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Review

Hepatitis C — new developments in the studies of the viral life cycle

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Hepatitis C virus (HCV) is a causative agent of chronic liver disease leading to cirrhosis, liver failure and hepatocellular carcinoma. The prevalence of HCV is estimated as 3% of the world population and the virus is a major public health problem all over the world. For over 16 years, since HCV had been discovered, studies of the mechanisms of the viral life cycle and virus-host interactions have been hampered by the lack of a cell culture system allowing the virus to be grown in laboratory conditions. However, in recent years some new model systems to study HCV have been developed. The major breakthrough of the last two years was the cell culture system for maintaining the virus in an adapted hepatocyte-derived cell line. This review describes the techniques and applications of most of the *in vitro* systems and animal models currently used for working with hepatitis C virus.

Keywords: hepatitis C virus, HCV replicons, HCV pseudoparticles, HCVcc-cell culture-derived

INTRODUCTION

Hepatitis C virus is a single stranded, positive-sense RNA virus belonging to the genus *Hepacivirus* in the *Flaviviridae* family. HCV has a very narrow host range and infects only humans and chimpanzees. HCV particle consists of a capsid enclosing single-stranded RNA genome, surrounded by an envelope derived from host cell membranes containing spike-like projections of viral glycoproteins. Naturally occurring HCV particles circulating in the blood of infected people are highly heterogeneous (Maillard *et al.*, 2001). According to recent data, the majority of viral particles are associated with lipoproteins (Thomssen *et al.*, 1993; Andre *et al.*, 2002) and such association correlates with the highest infectivity of HCV virions. Different forms of lipoprotein-associated HCV particles have been identified: simple low density lipoprotein associated HCV virions, lipo-viro-particles (LVP) (Andre *et al.*, 2002; 2005) and exosomes (Masciopinto *et al.*, 2004).

The genome of HCV contains short untranslated regions (UTRs) at each end of the viral RNA, which are required for replication and translation, and carries a long open reading frame encoding a polyprotein of about 3010 amino acids, which is co- and post-translationally processed by the host and viral proteases into 10 viral proteins (Bartenschlager & Lohmann, 2000). Core protein C and envelope glycoproteins E1 and E2 belong to the structural proteins building the viral particle. Downstream of the structural region there is a small, highly hydrophobic, integral membrane protein, p7, most probably involved in ion channel formation (Griffin *et al.*, 2003; Pavlovic *et al.*, 2003). The non-structural region of the polyprotein comprises six intracellular pro-

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Abbreviations: con-1, consensus genome 1; CMVp, cytomegalovirus promoter; EMCV, encephalomyocarditis virus; GBV-B, GB virus B; GFP, green fluorescent protein; HCV, hepatitis C virus; HCVpp, HCV pseudoparticles; HCVcc, HCV cell culture-derived; HIV, human immunodeficiency virus; IFN, interferon; IRES, internal ribosomal entry site; LTR, long terminal repeat; MLV, murine leukemia virus; NS, non-structural; PBMC, peripheral blood mononuclear cells; SCID, severe combined immunodeficiency; UTR, untranslated region.

teins NS2, NS3, NS4A, NS4B, NS5A and NS5B that are responsible for viral replication and polyprotein processing and are not included in the viral particle (Fig. 1). NS5B is the viral RNA polymerase responsible for replication of HCV genome. Apart from the polyprotein, expression of a novel HCV protein from an alternative reading frame overlapping the core gene has been reported (Walewski *et al.*, 2001; Xu *et al.*, 2001; Boulant *et al.*, 2003). The resulting 17-kDa protein is called the frameshift (F) or alternative reading frame (ARF) protein (Varaklioti *et al.*, 2002). The role of the F protein remains to be defined (Bartenschlager *et al.*, 2004).

Translation of viral polyprotein is dependent on an internal ribosomal entry site (IRES) localized in the 5′ UTR, which is an RNA structural element interacting directly with the 40S ribosomal subunit during translation initiation (Tsukiyama-Kohara *et al.*, 1992; Pestova *et al.*, 1998; Spahn *et al.*, 2001; He *et al.*, 2003; Boni *et al.*, 2005).

