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Removal of cell-bound lipoproteins: a crucial step for the efficient infection of liver cells with hepatitis C virus in vitro

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Abstract – Hepatitis C virus (HCV) is of major social, medical and economic importance. The prevalence of HCV is approximatively 1 % in most developed countries, and much higher in developing countries. HCV infection is the second major cause, after hepatitis B virus infection, for the generation of chronic liver disease and hepatocellular carcinoma. To date, the only reliable model for the study of HCV infection is the chimpanzee. Indeed, there is no robust in vitro infection system, yet. There is thus an urgent need for such an in vitro infection system in order to evaluate therapeutic agents. Here, a process is provided for infecting hepatocyte cell lines with hepatitis C virus in vitro. It is strongly suggested that cell-bound lipoproteins are playing a crucial role during the infection process. In order to obtain a robust infection, the cell-bound lipoproteins have first to be removed from their cellular receptor prior to the addition of viral inocula originating from human sera, the latter being made originally of a virus-lipoprotein complex. © 2001 Académie des Sciences/Éditions scientifiques et médicales Elsevier SAS

Hepatitis C virus / HCV / in vitro / infection / hepatocyte / lipoproteins / disease / hepatocellular carcinoma / cancer

Résumé – Enlèvement de lipoprotéines liées à la cellule : une étape clef pour l'infection efficace de cellules du foie avec le virus de l'hépatite C in vitro. Le virus de l'hépatite C (VHC) a une importance sociale, médicale et économique majeure. La prévalence du VHC est d'approximativement 1 % dans les pays développés, et beaucoup plus dans les pays en voie de développement. L'infection par ce virus est la seconde cause majeure de maladie chronique du foie ainsi que d'hépatocarcinome, après l'infection par le virus de l'hépatite B. À ce jour, le seul modèle disponible pour l'étude de l'infection par le VHC est le chimpanzé. En effet, un système d'infection in vitro efficace n'a pas encore été obtenu. Un tel système d'infection in vitro est donc urgent pour l'évaluation d'agents thérapeutiques. Ici est présenté un procédé pour infecter in vitro des lignées hépatocytaires par le VHC. Il est fortement suggéré que les lipoprotéines liées aux cellules jouent un rôle crucial dans les étapes d'infection. Afin d'obtenir une infection efficace, les lipoprotéines liées à leurs récepteurs cellulaires se doivent d'être enlevées avant l'addition de l'inoculum viral, surtout lorsque ce dernier

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provient de sérums humains et est originellement constitué d'un complexe virus-lipoprotéine. © 2001 Académie des Sciences/Éditions scientifiques et médicales Elsevier SAS

virus de l'hépatite C / VHC / infection / in vitro / hépatocyte / lipoprotéines / maladie / hépatocarcinome cellulaire / cancer

. Version abrégée

La biologie des virus des hépatites et la description des atteintes du foie par ces virus ont été résumées extensivement ailleurs [1–4]. Au moins sept agents viraux sont impliqués dans la genèse des hépatites, à savoir, les virus à hépatite A (VHA), B (VHB), C (VHC), D (VHD), E (VHE), G (VHG) et TT (TTV). Tous ces virus, à l'exception des VHA et VHE, conduisent à une infection chronique. Les infections par les virus de l'hépatite B et C sont les maladies virales prédominantes dans le monde, puisque 350 millions et 170 millions de personnes sont infectées par ces virus, respectivement [5–7]. Les VHB et VHC sont la cause majeure des maladies chroniques du foie, spécialement dans les pays en voie de développement. L'infection par les VHB et VHC peut conduire au cancer du foie (carcinome hépatocellulaire) [8–15].

A ce jour, le seul modèle animal validé pour étudier l'infection par le VHC est le chimpanzé [16], une espèce très protégée et d'utilisation très coûteuse. Ceci tient au fait qu'un système d'infection in vitro efficace et sûr est manquant. Ces dernières années, de nombreuses tentatives ont été faites pour propager le VHC dans des cellules eukaryotes in vitro. A cette fin, deux procédures ont été employées, à savoir soit l'infection des cellules avec un inoculum viral, soit la transfection des cellules avec des acides nucléiques [1, 3, 16–29].

