

# Detection of the negative-strand hepatitis C virus RNA in tissues: implications for pathogenesis

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## Abstract

The replication of hepatitis C virus (HCV) RNA is believed to occur via its transcription into a complementary, genomic-length RNA, the so-called negative-strand HCV RNA. This is based on the comparison with the replication of other members of the *Flaviviridae* family. Detection of the negative-strand HCV RNA in human tissues by semi-quantitative, strand-specific RT-PCR has contributed to the understanding of the HCV cell tropism and of the pathogenesis of HCV-associated disease manifestations. In particular, it was shown that the levels of intrahepatic HCV RNA are not correlated to the extent of the necroinflammation, but that a significant correlation was found with the liver steatosis. These results suggest that most liver disease associated with HCV infection is mediated by the host immune response. However, in some patients, most notably those infected with HCV genotype 3, HCV may cause a cytopathic effect, consisting in the lipid accumulation within hepatocytes. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Hepatitis C virus; Necroinflammation; *Flaviviridae* family; Steatosis

## 1. Introduction

The hepatitis C virus (HCV) is an enveloped virus belonging to the *Flaviviridae* family. The virus genome is a linear, single-stranded RNA of ~9600 nucleotides, which contains a single open reading frame encoding a polyprotein precursor of about 3000 aminoacids (Major and Feinstone, 1997). Since its genome functions as a messenger RNA for its polyprotein translation, it is also referred to as the positive-strand RNA. The repli-

cation of HCV RNA is believed to occur via its transcription into a complementary, genomic-length RNA, the so-called negative-strand HCV RNA. This is based on the comparison with the replication of other members of the *Flaviviridae* family that can be efficiently cultivated in vitro, thus allowing the characterization of the different steps of the viral replication (Chu and Westaway, 1985; Westaway, 1987; Gong et al., 1996). According to the model proposed for Flavi- (Chu and Westaway, 1985) and Pestivirus (Gong et al., 1996) replication, the viral genome is first transcribed into its negative-strand RNA. The double-stranded ‘replicative form’ (RF) RNA, consisting of the fully base-paired genomic- and negative-

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strand viral RNA's, functions as a recycling template for a semiconservative replication, where the progeny genomic-strand RNA is continuously transcribed and released for being incorporated in virions. Each nascent genomic-strand RNA molecule displace the previously synthesized genomic RNA in the 5'-3' direction, and each RF may direct the synthesis of one or more progeny genomes at the same time (Gong et al., 1996). The complex of RF and its associated nascent genomic-strand RNA(s) is referred to as replication intermediate.

The steps of the HCV replication have not been defined. This is mostly due to the fact the development of *in vitro* replication systems for propagation of HCV has been difficult, with reliable systems for efficient long term virus replication being poorly reproducible (Shimizu et al., 1992; Fournier et al., 1998). Thus, the HCV replication has been so far studied essentially in *in vivo* models, i.e. the experimentally infected chimpanzee and the naturally infected humans. These studies have to cope with the very low level of replication of HCV (Major and Feinstone, 1997), hence the need to detect its RNA by amplification-based procedures. To achieve so, both the reverse transcription-polymerase chain reaction (RT-PCR) and signal amplification procedures, such as the branched DNA assay (Detmer et al., 1996), have been used.

The scope of the present paper is to discuss some technical aspects of the specific detection of the negative-strand HCV RNA by RT-PCR as well as our personal experience deriving from the application of this procedure to the study of HCV pathogenesis and cell tropism. Several review articles can be found in the literature on technical and virological aspects of the strand-specific detection of HCV RNA (Lanford and Chavez, 1998; Sangar and Carroll, 1998).

## 2. The technical issue of strand-specificity

Some authors have reported on the detection of intrahepatic HCV RNA of genomic polarity (Di Martino et al., 1997; Terrault et al., 1997). Although the ratio of liver to serum HCV RNA

titers may be high (median 103, range 17.4–286) (Terrault et al., 1997), in some patients with high-level viremia the intrahepatic titer of genomic HCV RNA may be affected by the viral RNA of circulating virions, trapped in the liver tissue at the time of sampling. For this reason, we and others prefer to assess the HCV replication in liver and extrahepatic tissues by strand-specific RT-PCR.