Naturally occurring variants of HCV are classified into six major genotypes, numbered 1 to 6, and multiple subtypes. Additional variants, known as quasispecies, develop in infected individuals as a result of the high error rate of viral RNA polymerase. Despite the sequence diversity between the genotypes of about 30–35%, all of them share the same genome organization, replication cycle and ability to establish persistent infection (Simmonds, 2004). HCV infections are common worldwide. It is estimated that about 3% of the world population (170 million people) is infected with the virus and there are about 4 million carriers in Europe alone. HCV is the main etiological agent of chronic liver inflammation leading to cirrhosis and liver cancer. Probably as many as 70–90% of infected people fail to clear the virus during acute phase of the disease and become chronic carriers. In most cases (about 80%) acute hepatitis C is asymptomatic and about 20% of chronic carriers develop cirrhosis which, in up to 25% of cases, progresses into a fatal liver disease and liver cancer (WHO report, 2003). Different HCV genotypes account for diverse progression and severity of the disease. Genotype 1 is considered the most difficult to treat with current HCV therapy and subtype 1b is associated with the most severe disease progression and the highest probability of developing chronic infection and liver fibrosis. The genetic variability of hepatitis C virus, emerging with so many different genotypes, subtypes and quasispecies, makes it extremely difficult to develop a universal treatment and a vaccine that will protect against all HCV strains. Current HCV drug therapy is based on general antivirals, like interferon and the nucleoside analogue ribavirin. The best results are obtained with the combination therapy with pegylated interferon α (IFN-α) and ribavirin (Bretner, 2005; Pawlowska *et al.*, 2006). Depending on the viral genotype, the therapy is successful in about 40% of patients, with genotypes 1 and 4 being the most resistant to IFN treatment. Many infected people do not qualify for interferon therapy because of the serious side effects of the drug. In the developed countries, patients with HCV-related liver cirrhosis are qualified for liver transplantation (WHO report, 2003). These data indicate that HCV is a very serious global health problem and the need for new, more efficient therapeutic strategies, based on drugs specifically targeting the virus, is urgent and obvious. The development of new therapy is inseparably connected with the understanding of all possible aspects of the molecular virology of HCV infection.

METHODS TO STUDY HCV VIRUS

The possibilities to study hepatitis C virus were, until very recently, seriously limited by the lack of a cell culture system for growing the virus in laboratory conditions and a small animal model for *in vivo* experiments. For a long time the only approaches to study HCV were experimental infection of chimpanzees, observation of infected patients and comparison with other viruses, members of *Flaviviridae* family. Additionally, some important data about basic biochemical properties of viral enzymes and

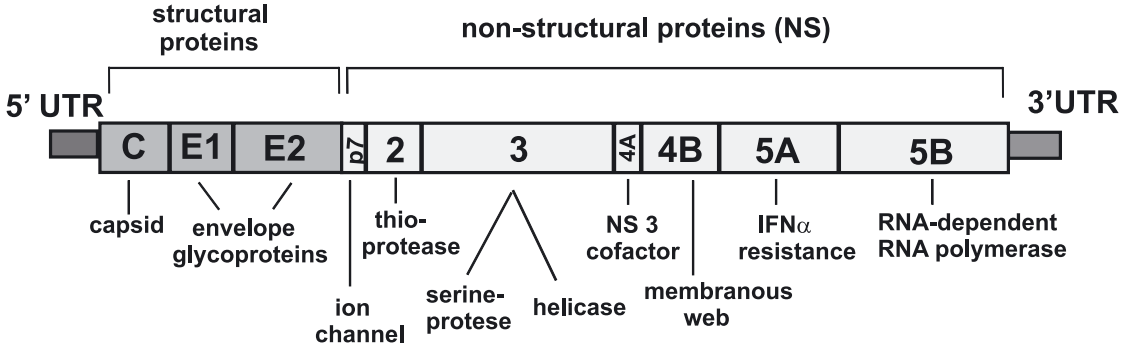

Figure 1. Structure of HCV genome and function of HCV proteins.

glycoproteins came from studies based on expression systems that produce viral proteins in different types of cells. Recently, many laboratories have been working on different systems, enabling the replication and growth of HCV in cell culture conditions. These attempts have resulted in the establishment of currently used models to study hepatitis C virus (Brass *et al.*, 2006):

***in vitro* models:**

- transient and stable expression systems
- HCV replicon systems
- retrovirus based HCV pseudo-particles (HCVpp)
- infectious HCV virus in cell cultures (HCVcc)

***in vivo* models:**

- experimentally infected chimpanzees
- murine models for HCV
- New World monkeys–marmosets infected with GBV-B virus.

Some of these model systems allow only limited studies of some aspects of the complex viral replication cycle. Nevertheless, while a detailed analysis of the HCV life cycle was hampered by a lack of an efficient viral culture, they contributed to a better understanding of the biology of the virus.

Transient and stable expression systems

Studies based on recombinant HCV envelope proteins produced in various expression systems had great influence on the current knowledge about the sub-cellular localization, folding, glycosylation and dimerization of E1 and E2 glycoproteins (Dubuisson *et al.*, 1994; Debuisson, 2000; Patel *et al.*, 2001 Deleersnyder *et al.*, 1997; Goffard *et al.*, 2005) and their interaction with major HCV receptors: CD81 and SR-B1 (Pileri *et al.*, 1998; Scarselli *et al.*, 2002). Recombinant HCV proteins proved to be very useful for both basic and advanced biochemical studies of protein structure and interactions with other viral or cellular proteins, and are still used in such type of studies. However, a recently developed HCV cell culture system enabled the analysis of HCV proteins in the natural environment during viral infection.

HCV replicon systems

A very important step forward in HCV research was the development of HCV replicon systems, designed to study viral RNA replication together with translation and maturation of viral proteins (Lohmann *et al.*, 1999). HCV replicons are self-amplifying, genetically engineered HCV genomes. They contain either complete genomic RNA of HCV or shorter sub-genomic fragments consisting of the minimal non-structural region from NS3 to NS5B of the genome (Fig. 2).