En ce qui concerne l'infection virale par le VHC, les niveaux de réplication obtenus sont très faibles et difficilement reproductibles, à l'exception cependant des cellules hépatocytaires primaires d'origine humaine dont l'accès est très contraignant.

Plusieurs récepteurs cellulaires se liant au VHC on été proposés: le récepteur CD81 [30, 31], ainsi que le récepteur LDL [32, 33].

Dans cet article, il est fortement suggéré que les lipoprotéines (provenant de la synthèse des hépatocytes eux-mêmes [34], ou étant présents dans le sérum utilisé pour la culture cellulaire), peuvent interferer avec et inhiber l'infection de cellules in vitro par un inoculum viral d'origine humaine. Lorsque les lipoprotéines liées aux cellules sont détachées de leurs recepteurs cellulaires respectifs avant l'addition de l'inoculum viral, on favorise une infection efficace des hépatocytes par le VHC.

1. Introduction

The biology of hepatitis viruses and the description of the associated liver diseases have been reviewed extensively [1–4]. At least seven viral agents are involved, namely, hepatitis viruses A (HAV), B (HBV) , C (HCV), D (HDV), E (HEV), G (HGV), and TT (TTV). All these viruses except for HAV and HEV cause chronic infections. Hepatitis B virus and hepatitis C virus infections are the most prevalent viral diseases in the world, since around 350 million and 170 million individuals are infected with these viruses, respectively [5–7]. HBV and HCV are the major causative viruses for chronic liver disease, especially in developing countries. Infection with HBV and HCV can lead to hepatocellular carcinoma (HCC) [8–15].

To date, mostly chimpanzees have been validated as reliable model for the study of the hepatitis C infection [16]. This is due to the lack of an efficient and reliable in vitro infection system. During the recent years, several attempts have been made in order to propagate HBV and HCV in eukaryotic cells in vitro. For this, two main procedures have been followed, namely: i) the infection of susceptible cells with a viral inoculum, and ii) the transfection of such cells with nucleic acids [1, 3, 16–29].

Several HCV-binding proteins have been suggested to mediate viral entry of HCV into cells; these include the CD81 receptor [30, 31], and the LDL receptor [32, 33]. Here, it is strongly suggested that cell-bound lipoproteins (originating from the synthesis of the hepatocytes themselves [34], or from the serum employed for the cell culture) might interfere with and/or inhibit efficient in vitro infection of cells with a viral inoculum of human origin. When the cell-bound lipoproteins are first removed from their receptor prior to the addition of the viral inoculum to the cells, HCV infection is facilitated.

2. Materials and Methods

2.1. Cells

HepG2 cells (American type culture collection; passage number over 150) were grown in Eagle's Minimal Essential medium (EMEM; Biochrom AG, Berlin, Germany) supplemented with 10 % fetal calf serum (FCS; PAA, Linz, Austria), 2 mM L-glutamine (Gibco-Life Technologies, Paysley, Scotland) and Earle's BSS (Gibco), and adjusted to contain 1 % non-essential amino acids (Amimed Bioconcept, Allschwil, Switzerland), 1 mM sodium pyruvate