The specificity of early procedures (Wang et al., 1992; Müller et al., 1993) used to detect the negative-strand HCV RNA has been questioned by subsequent work (Gunji et al., 1994; Lanford et al., 1994; McGuinness et al., 1994; Lanford et al., 1995; Lérat et al., 1996), based on the demonstration that excess, genomic-strand HCV RNA may act as a template for the synthesis of a false negative-strand. The explanations for this phenomenon vary, but it is most likely due to a false priming during the complementary DNA (cDNA) synthesis by the thermo-sensitive RT (probably mediated via a thermostable hairpin structure at the 5' non-coding region of HCV RNA) or to the failure to inactivate the RT during the PCR amplification. Several gimmicks have been proposed to overcome this lack of specificity: chemical modification of RNA 3' ends prior to RT (Gunji et al., 1994), use of tagged primers (Lanford et al., 1994), use of thermoresistant RT (rTth) (Lanford et al., 1995) or use of primers amplifying the core-encoding region of HCV (Lérat et al., 1996). Even when one of these approaches is followed, the specificity of the assay must be further confirmed. The use of synthetic, *in vitro* transcribed HCV RNA of either polarity has been proposed (McGuinness et al., 1994) but later questioned (Lanford and Chavez, 1998). In fact, failure to digest to completion the template cDNA used for the *in vitro* transcription results in the production of small deoxyribonucleotides which may prime the RT reaction. Thus, the classical experiment used to assess the strand-specificity of the RT-PCR, i.e. the titration to the endpoint of synthetic HCV RNA of either polarity in the presence of the 'good' primer (i.e. complementary) or of the 'wrong' primer (i.e. having the same sequence as the RNA to be reverse-transcribed) is of little meaning, since the primers resulting as end-prod-

ucts of the incomplete DNase digestion of the template may efficiently direct the synthesis of a HCV cDNA of either polarity, thus impairing the assessment of the strand-specificity.

We (Negro et al., 1998) and others (Lanford and Chavez, 1998; Agnello et al., 1998) have suggested that this limitation may be overcome by using an alternative specificity experiment. To assess the strand-specificity of the assay, we performed an RT-PCR amplification with the sense or, respectively, the antisense primer on genomic HCV RNA extracted from serum. In serum, negative-strand HCV RNA is mostly found at very low titers. The above experiment is sufficient to prove the strand-specificity of the RT reaction when the titration of both HCV RNA strands in a serum containing at least  $10^6$  HCV genomes/ml results in a difference between the genomic- and the negative-strand titer of at least six Logs. In other words, in the presence of a  $10^6$ -fold excess of genomic vs. negative-strand HCV RNA, there should be no appreciable amplification using the sense primer (Agnello et al., 1998; Negro et al., 1998). Unfortunately, a sample containing excess negative-strand HCV RNA vs. genomic-strand HCV RNA is not available in vivo. Thus, once the strand-specificity has been proven for the genomic-strand, one can only infer that the same level of specificity would reasonably apply to the amplification of the opposite, negative-strand HCV RNA.

### 3. Detection of negative-strand HCV RNA in chronic hepatitis C

One of the approaches used to study the pathogenesis of HCV-associated disease is to assess its replication in infected tissues and to establish anatomic-clinical correlations.

HCV RNA replication may be studied by *in situ* analysis techniques or by extraction-based procedures. The extraction-based techniques used to study the HCV replication in tissues include the Northern gel analysis and the strand-specific RT-PCR. Although extraction-based techniques do not allow to define the number and type of HCV-infected cells, they may provide nonetheless criti-

cal information to assess the presence and level of HCV replication in a given tissue.