The prototype subgenomic replicon (Lohmann *et al.*, 1999) was based on HCV genotype 1b of a con-1 clone (consensus genome 1), isolated from a patient with chronic infection. Since short RNA is known to replicate more efficiently than long one, all the structural region of the HCV genome was replaced with two heterologous elements, one of them

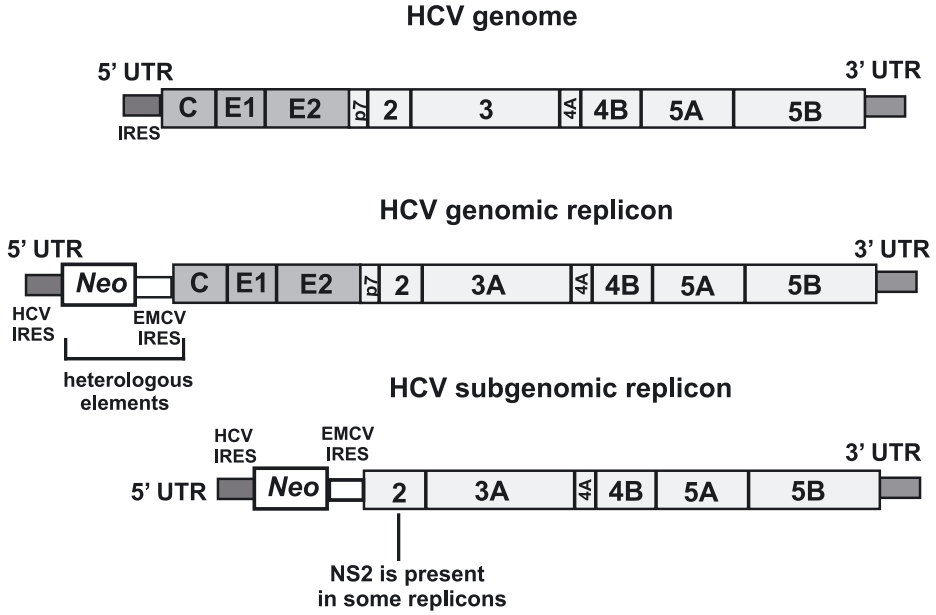

Figure 2. Structure of genomic and subgenomic HCV replicons.
Schematic representation of HCV genome and basic genomic and subgenomic replicons, *neo* gene allows for stable replication under antibiotic selection, in transient replicons the *neo* gene is usually replaced with a reporter gene coding for GFP or luciferase.

encoding neomycin phosphotransferase (*neo*^{r}), conferring the antibiotic G418 resistance, and the second one being the internal ribosome entry site (IRES) of encephalomyocarditis virus (EMCV). The resulting construct was a selectable, bi-cistronic RNA replicon, with the expression of the *neo*^{r} gene directed by HCV IRES, and the second cistron of HCV non-structural region translated under the control of EMCV IRES. Replicon RNA was generated by *in vitro* transcription from cDNA and transfected into Huh-7 cells. Upon G418 selection, Huh-7 cell clones were selected carrying high numbers of replicating HCV RNA and viral proteins, with an average of 1000–5000 replicons per single cell. Replicons maintained in G418-selected Huh-7 cell clones acquire certain single amino-acid substitutions, conserved among the cell clone that allow for efficient replication (Blight *et al.*, 2000). These substitutions, called cell-culture adaptive mutations, are found in all non-structural proteins, but most of them cluster to a central region of the NS5A gene. The most efficient replicons usually carry more than one mutation. The most potent substitutions enhance replication even 500-fold when introduced into the wild type HCV replicons (Krieger *et al.*, 2001). It is not clear how exactly these substitutions influence RNA replication, but most of them lead to modifications of the surface of the particular protein. It is believed that such modifications may affect viral interactions with cellular proteins, components of the replication machinery (Lohmann *et al.*, 2001; Bartenschlager *et al.*, 2003).

The replicon system made it possible, for the first time, to study genuine HCV RNA replication *in vitro* and to analyze structural aspects of the replication complex and translation of the viral polyprotein.

