupaia hepatocytes

To study the susceptibility of tupaia hepatocytes to HCV infection, 20 different tupaia cell cultures were incubated with sera from different HCV-infected patients. In these cultures, genomic HCV RNA was examined by nested RT-PCR of the 5′NCR, negative-strand HCV RNA by strand-specific r*Tth* RT-PCR and the selection of quasispecies in culture by sequence analysis of HCV HVR1 of the HCV E2 region.

Presence of genomic HCV RNA. Genomic HCV RNA was detected from 5 to 10 days post-inoculation in 70 % of primary tupaia hepatocytes cultures (data not shown). An example of three cultures is shown in Fig. 1. HCV RNA was not directly detected in any of the culture supernatants analysed.

As the high sensitivity of nested RT-PCR used to detect genomic HCV RNA in tupaia hepatocytes could amplify residual inoculum non-specifically bound to cells, we used primary hepatocytes from three rats infected under the same conditions as tupaia hepatocytes as a negative control. In cultured rat hepatocytes, genomic HCV RNA could be found 1–3 days after serum incubation (data not shown). Thus, based on PCR detection of the genomic RNA strand, it was not possible during the first 3 days of culture to distinguish signals from the residual inoculum from actual HCV infection of cultured cells.

Presence of negative-strand HCV RNA. HCV is a positive-strand RNA virus whose replication occurs via a negative-strand RNA intermediate. Since the positive strand is the only form of viral RNA present in the inoculum, detection of negative-strand HCV RNA in infected cells is indicative of active virus replication (Fournier *et al.*, 1998). Using the strand-specific r*Tth* RT-PCR assay, false priming of the incorrect strand is reduced by conducting cDNA synthesis at 70 °C with the thermostable r*Tth* enzyme (Sangar & Carroll, 1998). To assess the sensitivity and specificity of the method, synthetic positive and negative strands of HCV RNA encompassing the 5′NCR were used as standards in our assay. Using the negative strand-specific r*Tth* RT-PCR, we were able to detect 10^2 molecules of the negative-strand RNA, while a minimum
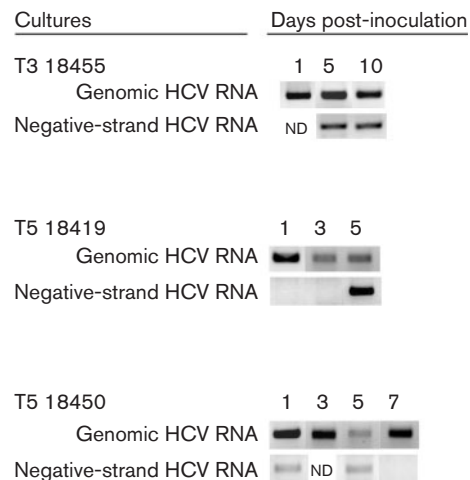
Fig. 1. Detection of genomic and negative-strand HCV RNA in tupaia primary hepatocytes. Cells were incubated 1 day after plating with different human inocula. Total cellular RNA was harvested at various times after inoculation and analysed for HCV genomic and negative-strand RNA using RT-nested PCR or r*Tth* RT-nested PCR, respectively. Representative examples of ethidium bromide staining of nested PCR products obtained by amplification of the 5′NCR from three different tupaia cultures are shown for genomic and negative-strand HCV RNA. All genomic HCV detection assays included negative controls consisting of tupaia mock-infected cells and water. RNA isolated from HCV-positive serum was used as a positive control. All negative-strand HCV RNA detection assays included positive- and negative-strand synthetic HCV RNAs as controls for strand specificity and sensitivity.

of 10^5 molecules of the positive strand was required to produce a signal (3 logs of specificity). Under these conditions (see Methods), no amplification band was obtained when using serum samples from infected patients, indicating that false priming of the positive strand was not taking place.

In the tupaia hepatocyte cultures with genomic HCV RNA that was detectable for more than 3 days (nine of 20 cultures), we analysed the negative-strand RNA to confirm HCV replication. In most cases, the replicative intermediate was detected at varying time points, from day 1 to 10 (Fig. 1).