*In situ* hybridization studies carried out in the chimpanzee have shown that hepatocytes infected with HCV (genotype 1a) have a normal morphology, suggesting the absence of a viral cytopathic effect (Negro et al., 1992). Although *in situ* staining techniques for HCV RNA and antigens may pose some specificity problems (Lau et al., 1996), these results have been so far confirmed by most authors using widely different approaches. A single *in situ* hybridization-based study showed the occasional presence of HCV RNA in steatotic hepatocytes (Haruna et al., 1993). The significance of this association, which suggests that HCV may in some cases cause a cytopathic effect, will be discussed below.

The Northern gel analysis has proven of little use in the study of the HCV pathobiology. It was successfully applied only to the study of genomic-length HCV RNA in total RNA extracted from large liver specimens obtained at the time of liver transplantation (Hu et al., 1995). In the same study, no appreciable amounts of subgenomic HCV RNA forms were detected. On the other hand, no genomic-strand HCV RNA was detected in extracts from 60 liver needle-biopsy specimens using  $^{32}\text{P}$ -labeled riboprobes covering 40% of the viral genome (Authors' unpublished observations), suggesting that human tissue specimens routinely obtained in the clinical setting are not suitable to this relatively insensitive analysis.

Relatively few papers have focused on the semi-quantitative detection of negative-strand HCV RNA in tissues by strand-specific RT-PCR in chronic hepatitis C. In a recent work (Negro et al., 1999), we studied the genomic- and negative-strand HCV RNA in the liver of 61 immunocompetent chronic hepatitis C patients. We found that the intrahepatic negative-strand HCV RNA titer was not correlated with the genomic HCV RNA level in the serum (although a correlation was found between the latter and the intrahepatic genomic-strand titer). This was a first indication that the negative-strand HCV RNA titer may not be a reliable indicator of the HCV replication rate, the latter being possibly affected by several variables other than the mere number of RF

molecules, either host- (intracellular factors) or virus-related (affinity of the replicase, presence of defective forms, relative excess of intracellular genomic-strand HCV RNA). Moreover, intrahepatic titers of both strands of HCV RNA were completely overlapping when patients were divided according to the response to  $\alpha$ -IFN treatment, although the pattern of response could still be predicted by the serum HCV RNA level and genotype. Again, this suggested that the titration of intrahepatic HCV RNA, may not provide additional information to the clinician with respect to the assessment of the HCV level in serum and genotype. However, these data should not necessarily lead to such pessimistic conclusions. The overall lack of correlation may also result in fact from the heterogeneity in the intrahepatic viral load in patients infected with different HCV genotypes. In patients infected with HCV types 1, 3 and 4, the genomic- and negative-strand HCV RNA titers in the liver were comparable with one another. On the contrary, titers found in the liver of patients infected with HCV genotype 2 were significantly lower when compared to titers found in patients infected with other genotypes, and this in spite of the fact that HCV viremia levels did not vary significantly according to the different genotypes. This difference in the intrahepatic HCV RNA titer observed in patients infected with genotype 2 was not due to a genotype-dependent efficiency of amplification. In fact, when we titrated to the endpoint a series of 10-fold dilutions of the genomic-strand HCV RNA extracted from seven sera (three with genotype 1, two with genotype 2 and one each with genotype 3 and 4), in which quantitation had been achieved by the bDNA assay, we found a sensitivity limit of 15 genomic-strand HCV RNA molecules per assay and this independently of the viral genotype. This experiment proved that the efficiency of our strand-specific RT-PCR was genotype-independent. One of the possible explanations to account for the low intrahepatic titers of HCV RNA in patients infected with HCV genotype 2 may be the presence of a significant contribution to the total viral load from extrahepatic sites. The presence of extrahepatic sites of HCV replication is now established (see below) and based upon sev-

eral lines of evidence (Lérat et al., 1996; Kao et al., 1997; Cabot et al., 1997; Navas et al., 1998; Mellor et al., 1998; Bronowicki et al., 1998), even though, in general, it seems to be low-level (Fukushima et al., 1996). Interestingly, infection with HCV type 2 has been associated, in some series, with clinically significant extrahepatic manifestations (such as cryoglobulins secretion) (Zignego et al., 1996; Leruez-Ville et al., 1998), sometimes among patients with little/minimal liver disease. So, we cannot exclude that, at least in some patients infected with HCV type 2, an extrahepatic HCV replication may prevail, affecting both the HCV disease expression and the correlation between serum and liver viral loads.

