Hepatitis C Virus (HCV) Occult Infection or Occult HCV RNA Detection?

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(See the article by Castillo et al., on pages 7–14.)

Hepatitis C virus (HCV) is the sole member of the Hepacivirus genus in the Flaviviridae family. The virus is ∼50 nm in diameter. Its envelope is derived from host membranes that surround a nucleocapsid containing a positive-sense, single-strand RNA genome of ∼9600 nt [1]. This is directed translated to a long polypeptide that is further processed into 10 different mature viral proteins. There is no integration of the virus into the host genome, as there is with some other RNA viruses, such as HIV. Replication of the HCV genome is believed to occur through the synthesis of an intermediate minus-strand HCV RNA driven by the viral RNA-dependent RNA polymerase. The main site of infection for HCV is the liver. This hepatotropism leads to the development of liver injury that can be monitored by measurement of the levels of serum aminotransferases. Despite a robust immune response in most individuals, hepatitis C is characterized by the chronicity of its infection. In chronically infected patients, liver fibrosis and cirrhosis develop, over decades, in 20%–30% of infected patients and, in some individuals, can eventually lead to hepatocellular carcinoma [2].

In general, the diagnosis and monitoring of HCV infection have been based on the serologic detection of antibodies to HCV, followed by the detection of HCV RNA. However, in the acute stages of hepatitis C, anti-HCV reactivity may remain undetectable for several months, even by the most sensitive assays, although the HCV RNA will be detectable. In transfusion-acquired hepatitis C, the mean time from transfusion to HCV RNA detection is 12.6 days (95% confidence interval, 2–22 days) [3]. In these cases and in source plasma and whole-blood donors who have acquired HCV infection, the median, or average, duration of the pre–anti-HCV viremic phase ranges from 41 to 61 days [4].

In most cases, EIA anti-HCV seroconversion occurs ∼1 week after elevation of the alanine aminotransferase (ALT) level. Rarely, an immunologically silent state of infection, which is thought to be due to persistently low levels of HCV RNA or to the immunocompetence of the host, has been known to persist for >2 years [5–7].

In considering whether a patient is infected with HCV, we have been indoctrinated with the principle that the absence of HCV markers in the plasma or serum of patients with liver disease eliminates that diagnosis. Perhaps the only exception to this preconceived axiom is observed during the first few hours of the eclipse phase of viral morphogenesis, when no infectious virus is detectable, or during the early phase of transition from negative to positive HCV RNA reactivity, during which the virus burden increases to detectable levels in the blood (see above). These serobiologic conventions began to disintegrate when Brechot and others (reviewed in [8]) discovered hepatitis B virus (HBV) DNA in the serum or liver of patients negative for hepatitis B surface antigen (HBsAg) who lacked detectable antibodies to hepatitis B core antigen (anti-HBc) and to HBsAg (anti–HBs). However, even when HBV was discovered in the liver and not in the serum [9], the sensitivity of the technology for detecting HBV DNA was questioned. Although completely negative seromarker test results for HCV-infected humans and chimpanzees have been observed transiently during follow-up, the causes of these rare events usually have been attributed to either collection, storage, and processing conditions; the varying sensitivity of the assays; or procedural testing errors.

The article by Castillo et al. [10] that appears in this issue of the Journal further confounds current diagnostic dogma regarding hepatitis C. Castillo et al. provide persuasive experimental evidence for oc-
cult HCV replication in the liver of some patients with long-standing liver-enzyme abnormalities of unknown etiology. From a design standpoint, the study was meticulously organized and executed, employing an extensive array of controls needed to confirm their observations. Serum samples from 100 patients in whom all currently known causes of liver disease were excluded and whose serum samples were negative for anti-HCV (by 2 different commercial EIAs) and for HCV RNA (by reverse transcriptase–polymerase chain reaction [RT-PCR]) were selected for evaluation. Early RT-PCR studies comparing plasma to serum, for the detection of HCV RNA, found reduced sensitivity with the use of serum [11], which was the principal source for samples examined in Castillo et al.’s study. Nevertheless, the lower limit of detection for their RT-PCR assay was 10 IU/mL, for HCV genotype 1b, the only strain of HCV found in the population that they studied. These patients had been followed at 3-month intervals, for a mean of 72 months, during which time sustained elevations of ALT, aspartate aminotransferase (AST), and/or γ glutamyl transferase (GGT) were present. An elevated ALT level in the presence or absence of AST and GGT abnormalities was found in 68 of these patients, whereas 32 displayed only an isolated GGT abnormality. With sophisticated technology, positive-strand HCV RNA was detected by RT-PCR in liver-biopsy specimens from 57 patients, whereas negative-strand HCV RNA sequences were identified by in situ hybridization in 48 (84%) of these patients. Because HCV replication occurs through the synthesis of a negative RNA–replicative intermediate, this observation implies ongoing HCV replication.