Quasispecies analysis. Analyses of the HVR1 deduced amino acid sequences from primary tupaia hepatocytes and from the corresponding inocula clearly showed that HCV undergoes genetic evolution during culture (Fig. 2). In most cases, the predominant HVR1 amino acid sequence found in tupaia hepatocyte cultures differed from the corresponding predominant sequence from the inoculum and this occurred as early as 24 h post-inoculation. Furthermore, we found similar changes in the sequences of different HCV-inoculated tupaia hepatocytes cultures (Fig. 2, amino acids in bold). Interestingly, similar changes

Fig. 2. Comparison and relative abundance of deduced amino acid sequences of HVR1 (underlined) obtained from five inocula (italics) and their corresponding cultures at different time points. Sequences are shown in decreasing order of abundance. Only amino acids differing from the top sequence are shown. Amino acids in bold indicate identity with the corresponding amino acid of sequences isolated from HCV-infected tupaia hepatocyte cultures and with the work of Zhao *et al.* (2002).

have also been reported in HCV sequences from tupaia hepatocyte cultures (Zhao *et al.*, 2002; shown in Fig. 2).

Quasispecies analyses in HCV-infected tupaia hepatocytes after 3–5 days of culture were not successful since HVR1 could not be amplified in these samples.

Differentiation status of cultured primary hepatocytes.
Our data indicated that tupaia hepatocytes are permissive to HCV infection but for only a short period, usually lasting less than 7–10 days. In order to analyse whether the loss of HCV replication could be associated with loss of the differentiated hepatocellular phenotype, we quantified the expression levels of albumin, CYP3A4 and vimentin. Albumin and CYP3A4 characterize the mature hepatocyte phenotype, while vimentin is induced during the dedifferentiation process. We found that tupaia hepatocytes dedifferentiated in culture, exhibiting a rapid decrease in the mRNA levels of albumin, a progressive reduction of CYP3A4 transcripts and a progressive increase in vimentin mRNA. Thus, by day 12, albumin mRNA was reduced to about 1/100 of initial values, CYP3A4 to less than half and vimentin expression, which was initially undetectable, showed strong upregulation (Fig. 3).

Effect of HCV on the antiviral and proinflammatory status of infected cells

To analyse whether an infectious inoculum could activate the endogenous interferon system and induce a cellular stress response in primary tupaia hepatocytes, we determined the expression of interferon-stimulated genes (ISGs) and the activation of NF-κB in these cells after incubation with an HCV-infected serum or a control serum sample.

Induction of ISGs in HCV-infected hepatocytes. Viral infection stimulates the synthesis of type I interferons, which induce a diversity of ISGs as a first-line defence against virus replication. After gene sequencing of the tupaia ISGs 2′5′-OAS, MxA, p56, IRF-7 and IRF-1 (see Methods), we determined their expression levels in HCV-infected hepatocytes. Determinations were performed in

Fig. 3. Changes over time in culture of hepatocyte differentiation (albumin and CYP3A4) and dedifferentiation (vimentin) markers in primary tupaia hepatocytes. The level of mRNA expression of each gene was determined by real-time RT-PCR and normalized to mRNA expression of GAPDH. Bars represent the standard deviation from five independent cultures.

cells harvested at 2, 4, 6, 12, 16 and 24 h after HCV or mock infection. As a positive control for ISG induction, we used tupaia hepatocytes infected with SFV, since we have previously observed that this virus replicates efficiently in tupaia liver cells (data not shown). We observed that MxA, p56 and 2′5′-OAS expression was induced 4 h after SFV and HCV infection and peaked at 24 h after infection, while IRF-7 and IRF-1 were induced at 2 h and peaked at 12 h (data not shown). Fig. 4 shows the relative expression of the ISG mRNAs in mock-infected, HCV-infected and SFV-infected hepatocytes at the peak of expression of these genes. Both viruses were able to significantly induce ISGs, although HCV induction was substantially lower than SFV in most cases.

NF-κB activation and induction of chemokines MCP-1 and CXCL2 (MIP-2) in HCV-infected hepatocytes.

NF-κB activation and the resulting nuclear translocation induces the expression of genes involved in the response to cellular stress. We analysed NF-κB-binding activity in the nuclei of tupaia hepatocytes incubated with HCV-positive or -negative serum. Interestingly, we found that NF-κB-binding activity was twofold higher (analysed by densitometry of autoradiograms) in the nuclei of HCV-infected cells than in control cells (Fig. 5a). As shown in Fig. 5(a and b), active NF-κB present in tupaia hepatocyte nuclei was composed mainly of p65/p65 homodimers and p50/p65 heterodimers, with one minor complex made up of p65 bound to an unknown element.