An important extension of the replicon system was the development of full length genomic HCV replicons as a potential tool to generate viral particles in cell culture. Although the replication of genomic replicons was very efficient, and viral proteins were produced, infectious viral particles were not assembled (Ikeda *et al.*, 2002; Pietschmann *et al.*, 2002; Bartenschlager *et al.*, 2003; Brass *et al.*, 2006). The fact that the full length HCV genomic replicons fail to produce infectious viral particles is caused most probably by the presence of cell-culture adaptive mutations. Moreover, the HCV RNA genomes containing such mutations are severely attenuated when transfected into the liver of chimpanzees in *in vivo* experiments (Blight *et al.*, 2002; Pietschman *et al.*, 2002). Despite this limitation, HCV replicons have successfully been used to study the mechanisms of replication and viral RNA translation (Bartenschlager *et al.*, 2003; Brass *et al.*, 2006). A large panel of different replicon systems has been generated, mostly derived from HCV genotypes 1a and 2a (Blight *et al.*, 2003; Kato *et al.*, 2003). Some of the replicons have been modified to visualize or quantify viral replication; these include replicons with green fluorescent protein (GFP) insertions in NS5A protein to track the replication complexes in living cells (Moradpour *et al.*, 2004), transient replication systems expressing easily quantifiable reporter genes like luciferase (Krieger *et al.*, 2001) and selectable replicons with luciferase (Vrolijk *et al.*, 2003) successfully used for measuring interferon levels in HCV patients and screening for anti-HCV compounds (Puerstinger *et al.*, 2007). Such replicons, containing reporter genes, are very useful tools in drug screening studies in respect to their influence on viral replication. The replicon system has also been used to characterize the assembly of HCV replication complex and the so called membranous web as a platform for viral replication (Gosert *et al.*, 2003; Hardy *et al.*, 2003; Lai *et al.*, 2003). With the use of cell clones that stably support high levels of HCV RNA replication, its influence on cell growth could also be studied. It has been shown that HCV replication does not have a cytopathogenic effect and is the most efficient in the log phase of the cell growth (Pietschman *et al.*, 2001). The replicon system has become one of the most important tools to study HCV RNA replication, pathogenesis and persistence. In the last few years replicons have been used to screen for resistance against selective antiviral compounds targeting mainly the viral NS3 protease and the NS5B RNA-dependent RNA polymerase (Lin *et al.*, 2005; Ma *et al.*, 2005).

Retrovirus based HCV pseudo-particles (HCVpp)

For a few years several laboratories have tried to develop a model to study HCV entry. A major advance has been achieved by the development of the HCV pseudo-particles (HCVpp) system (Bartosch *et al.*, 2003b; Drummer *et al.*, 2003; Hsu *et al.*, 2003b). HCVpp are recombinant viral particles containing a retroviral core surrounded by an envelope, bearing HCV glycoproteins E1 and E2. Pseudo-particles mimic the HCV virions in terms of cell entry pathways, as the early steps of infection like attachment, receptor binding and probably fusion are dependent on functional envelope HCV glycoproteins. Pseudo-particles are engineered to contain a reporter gene transcript, such as green fluorescent protein (GFP) or luciferase, enclosed in the retroviral capsid. Upon infection the reporter gene transcript is released into the target cell resulting in expression of GFP or luciferase (Fig. 3). Infected cells expressing the reporter gene can be detected and quantified with the use of very sensitive fluorescence methods. HCV pseudotyped retroviral particles are produced in HEK293 cells (a human embryonic kidney-derived cell line), typically after transfection of three