Another potentially meaningful observation was the detection of positive-strand HCV RNA in peripheral-blood mononuclear cells (PBMCs) from 40 (70%) of the 57 patients whose liver-biopsy specimens also were positive. It is generally accepted that virus will not be detected in PBMCs unless replication is occurring in the liver. Apparently, Castillo et al. did not examine the PBMCs for negative-strand HCV RNA, and thus we are left to wonder whether ongoing replication was occurring in these circulating cells. Other investigators have reported divergent results with regard to the presence of negative-strand HCV RNA in PBMCs. These discrepancies could be due to the small number of patients included in each report or to technical problems. In fact, data from Lerat et al. [12] suggest that negative-strand HCV RNA–bearing cells (e.g., monocytes or B cells) could be obscured by large numbers of noninfected cells such as T cells, the main cell subset recovered after ficoll-hypaque separation, and that a possible concentration-dependent variation of HCV RNA may occur in hematopoietic-cell subsets [13]. Although Castillo et al. did not characterize which PBMC population was infected by HCV, several other studies have implicated a number of cell lineages within the immune system, such as monocyte/macrophages and B cells [12, 14–16]. Interestingly, HCV infection is associated with B cell lymphoproliferative disorders, including mixed essential cryoglobulinemia and B cell lymphoma [17, 18]. That HCV infection plays a direct role in the genesis of these B cell lymphoproliferative disorders has been suggested and is supported by recent studies. However, the immunopathogenetic or molecular mechanisms for this phenomenon remain unknown. Dendritic cells, including cells that target immunologically privileged sites such as the central nervous system (CNS), also have been shown to carry HCV genomes [19]. Trafficking of mononuclear phagocytes across the blood-brain barrier has been demonstrated [20]. Such cells have been found to be permissive of HCV replication in vitro, but at a relatively low rate. In this regard, several groups have reported finding positive- and negative-strand HCV RNA sequences in cerebrospinal fluid and brain tissue from HCV-infected patients, some of whom displayed various CNS and peripheral-nervous-system manifestations of disease [21–24].

We cannot presume, simply because other sites were not investigated, that PBMCs are the only extrahepatic source of occult HCV infection. Nevertheless, this issue must be resolved if we are to more fully comprehend virus escape from treatment and de novo infection or reinfection after transplantation. Because the RT-PCR technique does not discriminate between infectious and noninfectious virus, what has not been convincingly demonstrated for HCV (or HBV) in these occult studies is whether the genomic material detected is infectious. This, of course, is critical to any concerns that we might have.

It has been estimated that, in chimpanzee liver samples, 1 negative-strand HCV RNA is present per 10 positive strands [25]. In these occult human infections, only ~5.4% of the hepatocytes displayed positive-strand HCV RNA, a frequency lower than that detected in liver-biopsy specimens from patients with chronic HCV infection. Despite this reduced estimate of infected cells, there must have been a relatively high copy number of plus-strand HCV RNAs per cell to allow Castillo et al. to genotype the virus in all of their RT-PCR–positive samples, because the lower limit for typing is ~20,000 IU/mL, depending on the parameters of the assay used [26].

The relatively low concentration of virus present in the liver-biopsy specimens and PBMCs from these seronegative occult HCV infections made preservation of the HCV RNA mandatory. Castillo et al. believe that part of the reason for their success in detecting HCV RNA in these tissues was due to methodology. In 2000, Castillo et al. and their associates published a paper extolling the virtues of rapidly freezing the liver-biopsy specimen after collection, to avoid degradation of nonencapsidated HCV RNA by cellular RNases [27]. They demonstrated that maintaining the liver samples at room temperature for ~3 min before freezing them in liquid nitrogen led not only to a decrease in the concentration of the virus but also to a reduction in the frequency of detection,
for both the positive- and negative-strand HCV RNAs. As expected, detection was significantly lower for the negative strand than for the positive strand, simply because the concentrations are so radically different.

We anticipated and hoped that only those patients with an elevated ALT level would be the ones to express positive-strand HCV RNA in the liver or PBMCs, so that a single biochemical test would suffice for identification of occult disease. However, Castillo et al. have informed us that 19 (33%) of the 57 RT-PCR–positive liver samples and 12 (30%) of the 40 RT-PCR–positive PBMC samples were obtained from seronegative patients displaying only an elevated GGT level. The distribution of the negative-strand results in the liver–biopsy specimens, which were based on biochemical abnormalities, provided comparable data. Thus, these data imply that clinicians must use both ALT and GGT to identify these occult HCV infections. Correspondingly, we also must assume that HCV is a noncytopathic virus and that clearance or injury depends on the immune response, because ALT is not invariably elevated in these patients.