(Gibco), 25 mM HEPES (Gibco), 1.5 $g \cdot L^{-1}$ sodium bicarbonate (Amimed), and antibiotics (100 $U \cdot mL^{-1}$ penicillin, 100 $mg \cdot mL^{-1}$ streptomycin; Gibco) (complete medium). After washing in phosphate buffered saline (PBS; per liter: NaCl 8 $g \cdot L^{-1}$, KCl 0.2 $g \cdot L^{-1}$, KH_2PO_4 0.2 $g \cdot L^{-1}$, Na_2HPO_4 (2 H_2O) 1.44 $g \cdot L^{-1}$) and trypsinization (Trypsin-EDTA; Gibco), the cells were either directly seeded at 25 to 50 % confluency into new culture flasks, or the cell monolayer was trypsinized as above and the cells were washed thereafter once in complete medium before seeding into new culture flasks. This supplementary wash after trypsinization is optional according to the proposed protocol for the trypsinization of the HepG2 cells, but it revealed that the cells were growing as a true monolayer after their seeding into the new culture flasks. Cultures were observed using a phase-contrast microscope. The doubling time was around 8 to 10 days under the employed conditions. The totality of the cell culture medium was changed every 2 to 3 days.

2.2. Infection of cell cultures

The cells (7×10^5 to 1×10^6) were grown in 6-well plates (Costar) until confluency in 4 mL of complete cell culture medium as described above. For infection, the cells were first washed with PBS and then treated with high molecular weight dextran sulfate (AppliChem, Darmstadt, Germany) at 10 $mg \cdot mL^{-1}$ in PBS during an incubation period of 5 to 10 min. This incubation with dextran sulfate carried out the removal of the cell-bound lipoproteins [35]. After this incubation step, the cells were extensively washed with PBS to remove traces of the dextran sulfate-lipoprotein complex. The viral inoculum was then added to the cells for an incubation period of 45 min to 1 h. The viral inoculum used consisted of patient's serum titrated to provide up to 10 HCV genome equivalents per cell either undiluted or diluted in a final volume of 500 μL of PBS. After the infection period, the viral inoculum was removed, and the cells were extensively washed with PBS. The cells were then incubated in complete cell culture medium. Usually, the complete cell culture medium was routinely changed after an overnight incubation in order to remove unbound or loosely bound input virus.

Alternatively, a higher efficiency of infection was achieved if a portion of the infection process described above was performed at lower temperatures, for example at 4 °C or on ice. In this respect, the cells in complete medium were firstly placed on ice for an incubation period of 10 min. The cells were then washed with ice-cold PBS and treated on ice with dextran sulfate, as described above. After the removal of the dextran sulfate-lipoprotein complex, the cells were washed extensively with PBS, and the viral inoculum was added to the cells. The cells were then incubated with the viral inoculum by placing them directly at 37 °C for 45 min to 1 h, or the cells were kept for a while at room temperature for 10 min, before being kept at 37 °C. After the incubation, the viral inoculum was removed, the cells were washed as described above, and then incubated with complete cell culture medium.

Since the binding of lipoproteins to the LDL receptor is a calcium-dependent process [36–39], the calcium chelator ethylene glycol-bis (beta-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA; ethylene-bis(oxyethylenenitrilo) tetraacetic acid; egtazic acid) at 1 mM was employed concomitantly with dextran sulfate.

The HCV-containing supernatant, originating from HepG2 cultures which had been infected in a previous experimental process, were employed for re-infection experiments in two successive passages.

2.3. Maintenance of infected cell cultures

For long-term infection experiments with HCV, about 50 % of the cell culture supernatant was replaced every 3 to 4 days. The cells were splitted in new tissue culture flasks every 3 weeks.

2.4. Detection of expressed viral components

For the initial qualitative quantitation of the HCV RNA present in the infected cell culture supernatants, the Amplicor HCV Monitor test (Roche) was routinely employed. Viral particles were not concentrated. To keep a convenient concentration of proteins in the samples in order to get accurate results, a routine procedure was taken into consideration: the cell culture supernatants from mock-infected and HCV-infected cell cultures were mixed with virus-negative human serum. Usually 50 % to 90 % of serum (final concentration) was present in the samples to be analysed. Negative controls were performed with virus-negative sera. Positive controls consisting of human plasma or sera from patients infected with HCV and with known viremia were processed in a similar way than the samples from the culture medium obtained from mock- and HCV-infected cells. The results were obtained in the form of an index: values below 0.2 did indicate the absence of viral RNA in the analysed samples. Index values over 0.2 did reveal the presence of viral RNA in the sample tested, while index values of around 3.5 to 3.8 were suggestive of the saturation of the analyzed sample with HCV RNA. For the quantitation of the viral genome equivalents in the viral inoculums and in the cell culture supernatants, the Quantiplex 2.0 Assay (Chiron) was used. The detection limit of this test is 200 000 genome equivalents$\cdot mL^{-1}$ (31746 $IU \cdot mL^{-1}$).