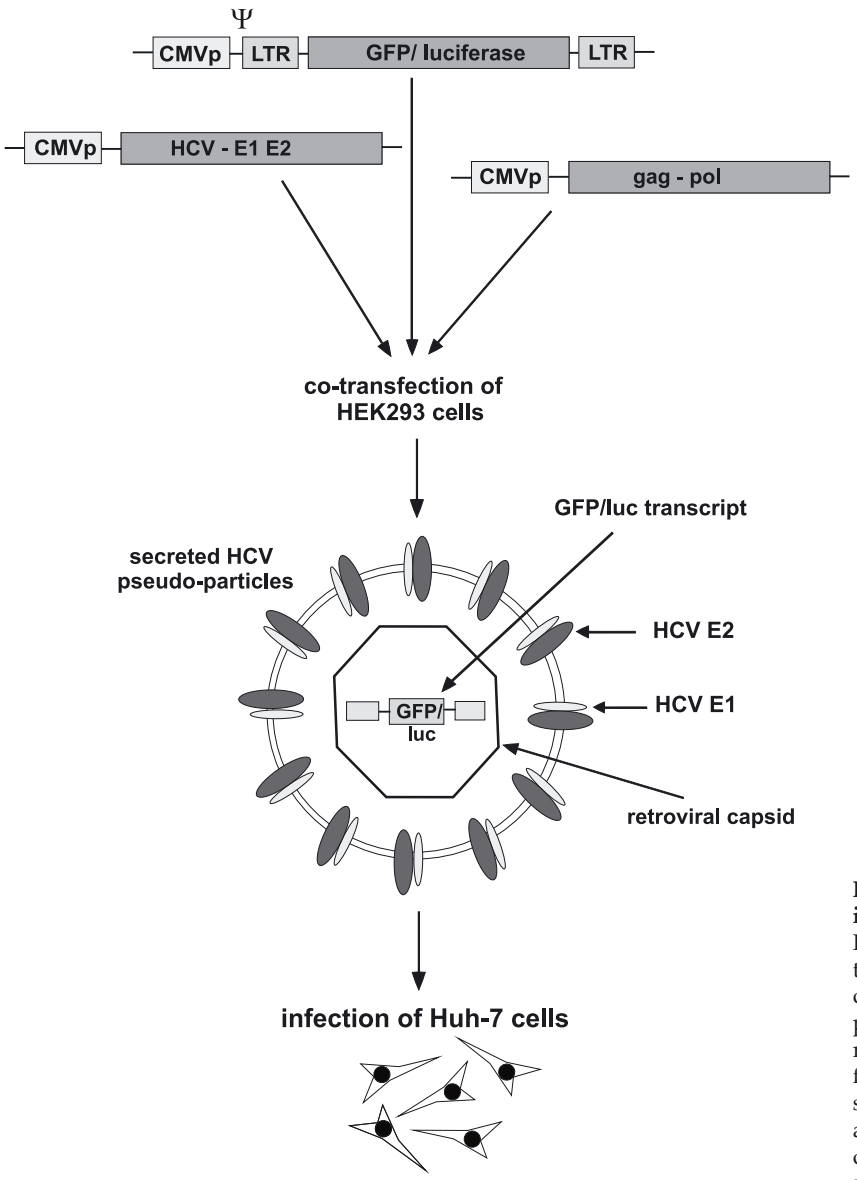

Figure 3. Generation of HCVpp for infection assay.
Hek 293 cells are cotransfected with three independent expression vectors coding for: 1. HCV E1 and E2 glycoproteins, 2. retroviral gag and pol, 3. reporter protein (GFP or luciferase) flanked by the retroviral genome LTR sequences containing transcript packaging signal – Ψ. Culture supernatant containing HCV pseudoparticles is used for infecting Huh-7 cells.

independent DNA constructs containing the *gag* and *pol* genes of the retrovirus, a packaging/reporter gene construct and HCV glycoproteins (Fig. 3). Viral capsids composed of retroviral proteins and containing two copies of the retroviral transcripts including the reporter gene are assembled inside transfected cells. Such particles are subsequently transported to the cell surface, where they acquire an envelope by budding at the cell membrane. The envelope of the newly formed particles contains HCV glycoproteins E1 and E2 derived from the host cell membrane (Bartosch *et al.*, 2003b; Op De Beeck & Dubuisson, 2003; Diedrich, 2006).

Retroviruses are very suitable vectors for the construction of pseudotyped viruses because they possess a natural ability to incorporate a number of cellular proteins into the viral particle, and their genomes tolerate large insertions of genetic markers. The viruses used in the HCVpp system are mainly MLV (murine leukemia virus) and HIV (human immunodeficiency virus). These viruses are extensively studied, well characterized and efficiently assemble in cell cultures. The retrovirus-based pseudo-particle system is relatively safe to work with because the defective viral genome canot replicate inside the infected cell. The only manifestation of infection is expression of the reporter gene. HEK293 cells were chosen as the platform for the assembly of HCV pseudotyped viruses because they are easy to transfect and accept large amounts of foreign DNA. In the infection assay, pseudo-particles assembled in HEK293T cells and released into the culture medium are subsequently used for infecting hepatocytes of the Huh-7 human hepatoma cell line. Upon the infection, retroviral transcripts

are released into the target cells and the reporter gene is expressed. Infectivity mediated by HCV glycoproteins is reflected by the number of cells expressing the reporter gene. HCV pseudo-particles infection is neutralized by HCV glycoprotein E2-specific monoclonal antibodies and serum from chronically infected patients. HCVpp infectivity is restricted primarily to human hepatocytes and hepatocyte-derived cell lines, proving the specificity of the system and the role of the E1 and E2 glycoproteins in HCV cell entry (Bartosch *et al.*, 2003a; Hsu *et al.*, 2003; Op De Beeck *et al.*, 2004).

Although the HCV pseudo-particle system has been developed only recently, it has already shed some light on the early steps of HCV infection. Several molecules have been proposed as potential HCV receptor candidates, such as the tetraspanin CD81 (Pileri *et al.*, 1998), the scavenger receptor class B type 1 (SR-B1) (Scarselli *et al.*, 2002; Voisset *et al.*, 2005; Dreux *et al.*, 2006), the low density lipoprotein (LDL) receptor (Agnello *et al.*, 1999; Monazahian *et al.*, 1999; Andre *et al.*, 2002) and nectins L-SIGN and DC-SIGN (Lozach *et al.*, 2003; 2004). The HCVpp system has been widely used for characterization of some candidate receptors for HCV (Cocquerel *et al.*, 2006; McHutchinson *et al.*, 2006) and their interaction with the E1E2 glycoproteins. It has been revealed that none of the putative receptor molecules alone is sufficient to restore infectivity of HCV pseudo-particles in non-permissive cells and infection with HCVpps requires a set of co-receptors that include both CD81 and SR-B1 (Bartosch *et al.*, 2003c). Considering the great heterogeneity of HCV virions, it can be assumed that different particles might infect cells using different mechanisms and receptors (Diedrich, 2006). Infection with HCV pseudo-particles differs from the naturally occurring infection in humans because HCV pseudotypes do not associate with lipoproteins. Thus, some aspects of HCV entry, such as the lipoprotein mediated infectivity or the role of LDL receptor in the attachment, could not be studied. However, a number of very interesting findings came recently from the HCVpp studies. The glycosylation status of HCV E1, E2 has been shown to be crucial for the infectivity of pseudo-particles (Goffard *et al.*, 2005) and some conserved residues involved in CD81 interaction have been identified (Owsianka *et al.*, 2006). HCV pseudo-particles have also been used to study the humoral immune response in humans and chimpanzees (Bartosch *et al.*, 2003a; Meunier *et al.*, 2005).