The chemokines MCP-1 and CXCL2 (MIP-2) are NF-κB-responsive genes (Walpen et al., 2001; Wang et al., 2000) whose expression is upregulated in the liver of patients with chronic hepatitis C and in other forms of virus-induced liver damage (Bowen et al., 2002; Muhlbauer et al., 2003). It is not known whether the virus per se is able to modulate the expression of these inflammatory mediators by parenchymal cells. Therefore, we wished to study whether chemokine gene expression was induced in tupaia hepatocytes after incubation with HCV-positive serum. We found that the mRNA levels of both MCP-1 and CXCL2 (MIP-2) were significantly increased at 24 h in tupaia hepatocytes sustaining HCV replication compared with mock-infected cells (Fig. 6), indicating that HCV may directly activate chemokine gene expression in infected liver cells. In these experiments, we used as controls primary hepatocytes from rats incubated for 24 h with the infectious serum or with normal control serum. In these samples, we did not observe any changes in the expression of NF-κB-inducible chemokines (data not shown), suggesting that the induction of chemokine expression did not result from the mere attachment of virions to the cell membrane, but rather from the penetration and replication of the virus in the cell.

DISCUSSION

In a previous study, we have shown that tupaias (T. belangeri chinensis) can be infected with HCV, although the infection is barely productive and needs to be facilitated by
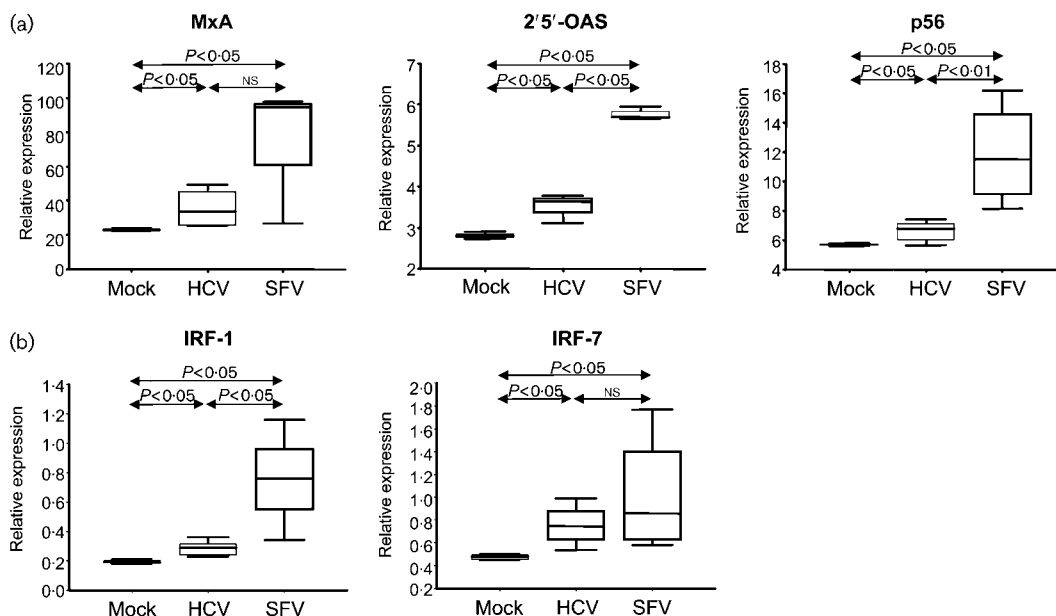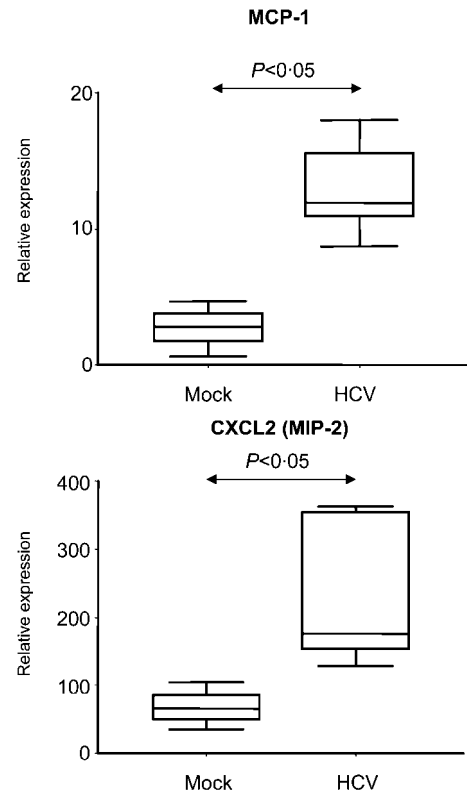