In the same work, no correlations were found between the liver disease grading and staging scores and the HCV load, as assessed either in the serum or in the liver, suggesting that higher levels of HCV replication are not necessarily associated with a more severe liver disease. These findings are a strong argument in favor of the notion that HCV-associated liver disease is mostly mediated by the host immune response.

We concluded that the detection of the negative-strand HCV RNA in any given tissue provides a reliable, qualitative evidence of the presence of HCV replication in its target organ. For the time being, and in the absence of larger studies on bigger numbers of liver samples from patients infected with all different HCV genotypes, the quantitative measurement of viremia remains the clinically meaningful 'golden standard' for assessing the level of HCV replication.

#### **4. Negative-strand HCV RNA and recurrent hepatitis C after liver transplantation**

We studied 23 patients who underwent an orthotopic liver transplantation (OLT) for end-stage, HCV-related cirrhosis (Negro et al., 1998). All of them had recurrent HCV infection early after OLT, since HCV RNA was detected in serum of all patients tested during the first week after OLT. Six patients, however, did not develop recurrent hepatitis: all six had an initial episode of rejection controlled by treatment but progressing

to chronic rejection in two. Two further patients, among those considered in that study, developed recurrent hepatitis, but subsequent liver biopsies showed only minimal changes. Thus, only among the remaining 15, histological signs of recurrent hepatitis persisted throughout an average follow-up of 108 weeks after OLT. Serial liver biopsies were thus available from patients with or without recurrent hepatitis, and we could titer both strands of HCV RNA in the liver and correlate the results with the histological features.

The negative-strand HCV RNA was detectable in the liver as early as 7 days after OLT. Although there were wide variations of the intrahepatic HCV RNA levels, even within the same patient, the intrahepatic negative-strand HCV RNA titer tended to increase over time, at least up to the first 6 months from OLT. This increase appeared independent of the presence of recurrent hepatitis. Later during the follow-up, the titers were no longer correlated with the time from OLT.

The most important observation in this setting was that the levels of negative-strand HCV RNA in specimens without hepatitis were comparable to those seen in specimens with recurrent hepatitis and were unrelated to the liver disease grading and staging scores. Indeed, some livers contained very high titers of HCV RNA, in spite of the total absence of virally-induced liver damage, and the same held true for viremia levels. Moreover, the intrahepatic HCV replication occasionally preceded by several months the recurrence of the hepatitis. These results, put together, show that HCV may replicate without inducing necroinflammatory changes in the liver, and suggest that the pathogenesis of the recurrent hepatitis C after OLT may be due to a loss of immunological tolerance to HCV proteins.

## 5. HCV and steatosis of the liver

A peculiar histopathological feature associated with chronic hepatitis C is the steatosis of the liver (Scheuer et al., 1992). The causes underlying the fat accumulation in chronic hepatitis C patients may encompass obesity, drugs, alcohol, diabetes and concomitant infections. However, even when

all of these causes are carefully excluded, a significant proportion of patients persistently infected with HCV may still have a fatty liver. The proportion of patients infected with HCV who have a steatosis may be as high as 50%, to the point of being considered as diagnostic (Czaja and Carpenter, 1993).

These observations suggest that the lipid accumulation in hepatocytes may be due, at least in some patients, the expression of a cytopathic effect of HCV. As mentioned above, a single *in situ* hybridization-based study had already showed the occasional presence of HCV RNA in steatotic hepatocytes (Haruna et al., 1993), but the real significance of this association had not been studied in further detail.

To verify the hypothesis that HCV may be directly involved in the lipid accumulation, we titrated both strands of HCV RNA in the liver of a population of hepatitis C patients without other risk factors for a fatty liver. The occurrence and severity of liver steatosis was not only compared with the intrahepatic viral load, but also with the viremia level, the HCV genotype, and the response to antiviral treatment. The study population included 70 immunocompetent chronic hepatitis C patients and 31 patients with recurrent hepatitis C after OLT (Rubbia-Brandt et al., 2000).