Infectious HCV virus in cell cultures (HCVcc) — a breakthrough in HCV research

The development of replicon systems and generation of HCV pseudo-particles has brought substantial information about HCV replication and cell entry. However, in these experimental systems, the later stages of infection like the spreading of the virus and release of the viral progeny could not be analyzed.

In the past years many attempts have been made to establish a cell culture system supporting HCV replication. Many systems were based either on the infection of human or chimpanzee primary hepatocytes (Iakovacci *et al.*, 1993; Lanford *et al.*, 1994; Fournier *et al.*, 1998; Rumin *et al.*, 1999) and human hepatocyte-derived cell lines (Dash *et al.*, 1997; Seipp *et al.*, 1997; Ikeda *et al.*, 1998; Kato *et al.*, 1996; Song *et al.*, 2001) with HCV particles from patient's serum, or on the cultivation of cells derived from chronically infected individuals. Several groups have also shown that HCV is able to infect a variety of lymphoid cell lines in culture, including several T-cell lines (MacParland *et al.*, 2006; Mizutani *et al.*, 1996a; 1996b; Nakajima *et al.*, 1996; Shimizu *et al.*, 1992; 1993), B-cell lines (Bertolini *et al.*, 1993; Sung *et al.*, 2003; Valli *et al.*, 1995) and peripheral blood mononuclear cells — PBMCs (Cribier *et al.*, 1995; Laskus *et al.*, 1997; Pham *et al.*, 2005). The cell-culture-produced virus could be transmitted to naïve cells by co-cultivation (Shimizu & Yoshikura, 1994) and *in vivo* infectivity after inoculation of a chimpanzee with B-cell culture produced virus was reported (Shimizu *et al.*, 1998). However, the major drawbacks of those systems were poor reproducibility and inefficient HCV replication that could be measured only with very sensitive detection methods (Bartenschlager & Lohmann 2000). Moreover, stable long-term virus production could hardly be achieved. Nevertheless, some important questions mostly about the genomic variability of HCV and selection of lymphotropic HCV variants were addressed by these studies (Sugiyama *et al.*, 1997; Rumin *et al.*, 1999; Revie *et al.*, 2006). Lymphoid cell cultures were also employed in the first neutralization assays to test anti-HCV antibodies (Shimizu *et al.*, 1994; 1996), studies of antiviral activity of α, β interferons and first HCV antisense nucleotides (Mizutani *et al.*, 1995; 1996b). Studies based on T- and B-cell lines not only have shed light on many aspects of HCV infection but also indicated that these non-hepatic cells can possibly function as a reservoir of the virus.

What revolutionized HCV research was the cell culture HCV virus production system, based on the transfection of human hepatoma cell line Huh-7 with genomic RNA derived from a cloned HCV genome (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). The starting point of this new system was the isolation in 2001 by the group of Takaji Wakita of an HCV genotype 2a strain JFH-1 from a patient with fulminant hepatitis (Kato *et al.*, 2001). In the first series of experiments,

the JFH-1 isolate was used for the development of a new subgenomic replicon which, as it was soon demonstrated, could efficiently replicate in a variety of cell lines (Huh-7, Hep-G2, IMY-N9 and non-hepatic cells) in spite of the lack of adaptive mutations (Kato *et al.*, 2003; 2005; Date *et al.*, 2004). In the following years Wakita and other researchers proved that replication of JFH-1 complete genome in human hepatoma cell line Huh-7 leads to the secretion of infectious viral particles. Cell-culture-produced virus was infectious for Huh-7 cells and the virions were physically similar to natural HCV isolates. However, attempts to infect cell lines other than Huh-7 were not successful. The new HCV cell culture system generates different types of viral particles that are able to associate with lipoproteins. Thus, the lipoprotein-mediated infectivity of HCV and release of viral particles from infected cells could be studied (Diaz *et al.*, 2006; Gastaminza *et al.*, 2006; Lindenbach *et al.*, 2006) which was not possible in any of the previous *in vitro* models. Infectivity of HCVcc was neutralized by CD81 receptor-specific antibodies and immunoglobulins from chronically infected patients. Infection was sensitive to interferon treatment and limited to hepatoma cell lines, proving specificity and selectivity of the infection. Moreover, cell-culture-generated HCV was infectious for chimpanzees, generating disease symptoms identical to those observed for human-derived HCV virus (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). As determined in studies of J6/JFH-1 chimeric virus a determinant of the infectivity of JFH-1 clone was localized in the NS region (NS3–NS5B) of the HCV genome (Lindenbach *et al.*, 2005). In future it will be very interesting to find out which particular gene or region is responsible for the infectivity of JFH-1 in cell culture.