3. Results

. Conditions for infection of eukaryotic cells with hepatitis C virus in vitro

The initial aim was to establish an in vitro infection procedure that would not require an overnight incubation and could be performed within one hour and which

Table I. Effect of in vitro infection conditions on expression of HCV RNA. The human hepatoma cell line HepG2 was pretreated with dextran sulfate and infected with HCV-containing inoculums originating from human blood, where indicated. Cell culture supernatants were analyzed for HCV genome equivalents 5 to 8 days post infection. Indexes of 0.2 and 3.8 denote cutoff value and saturation of the analysis with HCV genome equivalents, respectively.

Conditions for infection of cells with hepatitis C virus in vitro	
Treatment	Analysis of the cell culture supernatants, (Indexes COBAS Amplicor, Roche)
Mock infection	0.02–0.2
No dextran sulfate treatment, viremic serum onto cells at 37 °C	0.02–0.2
No dextran sulfate treatment, viremic serum onto cells at 4 °C	0.2–0.5
Dextran sulfate treatment, viremic serum onto cells at 37 °C	0.5–2.5
Dextran sulfate treatment, viremic serum onto cells at 4 °C	> 2.5 (up to 3.8)

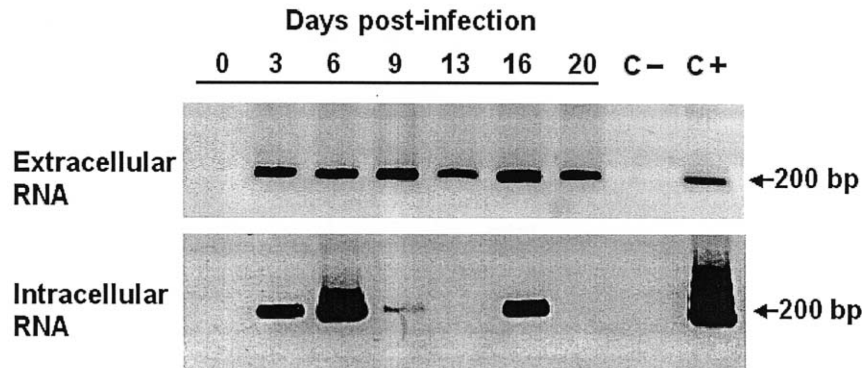

Figure 1. Kinetics of detection of positive-strand HCV RNA in supernatants and cells of HepG2-infected cells. Infection was performed as described in Materials and Methods using human serum positive for HCV. Half of the cell culture medium was changed after 3, 6, 9, 13 and 16 days. At the times indicated, the single-round PCR products were obtained by amplification of the 5′ noncoding region and analyzed by agarose gel electrophoresis followed by staining by ethidium bromide. Negative control (C-) included PCR amplification from cell culture medium and mock-infected cells at the beginning of the infection. RNA isolated from HCV-positive serum and from HCV-positive cells originating from liver biopsies was used as positive controls (C+) for positive-strand RNA detection. PCR amplification of HCV RNA from the cell culture supernatants (higher panel) and from the cells (lower panel).