Fig. 4. Quantification of ISG mRNA expression in primary tupaia hepatocytes infected with HCV or SFV or mock-infected, using real-time RT-PCR and the LightCycler System, as described in Methods. Results were normalized against levels of GAPDH mRNA. (a) Relative expression of MxA, 2′5′-OAS and p56 at 24 h. (b) Relative expression of IRF-1 and IRF-7 at 12 h after infection. Data correspond to triplicates of three mock-infected cases, six HCV-infected cases and three SFV-infected case. NS, Not significant.
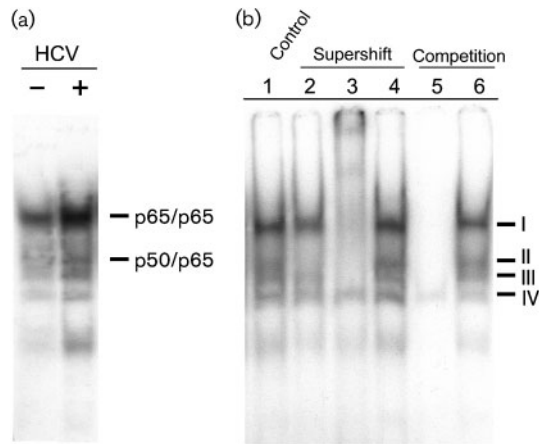
Fig. 5. NF-κB-binding activity in tupaia hepatocytes assayed by EMSA. (a) NF-κB activation of p65/p65 and p50/p65 was observed in tupaia primary hepatocytes infected with HCV (+) compared with mock-infected cells (−). (b) Supershift and competition experiments were performed to identify NF-κB subunits in tupaia hepatocytes and the specificity of the bands. Lane 1, nuclear extract from tupaia cultured hepatocytes incubated with κB-labelled probe (control lane); lanes 2–4, supershift assay with antibodies specific for different members of the NF-κB family (lane 2, anti-p50; lane 3, anti-p65) and the non-specific antibody c-Jun (lane 4). Complexes I, II and III shown in lane 1 are supershifted with anti-p65 antibody (lane 3) and complex II is supershifted with anti-p50 antibody (lane 2), indicating that complex I is composed of p65/p65 homodimers, complex II of p50/p65 heterodimers and complex III of p65 and another uncharacterized subunit. Lanes 5 and 6, competition experiments. Unlabelled specific κB (lane 5) or non-specific AP-1 (lane 6) oligonucleotides were incubated with nuclear extracts and the labelled probe. Specific bands (complexes I, II and III) showed decreased intensity in lane 5 with respect to lane 6. The non-specific band (complex IV) showed little change in intensity in either lanes.

Fig. 6. Quantification of chemokines MCP-1 and CXCL2 (MIP-2) mRNA expression in mock-infected and HCV-infected tupaia hepatocytes. Results are represented as mRNA relative expression after normalization of the data to the expression level of the GAPDH mRNA. Data correspond to triplicates of three mock-infected cases and six HCV-infected cases.

immunosuppression with total body irradiation (Xie *et al.*, 1998). In the present work, we have shown that HCV can infect and replicate in cultured primary tupaia hepatocytes, confirming the findings by Zhao *et al.* (2002), who demonstrated the possibility of culturing HCV using primary hepatocytes from these animals.

In our *in vitro* system, cells were maintained overnight in the presence of the infectious sample (50 μl in 1 ml culture medium) and then washed repeatedly and cultured for 3 weeks, changing the medium every 2–3 days. Despite careful washing, the viral genome from the inoculum can remain non-specifically bound to the cells for a period of time. Using control rat hepatocytes, we found that the genomic HCV RNA strand could be detected for a maximum of 3 days. In contrast, HCV RNA could be detected from 5 to 10 days in most of the tupaia hepatocyte cultures. Moreover, the replicative intermediate, which is not found in the inoculum, could be detected in tupaia

hepatocytes at various time points in different culture experiments. However, the level of HCV replication in tupaia liver cells was low and we were not able to detect genomic HCV RNA by RT-PCR in the culture supernatants. These data are in agreement with reports of HCV infection of adult human primary hepatocytes (Castet *et al.*, 2002; Fournier *et al.*, 1998) or human dendritic cells (Navas *et al.*, 2002), where no direct evidence for release of virus particles in the culture medium was observed. It seems, therefore, that the secretion of viral particles to the extracellular environment is minimal and, indeed, Zhao *et al.* (2002) were able to detect HCV RNA in the supernatant of tupaia hepatocytes only after vigorous concentration of the virions present in the medium.