In the optimized protocol for producing infectious HCV virions in cell culture, the first step is the transfection of *in vitro* transcribed JFH-1 or chimerical (JFH-1 and other clones) HCV RNA into the Huh-7-derived cells. Transcripts from the cDNA derived from the viral RNA induce infection when introduced into a permissive cell (Gale & Beard, 2001). This was based on the observation that *in vitro* transcribed HCV RNA is infectious when transfected into the liver of chimpanzees (Kolyhakov *et al.*, 1997; Yanagi *et al.*, 1997). Infectious viruses are obtained from cell culture supernatants and infectivity is determined by indirect immunofluorescent staining of infected cells for the viral NS5A protein (Fig. 4). This system yields viral titers of 10^4–10^6 infectious units per ml of culture supernatant. Infection spreads throughout the culture within a few days after inoculation at low multiplicity of infection (moi) and the virus can be serially passaged without loss of infectivity (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). The replication of HCV is the most efficient in the highly permissive Huh-7.5 cells derived from an HCV replicon-harboring Huh-7 cell line selected for the highest HCV replication efficiency (Blight *et al.*, 2002). The new Huh-7.5 cell line with the replicon removed by γ-interferon treatment is ideal for robust HCV replication and produces much higher viral titers than the original Huh-7 cell line (Lindenbach *et al.*, 2005; Zhong *et al.*, 2005).

Initially the HCV cell culture system was limited by the dependence on two particular structural gene sequences (JFH1 and J6), both belonging to the genotype 2a. This was a major problem in comparative studies including multiple genotypes of HCV. Further construction of chimeric genomes of different genotypes was necessary to obtain cell culture derived infectious viruses representing, in terms of the structural genes, genotypes other than 2a. This has resulted so far in new functional chimeras representing genotypes 1a (H77 isolate) and 1b (con1 isolate) (Pietschman *et al.*, 2006). For these constructs, an efficient infectious virus production was obtained. However, the JFH1 virus still appears to be unique among the strains of HCV in terms of its ability to cause productive infection in cell culture.

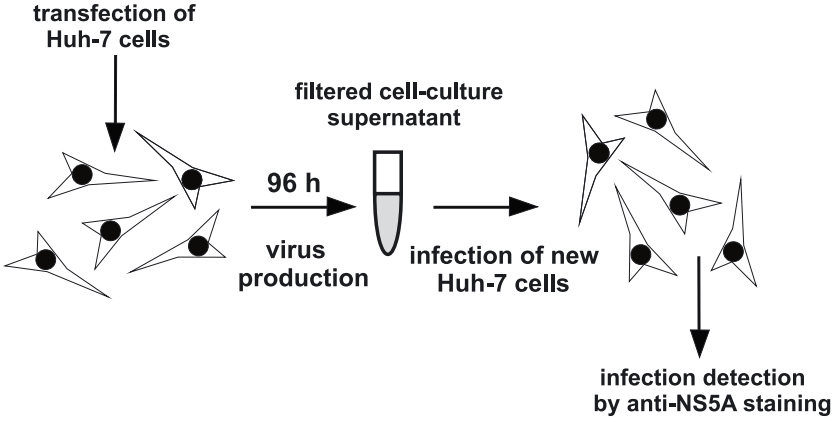

Figure 4. Overview of production of infectious HCV in cell culture.
Upon transfection of Huh-7 cells with *in vitro* transcribed HCV RNA and 96 h incubation cell culture supernatant containing HCV viral particles is collected and used for subsequent infection of naïve Huh-7 cells. HCV infection is detected by anti-NS5A staining.

As an extension and modification of the HCVcc system, modified HCV genomes expressing luciferase as a reporter gene were constructed (Wakita *et al.*, 2005; Tscherne *et al.*, 2006). With the urgent need for the improvement of HCV drug therapies, this new approach may be useful for testing current and future antiviral compounds.

Experimentally infected chimpanzees

Chimpanzees, as the only animals susceptible to HCV infection, have commonly been used in the initial studies on non-A non-B hepatitis and they are continuing to play an essential role in many aspects of HCV research. Studies in chimpanzees included the characterization of infectious sera, analysis of the course of the disease and viral transmission, host immune response studies, infectivity studies and testing of anti-HCV compounds and vaccine candidates (Bassett *et al.*, 1999; Gale & Beard, 2001; Bukh *et al.*, 2001; Lanford *et al.*, 2001). The chimpanzee model, however, has some serious limitations and disadvantages. Most importantly, the availability of the animals is very limited. They are on the list of endangered species, very expensive and difficult to handle. Furthermore, chimpanzees do not respond to HCV infection exactly in the same way as humans. The major difference is in the frequency of chronic infection, which occurs in approximately 75% of the cases in humans, while only 30–50% of infected chimpanzees develop chronic hepatitis. Human disease can progress to liver cirrhosis and fibrosis, which does not happen in chimpanzees. Unlike in humans, high viral clearance (over 60%) is observed in chimpanzees (Basset *et al.*, 1999; Bradley, 2000; Major & Feinstone, 2000; Thomson *et al.*, 2003). These limitations of the chimpanzee model stimulate the search for alternative animal models for HCV.