would favor infection with hepatitis C virus. Initial infection conditions employing the hepatoma cell line HepG2 revealed that very few HCV genome equivalents, as determined by qualitative analyses, were produced in the cell culture supernatants 5 to 7 days post-infection. However, when the cells were kept at 4 °C or on ice during a 1 h infection period, an increase in the amount of genome equivalents present in the cell culture supernatants was obtained, as compared with the similar incubation period performed at 37 °C. This observation led to hypothesize that the adsorption and/or the penetration of the virus into the target cells was probably a very fast process, possibly involving a membrane receptor having a high turnover rate at 37 °C. It was also speculated that the binding of the virus to its putative cellular receptor(s) would somehow be hampered by physiological molecules that would be cell-bound and/or present in vast excess in the serum employed as the viral inoculum. Indeed, when the cell-bound lipoproteins were removed by using dextran sulfate prior to the addition of the viral inoculum onto the target cells, higher levels of HCV RNA were detected in the cell culture supernatants (*table I*). In order to exclude the possibility that the genome equivalents measured in the cell culture supernatants were indeed made of input virus, quantitative measurements of the viral titers in the cell culture supernatants were then performed. This revealed that during long-term experiments lasting up to three months, a two logarithmic_{10} increase of output virus was obtained, when compared to the input virus. We determined, whether the established hepatocyte cell line HepG2 could be infected with human serum positive for HCV. *Figure 1* shows the detection of extracellular and intracellular positive strand RNA in cell cultures up to day 20 after infection. Viral RNA was detected in the cell supernatant from day 3 up to day 20. The level of positive strand in the infected cells appeared to fluctuate, showing two peaks of detection at days 6 and 16 post-infection. Negative strand RNA was only detected intermittently in delayed time points (not shown). To further assess, whether the released viral particles were infectious, re-infection experiments were performed using supernatants from the previous infectious cell round. This revealed that naive HepG2 cells could be infected with HCV in two successive passages (*table II*). Under the conditions employed, the secretion of viral particles, as revealed by the detection of the viral RNA in the cell culture supernatant, was a very slow but permanent process; HCV RNA could be detected in the cell supernatants up to four months. Viral sequences

Table II. Kinetics of HCV RNA production during reinfection of long term HepG2 cell cultures with cell culture supernatants positive for HCV. HepG2 cells growing in 6-well plates in 4 mL cell culture medium were initially infected with HCV at around 3 genome equivalents per cell (1.5 MEq per well). The cell culture medium was changed every three to four days. After three weeks of incubation, the cells were trypsinized, washed, and passed into new 6-well plates at a splitting ratio of 1:3. The cells were then long-term incubated for around 6 weeks without trypsinization, after which time the cell culture supernatant (inoculum A) was collected and employed as an inoculum for a first round of reinfection of naive HepG2 cells. After two successive trypsinizations of the cells every three weeks, the cell culture supernatant (inoculum B) was collected 90 days after the onset of the first round of reinfection, and employed for a second round of reinfection of naive HepG2 cells. Results are given as million genome equivalants (MEq) per milliliter of cell culture by employing the Quantiplex HCV RNA 2.0 assay (Chiron).

	MEq/mL (full medium change)	MEq/mL (half medium change)
Reinfection I (Inoculum A)	41.7	41,7
Day 60	0.285	0,270
Day 75	0.315	0,293
Day 90	0.274	< 0.2
Reinfection II (Inoculum B)	0.364	0,364
Day 4	< 0.2	0,315
Day 25	0.414	< 0.2

were not detected in the cell culture supernatants from uninfected and mock-infected hepatocytes.

4. Discussion

The current 'state-of-the-art' model for the replication of HCV in tissue culture is based on the use of established hepatocyte cell lines, such as the HepG2 and the Huh-7 cell lines [16]. The method of choice does employ the transfection of in vitro transcribed hepatitis C virus RNA replicons in the hepatoma cell line HuH-7 [20, 40]. However, this progressively more efficient transfection procedure does not represent by any means an infection model. Although replicon-based assays do provide an in vitro subgenomic replication system which is precious for the screening of antiviral molecules, it is not relevant for the study of the early steps of infection and the evaluation of adsorption and internalization for the sake of receptor evaluation, and finally for neutralization studies and prophylaxis.