Among the 70 immunocompetent chronic hepatitis C patients, a steatosis was seen in 28 cases (40%), sometimes involving as many as 70–80% of hepatocytes (Fig. 1). Steatotic patients were more likely to be infected with HCV genotype 3, and patients with HCV genotype 3 had higher average scores of steatosis than patients with other genotypes. Among the 31 patients who underwent an OLT for HCV-related cirrhosis a steatosis of the graft was observed in 13 patients, and its score again correlated with the HCV genotype 3.

The steatosis score did not correlate with the serum HCV RNA level, either among the immunocompetent patients or the OLT recipients. Among the former ones, however, a significant correlation was found between the steatosis score and the liver HCV RNA titer, both genomic- and negative-strand, especially in patients with geno-

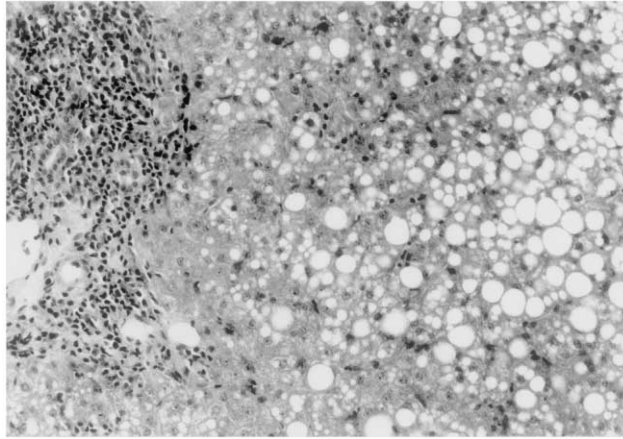


Fig. 1. Microphotograph of a liver taken from a chronic hepatitis C patient infected with HCV genotype 3, showing a severe steatosis affecting ~70% of hepatocytes. Hematoxylin and eosin staining. Original magnification 100 × .

type 3 (Fig. 2). Among OLT recipients, the correlation was still significant for both strands of HCV RNA, but only when the 18 liver biopsies from patients with genotype 3 were considered.

The steatosis score was also correlated with the scores of lobular and portal/periportal necroinflammation as well as with the fibrosis score, but only among immunocompetent patients, whereas this correlation was lost after OLT.

The response to antiviral therapy was accompanied by the disappearance of steatosis from the liver. Two immunocompetent sustained responders to  $\alpha$ -IFN had moderate to severe steatosis before treatment and were all infected by HCV genotype 3: the control liver biopsy performed 1 year after the end of treatment in two of them showed not only the absence of necroinflammation, but also the complete disappearance of the steatosis. Among OLT recipients who developed recurrent hepatitis, response to  $\alpha$ -IFN treatment was paralleled by disappearance of steatosis from the liver biopsy, and HCV relapse after the end of therapy was accompanied by reappearance of a fatty liver (Rubbia-Brandt L, Quadri R, Negro F, unpublished).

In conclusion, we have three pieces of evidence supporting the relationship between HCV and liver steatosis: (1) the association with HCV genotype 3, suggesting the presence of specific viral sequences peculiar of this viral type, in agreement

with data reported also by other investigators (Mihm et al., 1997; Adinolfi et al., 1999; Jonsson et al., 1999); (2) the correlation between genomic- and negative-strand HCV RNA titer in the liver and severity of the fatty accumulation; and (3) the concordance between response to antiviral treatment and disappearance of steatosis from hepatocytes.