Murine models for HCV

The chimpanzee model, in which the development of chronic liver disease is extremely rare, cannot be used for studies of liver pathology. To examine the influence of HCV on the liver in an animal model, two types of mouse HCV models have been established:

1. transgenic mice that express HCV proteins in the liver from tissue-specific promoters,
2. mice with chimeric human livers (engraftment of human liver tissue into transgenic, immunocompromised mice).

In the first model, HCV proteins are expressed individually or collectively from different promoters. This model has been used mostly to characterize such liver pathology manifestations as hepatocyte injury, steatosis and hepatocellular carcinoma induced by HCV proteins (reviewed by Gale & Beard, 2001).

The chimeric mice which give a possibility to study liver pathology directly in the human liver tissue seem to be a more accurate model for HCV-induced liver failure. In this model, SCID (severe combined immunodeficiency disease) mice with induced liver failure are engrafted with the human liver tissue. In SCID mice the humoral and cellular immune systems fail to mature, making them one of the best animal models for tissue transplants (Custer *et al.*, 1985). Human liver tissue is typically engrafted to transgenic *scid*/Alb-uPA mice carrying a tandem of murine urokinase genes under the liver-specific albumin promoter. Urokinase overproduction causes liver failure at 2–3 weeks of age and animals are rescued by the human liver transplant leading to repopulation of the mouse liver with human hepatocytes. The resulting chimeric mice are effectively infected with human serum-derived HCV of different genotypes and produce virus that is infectious to other animals (Mercer *et al.*, 2001). As a modification of the HCV mouse model, a novel non-infectious efficacy model for evaluating antiviral compounds has been developed. In this model, Huh-7 cells carrying an HCV replicon were implanted into the liver of SCID mice. The replicon contained the luciferase reporter gene allowing for monitoring the viral replication using non-invasive whole body imaging (Zhu *et al.*, 2006). Those newly developed models are very useful in *in vivo* tests of new compounds potentially inhibiting viral replication and preventing infection, both in drug evaluation and vaccine development studies (Ilan *et al.*, 2002; Hsu *et al.*, 2003a; Kneteman *et al.*, 2006). However, technical difficulty in generating animals and high costs of the experiments are serious limiting factors preventing the use of those models for routine studies.

New World monkeys — marmosets infected with GBV-B virus

An interesting surrogate model for HCV research is the GB virus B (GBV-B). GBV-B is an enveloped, positive-sense RNA virus belonging to the *Flaviviridae* family, phylogenetically most closely related to HCV (Bukh *et al.*, 1999). There is a high degree of structural and biochemical homology between the GBV-B and HCV replication processes (Sbardellati *et al.*, 2001; Hope *et al.*, 2002). GBV-B causes hepatitis in small New World primates such as tamarins (genus *Saguinus*) and marmosets (genus *Callithrix*) and replicates efficiently in cultures of primary hepatocytes of these species (Bukh *et al.*, 1999). The ability of GBV-B to replicate in cell culture makes it possible to grow and study the virus in laboratory conditions. Marmosets are suitable as

model organisms, relatively easy to breed in captivity and already regularly used for drug metabolism, pharmacokinetics, and toxicology studies in drug development, making them an ideal alternative HCV model (Bright *et al.*, 2004).

SUMMARY

The recent technical advances in cell culture systems, replicon and infection assays, have contributed to many important discoveries giving insight into the mechanisms of HCV infection.

New small-animal models (chimeric mice) have emerged which facilitate studies of liver pathology associated with viral infection and testing of new potential antiviral drugs.

The establishment of the cell culture system for HCV opens a new era in the studies of this virus. The system based on the JFH-1 clone has serious limitations: only one strain of HCV genotype 2a (not the most common genotype) can be propagated in a very specific type of cells. The cell-culture grown viral particles are more homogenous and less infective than the virus generated from experimentally infected animals, which may be due to the lower association with lipoproteins (Maillard *et al.*, 2006). However, this is the first true cell-culture system which allows the application of classical virological methods to study many aspects of the viral life cycle, including viral assembly, egress and spread, which have previously been unapproachable. Understanding the molecular virology of hepatitis C virus will be very helpful in identifying new specific targets for antiviral therapy.

All the new methods constitute a solid platform for researchers to study different aspects of HCV biology, including host-virus interactions, very important for the development of new antiviral strategies. Hepatitis C virus, since its discovery in 1989 (Choo *et al.*, 1989), has been a subject of extensive research. Taking into account how much was achieved in the past without such suitable and reliable research tools, it seems highly likely that in the near future hepatitis C virus will become a well known pathogen, with an effective treatment perspective for infected people. With the advances in the understanding of HCV virology and the mechanisms of its genetic variability, it will hopefully become possible to design a universal vaccine against this dangerous human pathogen.

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