Altogether, there is so far no appropriate in vitro infection system for HCV. It was hypothesized that some crucial events leading to the infection of eukaryotic cells with HCV may lie at the level of the cellular receptor(s) of the virus. Several HCV-binding proteins have been suggested to mediate viral entry of HCV into cells; these include the CD81 receptor [30, 31], and the LDL receptor [32, 33]. Interestingly , the majority of infectious, positive-strand hepatitis C virions in human sera are associated with β-lipoproteins [41, 42]. The hypervariable region HVR1 in the envelope glycoprotein E2 is involved in interactions with negatively charged molecules such as lipids, proteins, or glycosaminoglycans [43]. The viral entry of HCV might involve receptor-mediated endocytosis via the LDL receptor, and a similar mechanism of entry might also apply for other enveloped viruses [44, 45]. The receptor-mediated endocytosis and the lipoprotein metabolism in itself have been extensively reviewed [34, 36, 46, 47]. The internalization and recycling of the cell surface receptors is a fast process, and the lipoprotein metabolism does also involve divalent cations in the case of the LDL receptor [36, 47]. The working hypothesis for this study did thus speculate that lipoproteins might be involved in the viral entry of HCV into cells, and that cell-bound lipoproteins might hamper efficient binding of the viral particles to the low density lipoprotein receptor in vitro.

It is known that several precipitation procedures can be employed for the precipitation of high-density lipoprotein cholesterol from human blood and for fractionation of human blood in order to retain the high density lipoprotein (HDL) cholesterol in the supernates after the precipitation of both the low density lipoproteins (LDL) and the very low density lipoproteins (VLDL) [48–51]. In these studies, human blood was mixed with either heparin or dextran sulfate, and the resulting solution was centrifuged to sediment the insoluble lipoproteins. A related procedure was also applied for the removal of cell-bound low density lipoproteins from the hepatocyte cell line HepG2 [35].

It was thus clear that lipoproteins bound to both the LDL receptor and to HCV were certainly playing a crucial role for the virus entry into the target cells. A general scheme for the in vitro infection of eukaryotic cells is illustrated in

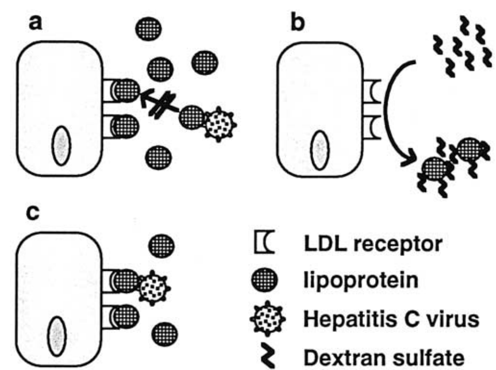

Figure 2. Model for the infection of eukaryotic cells with hepatitis C virus in vitro. Removal of cell-bound lipoproteins from the low density lipoprotein (LDL) receptor might be the crucial step for efficient hepatitis C virus infection in vitro. **a.** the binding of the HCV-lipoprotein complex to the LDL receptor is hampered in vitro by the cell-bound lipoproteins and by the vast excess of free lipoproteins present in the human blood. **b.** prior to HCV infection, the cell-bound lipoproteins are removed from the LDL receptor by using dextran sulfate, thus generating free LDL receptors. **c,** lipoprotein-free LDL receptors can bind the HCV-lipoprotein complex, thus allowing adsorption and penetration of HCV into target cells. A similar involvement of lipoproteins might take place during the infection of cells with HBV.