Castillo et al. presented some intriguing histologic data with regard to the possible association of occult HCV infection with the development of chronic liver disease. Among their 100 seronegative patients with persistently elevated levels of liver enzymes who were followed for a median of 6 years, increased fibrosis (META VIR score, ≥F1) was observed in 17.5% of those individuals with occult HCV, compared to 2.3% of those without intrahepatic HCV RNA (P = .02). Conversely, >82% of the patients with positive-strand HCV RNA in their liver–biopsy specimens had only minimal changes, steatosis, or no fibrosis. Further analysis showed that, although 8.8% of the patients with occult HCV infections had severe fibrosis or cirrhosis (META VIR fibrosis score, ≥3), this was not statistically significant. Additional information, supplied by I. Castillo in a personal communication, confirmed that no correlation was observed between the severity of the histologic findings and the presence of negative-strand HCV RNAs, as might be inferred immunopathogenetically.

Until other investigators can confirm the provocative data presented by Castillo et al., we are left to speculate about the significance of their observations. A number of papers published during the past decade, most of which have dealt with detection of occult HBV DNA either in patients displaying seromarkers of immunity—that is, patients who are negative for HBsAg but positive for anti-HBs and anti-HBc—or in patients not displaying seromarkers, have slowly moved us from cautious affirmation to full acceptance that such an event can occur. The discovery of HBV DNA in the liver of patients who had apparently cleared their infection 30 years earlier, as well as the detection of specific T cell responses that depend on continuous antigen expression, suggest that an epiphenomenon of universal scope might exist [28–30]. Perhaps this mechanism assists the host in preserving lifelong immunity.

With HBV serving as a background to the current data, what have we learned? Before Castillo et al.’s study, we would have predicted that patients with ALT abnormalities and evidence of increasingly severe histologic damage would be more likely to demonstrate replicative intermediates in corresponding liver–biopsy specimens. This was not observed. Whether this is a concentration phenomenon or a virus-host event cannot be ascertained from the data, but it is perplexing and remains unresolved. Castillo et al. do not explain why they were not able to detect core protein in liver–biopsy specimens from patients with negative-strand HCV RNA. Because they assume that the minus strand is representative of replicating virus, why were specific viral proteins not being expressed? Correspondingly, if synthesis of these proteins was occurring, why were the patients not positive for anti-HCV? And, if genotyping requires relatively high concentrations of HCV RNA, why was virus not detectable in the blood of these individuals? Taken together, these considerations could lead us to postulate that Castillo et al. may have been detecting a negative-strand HCV RNA from a defective virus and that release of HCV into the blood might be compromised. Alternatively, a finite amount of antigen might be necessary to facilitate a recognizable immune response.

It is believed that during chronic infection the viral load accurately reflects hepatic replication. Under normal circumstances, the serum half-life of free virions is estimated at 3 h, with a viral production rate of 10^12 virions/day [31]. Elimination of infected cells by immune clearance is slower, occurring in 2–70 days. Although the serum half-life of circulating virus should not be influenced by these occult infections, the viral production rate and elimination of infected cells are probably muted. The consequences of plasma dilution and/or precipitation with cryoglobulins also may be factors in generating a nondetectable reaction.

In addition to the liver, hematopoietic tissue may be the site of occult lifelong maintenance of replicating virus, as has been suggested by Castillo et al. and other studies in which virus is detected in PBMCs or stem cells. These observations parallel those seen in occult HBV infection. Although the notion is still somewhat controversial, the detection of negative-strand HCV RNA in PBMCs implies that these cells may be the site for viral replication—and a reservoir for reactivation under immunosuppressive conditions (e.g., drug therapy associated with transplantation) [32].

So what is the take-home message in the Castillo et al. study, if we assume that their data can be confirmed? Given the proper technological conditions for detecting HCV RNA, as well as the improvements that they have recommended for sample processing, >70% of occult HCV infections could be detected by the less invasive approach of PBMC analysis in a seromarker-negative person with an ALT and/or GGT abnormality. Detection of HCV in
PBMCs is evidence for its replication in the liver of these seronegative patients. Perhaps the use of a whole-blood RNA–extraction method [33] might be applicable in these patients, although some controversy exists as to whether it offers any advantages over current methodology [34].

Castillo et al. looked only at seronegative patients with persistently abnormal levels of liver enzymes. They did not evaluate individuals at high risk for HCV infection, regardless of HCV RNA, anti-HCV, or ALT/GGT status (e.g., injection drug users or multiply-transfused patients) or patients who had resolved their infection either spontaneously or as a result of treatment. However, despite such knowledge, the broader issue is whether blood, PBMCs, stem cells, or the liver are capable of transmitting virus, leading to an infection in another individual. Until this risk is demonstrated, perhaps we should call this phenomenon “occult detection of HCV RNA,” rather than “occult HCV infection.”

References