As mentioned above, in our system the presence of HCV RNA in cultured cells was limited to about 7 days (and never beyond 10 days). Cell viability was well preserved for longer periods, but cultured cells experienced a dedifferentiation process manifested by a rapid decline in the expression of hepatocyte-specific genes such as albumin and CYP3A4 and an increase in the dedifferentiation marker vimentin. It seems possible that the loss of the differentiated phenotype

might be responsible for the cessation of virus replication. The role of the hepatocyte phenotype in maintaining HCV in culture has been suggested by other authors (Iacovacci *et al.*, 1997; Rumin *et al.*, 1999). Rumin *et al.* (1999) reported that HCV replication in cultured human hepatocytes was strongly dependent on the composition of the culture medium. These studies showed that the presence of DMSO, hydrocortisone and normal human serum (as in the conditions used in the present study) resulted in more efficient HCV replication. It seems possible that the development of improved methods directed towards preserving both viability and differentiation of cultured hepatocytes could enhance the ability of the cells to sustain HCV replication.

Characterization of HCV variants isolated from tupaia hepatocytes and from the corresponding inocula showed that the virus undergoes genetic evolution in culture. It is important to note that some of the predominant variants detected in tupaia hepatocytes was not found in the inocula, a finding that confirms the ability of HCV to replicate in tupaia liver cells. Furthermore, we found coincident changes in cultures of cells infected with different inocula. These same specific amino acid changes were also found by Zhao *et al.* (2002), suggesting a strong selection favouring sequences able to adapt to the biology of tupaia cells. We noticed that this adaptation occurred very early during culture, being observed during the first 24 h after infection of the cells, indicating a surprising plasticity of the viral quasispecies. The selection of specific variants for different cell types has already been reported by several groups who described lymphotropic and hepatotropic HCV quasispecies in primary cultures (Laporte *et al.*, 2003; Navas *et al.*, 2002), as well as in cell lines (Kato *et al.*, 1998; Nakajima *et al.*, 1996). In our study, in addition to the selection of tupaia-tropic HCV variants, we found a marked decrease in quasispecies complexity over time in culture. This fact has also been described for HCV propagated in primary human hepatocytes (Rumin *et al.*, 1999). Nevertheless, this group found that most mutations in the HVR1 region were silent, occurring in the third codon position, while in tupaia hepatocytes the nucleotide changes observed were usually accompanied by amino acid changes, suggesting a strong evolution of the viral genome in the tupaia cells.

In the present work, we showed that HCV infection of cultured liver cells could increase the expression of the ISGs $2'5'$-OAS, MxA, p56, IRF-7 and IRF1, further supporting the existence of a productive infection of tupaia hepatocytes in culture. However, the ability of HCV to induce interferon-sensitive genes was blunted compared with that of SFV. This mild activation of the interferon system may be due to either the low level of replication of HCV in tupaia hepatocytes or to an intrinsic ability of the virus to blunt the interferon response.

We also provided evidence that HCV can activate NF-κB in isolated hepatocytes. Activation of this transcription factor has been detected in cells transfected with plasmids encoding NS5 and in cells stably expressing HCV subgenomic replicons (Gong *et al.*, 2001; Waris *et al.*, 2003). However, under these conditions the intracellular levels of viral proteins are very high and are not representative of those found in naturally infected hepatocytes. In natural infection, we found that NF-κB could be detected in hepatocyte nuclei in liver biopsies of patients with chronic hepatitis C (Boya *et al.*, 2001). However, it was not clear whether the viral infection per se or the effect of proinflammatory cytokines produced by the mononuclear cells of the inflammatory infiltrate were responsible for NF-κB activation in hepatocytes. Our present data indicate that HCV can directly activate NF-κB in hepatocytes and that this effect is followed by upregulation of NF-κB-responsive genes such as MCP-1 and CXCL2 (MIP-2). Expression of these chemokines by infected cells would induce a proinflammatory status in HCV-infected cells and attraction of immune effectors to the liver. This effect may play a role in the initiation of the inflammatory response and in the immune elimination of the infected cells.

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