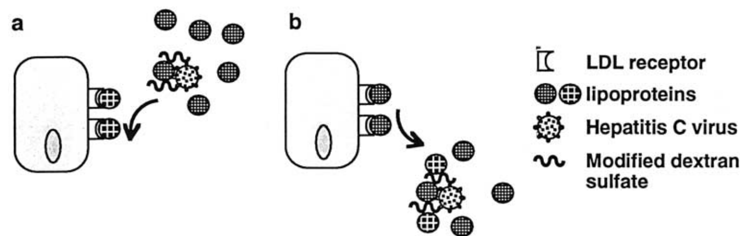

Figure 3.a. Model for the inhibition of hepatitis C virus infection in vivo. Therapeutic, modified dextran sulfate molecules in the blood should preferentially bind to the hepatitis virus-lipoprotein complex, but not to free lipoproteins **b.** Upon removal of the cell bound lipoproteins by the hepatitis C virus-lipoprotein complex, if any, the free lipoproteins can bind to the LDL receptor. Binding of the hepatitis virus-lipoprotein complex to the LDL receptor is thus inhibited.

figure 2. Removal of the cell-bound lipoproteins prior to the addition of the viral inoculum on the cells could well be therefore a crucial step for the efficient infection of eukaryotic cells and established cell lines with hepatitis C virus in vitro. To this end, the cell-bound lipoproteins should first be removed from the LDL receptor prior to the addition of the viral inoculum to the cells. The removal of the cell-bound lipoproteins can easily be achieved by treating the cells with the synthetic glycosaminoglycan and polyanionic heparin analogue, dextran sulfate [35]. The viral inoculum, composed of HCV-positive human serum, is then added to the cells to allow the binding of the HCV-lipoprotein complex to the LDL-free LDL receptors. Indeed, it has been shown that beta-lipoproteins competitively inhibit the infection of hepatocytes with HCV through the low density lipoprotein receptor in vivo [52].

A crucial event leading to a more efficient infection of eukaryotic cells with HCV may thus lie on the removal of the lipoproteins bound to the LDL receptor. In this respect, it is tempting to speculate that the proposed infection procedure might also apply for other enveloped viruses bound to lipoproteins in human serum, for example, dengue virus belonging to the Flaviviridae family [53].

It might be envisioned to modulate or even inhibit HCV infection in vivo by employing therapeutic, modified dextran sulfate molecules (*figure 3*). These modified dextran sulfate molecules might preferentially bind to both hepatitis C virus and to the virus-bound lipoproteins, thus leading to the inhibition of the binding of the lipoprotein-hepatitis C virus complex to the target cells, thus allowing solely the binding of the free lipoproteins to the LDL receptor.

The intermittent detection of the negative-strand RNA in liver cells has been reported in the chimpanzee and human in vitro culture models, as well as during experimental and natural infection [22, 24, 54–57] The HCV replication might thus have tightly regulated, intrinsic cycles. Indeed, the replication of the viral RNA does initially require the so-called IRES-mediated, cap-independent translation [58] of the genomic, positive-stranded RNA, a very inefficient process in nature [59]. The translated viral polymerase is then needed to transcribe the replicative intermediate, negative-stranded RNA. Thus, the cap-independent translation of the HCV RNA in vitro might be hampered, or strongly inhibited, due to the lack of additional growth factors and/or hormones [60, 61]. Moreover, amino acid status in the cell culture might also play a role in the IRES-mediated translation of the HCV RNA, as for the IRES-mediated translation of a mammalian mRNA [62].

Besides the transfection of cells by employing the replicon system which can now be used for the need of screening of antiviral candidates active against the viral protease, helicase and RNA polymerase, there is a major need for a true in vitro infection system for HCV. This will help for studying the virus-host cell receptor interactions which are the clue for the evaluation of T and B cell neutralizing epitopes and which are therefore mandatory for the development of vaccines.

The infection procedure described above might also allow the fine characterization of the viral infection at the cellular and molecular levels. Time-course studies for the detection of the nucleic acids and the viral polypeptides during the course of the HCV infection in vitro are currently in progress. An in vitro infection procedure might thus undoubtly reduce and spare the use of animals and especially chimpanzees for viral infection studies and for the screening of antiviral compounds. The relevance of an adequate in vitro infection system for the agent of hepatitis C virus, which constitutes a global health problem, deserves special emphasis.

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