We have underlined above that the titer of negative-strand HCV RNA may not be a precise

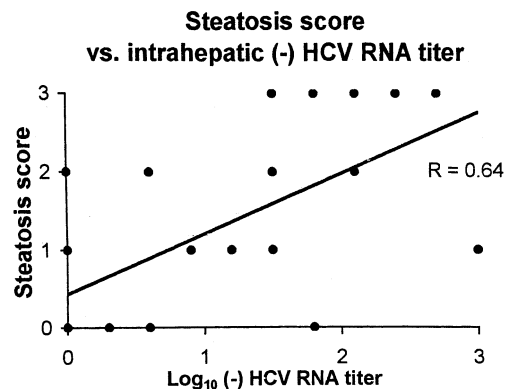


Fig. 2. Correlation between the intrahepatic minus-strand HCV RNA titer and score of liver steatosis in 18 chronic hepatitis C patients infected with HCV genotype 3. Scores are defined as follows: absent or minimal (<1% of hepatocytes) (score 0), mild (<30% hepatocytes involved) (score 1), moderate (between 30 and 60% of hepatocytes involved) (score 2) or severe (>60% of hepatocytes involved) (score 3).

measure of the HCV replication rate, at least not any better than the viremia. However, the good correlation (at least among patients infected with genotype 3) between intrahepatic HCV RNA levels and steatosis score suggests a causal link between the two (not necessarily between steatosis and HCV replication). Recently, the degree of steatosis was also correlated with the amount of core protein expression in the liver, as assessed semi-quantitatively (Fujie et al., 1999). This raises the following question: what is the HCV product responsible for the lipid accumulation? Is it a viral protein or the viral RNA itself? Mice made transgenic with the entire core-encoding gene of HCV are characterized by massive liver steatosis in some (Moriya et al., 1997) but not other cases (Kawamura et al., 1997; Matsuda et al., 1998; Pasquinelli et al., 1997). In vitro, an interaction between the HCV core protein and the apolipoprotein AII was suggested by a double immunofluorescence staining followed by confocal microscopy (Barba et al., 1997) and confirmed by yeast hybrid experiments (Sabile et al., 1999). Whether the expression of the HCV core protein directly leads to a derangement in lipoprotein secretions from hepatocytes in vivo remains to be seen. To further complicate the issue, it has to be noted that the HCV genotype associated with the fatty accumulation in both transgenic mice (Moriya et al., 1997) and in vitro (Barba et al., 1997) is 1b rather than 3, as reported by us (Rubbia-Brandt et al., 2000) and other investigators (Mihm et al., 1997; Adinolfi et al., 1999; Jonsson et al., 1999). To further investigate the mechanism of fatty accumulation in chronic hepatitis C patients, we then selected 23 patients with genotype 3 (19 immunocompetent chronic hepatitis C patients and four OLT patients at the time of the maximum severity of steatosis after OLT) for analyzing the sequence of the complete nucleocapsid-encoding region. The different consensus aminoacid sequences were then aligned and their heterogeneity compared with the severity of steatosis and titer of minus-strand HCV RNA in the liver (Abid et al., 2000). Sequences from patients with severe steatosis, infected with HCV genotype 3 and high level of intrahepatic HCV RNA did not show any specific single aminoacid

substitution, as compared with sequences obtained from patients with low degree or no steatosis but comparable titers of intrahepatic minus-strand HCV RNA. Thus, our sequence analysis is not consistent with the hypothesis that the HCV core protein is the sole responsible for the fatty accumulation, since we could not definitely assign any given aminoacid sequence with the steatotic phenotype. Given the massive steatosis seen in most of our patients, we believe it unlikely that poorly represented viral variants (viral quasispecies) may be responsible for the steatosis, although this may well be the only explanation of a direct cytopathic effect mediated by the HCV core protein.

To reconcile the literature data, we have some hypotheses: (1) the HCV protein responsible for the steatosis in the human infection is not the nucleocapsid, the published data on genotype 1b being valid only in the experimental models; (2) the HCV core protein may induce the steatosis by default, but other viral protein(s) (mostly among HCV genotypes non-3) may prevent from the fatty accumulation; (3) some polymorph host cofactors may modulate the degree of the fatty accumulation induced by the HCV core protein; (4) the steatosis is directly caused by the viral RNA (either the genomic- or the negative-strand), possibly via an interaction with a host RNA. Whether and how these interactions indeed occur in vivo is presently matter of speculation.

What seems important is that the steatosis may be associated with a progressive liver disease, as shown by the following: (1) the degree of the steatosis is correlated with the liver disease grading and staging, at least in immunocompetent chronic hepatitis C patients (Rubbia-Brandt et al., 2000); (2) after OLT, where steatosis of the graft is a specific hallmark of HCV recurrence (Baiocchi et al., 1998), the fatty accumulation seems to be the earliest predictor of fibrosis (Pelletier et al., 2000). Whether the steatosis is a condition favoring the liver disease progression per se or simply a byproduct of a pathogenic event independently leading to the fibrosis and possibly to oncogenesis is at presently unknown.

In conclusion, the titration of intrahepatic genomic- and negative-strand HCV RNA shows

that the hepatocyte steatosis seen in chronic hepatitis C may be caused by HCV, thus representing the morphological expression of a cytopathic effect of HCV genotype 3. The fine details of the interaction between an hitherto unknown viral product and the host lipid metabolism have still to be clarified.

## 6. Extrahepatic sites of HCV replication

In the context of the search for an extrahepatic reservoir of HCV replication, the strand-specific detection of the negative-strand HCV RNA has contributed to clarify which may be the major sites of HCV tropism. Peripheral blood mononuclear cells (PBMC) have been shown to harbor low-titer, HCV replication intermediate RNA (Lérat et al., 1996; Kao et al., 1997; Mellor et al., 1998; Bronowicki et al., 1998), and the HCV replication in these cells is further confirmed by the analysis of the viral quasispecies (Cabot et al., 1997; Navas et al., 1998). Different subsets of circulating leukocytes have been analyzed, and HCV replication seems to be confined to polymorphonuclear leukocytes, monocytes/macrophages and B (but not T) lymphocytes (Lérat et al., 1998).

This is important, because it has been argued that the PBMC reservoir may be involved in the graft reinfection by HCV after liver transplantation. Moreover, the HCV replication in cells involved in the immune response may have dramatic consequences as far as pathogenesis and oncogenesis are concerned. It has been shown that HCV may interact *in vitro* with the apoptotic cascade, leading to a resistance to the apoptotic stimulus (Ray et al., 1996, 1998; Marusawa et al., 1999). If this interaction occurs in lymphocytes and other cells of the immune system, this may lead to a loss of immune competence. Defects in the control of the immune system homeostasis by apoptosis are relevant for the pathogenesis of both autoimmune and lymphoproliferative disorders (Nagata, 1997), which are both frequently associated with HCV infection (Ferri et al., 1997). HCV does not seem, however, to replicate in B-cell non-Hodgkin lymphoma tissue (Ascoli et al., 1998; Rubbia-Brandt et al., 1999).

The presence of HCV RNA in PBMC has been questioned by other investigators (Laskus et al., 1997; Lanford et al., 1995). The disparate results reported in the literature may be due to the difficulties in detecting minute amounts of negative-strand HCV RNA in such cells, as suggested by the fact that negative-strand HCV RNA becomes more easily detectable in PBMC of immunosuppressed patients, who are characterized by higher HCV replication levels, as in case of confection with HIV-1 (Laskus et al., 2000) or after liver transplantation (Radkowski et al., 1998).

Other organs seem to support low-level HCV replication, such as bone marrow, lymph nodes, spleen, pancreas, thyroid, adrenal glands (Laskus et al., 1998). On the contrary, no negative-strand HCV RNA has been found in kidney, lung, muscle, spinal cord (Laskus et al., 1998), skin affected by vasculitis secondary to mixed cryoglobulinemia or lichen ruber planus (Mangia et al., 1999). Extrahepatic manifestations are frequent in HCV infection, but any correlation between HCV replication at extrahepatic sites and disease expression is at present premature. Taken together, the above results seem to confirm that the liver is the major reservoir of HCV replication, in agreement with indirect, kinetics data from the liver transplantation model (Fukumoto et al., 1996), and that extrahepatic sites contribute little to the total viral load (reviewed in Negro and Levrero, 1998). However, the low-level replication of HCV in these compartments may significantly affect the HCV disease expression, even though the evidence in favor of this is at present exclusively experimental.

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