Natural history of hepatitis C virus among injecting drug users
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Citation for published version (APA):
Beld, M. G. H. M. (1999). Natural history of hepatitis C virus among injecting drug users

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Chapter 8

General discussion
diphtheria toxin, in individual patients infected with HCV who remained HCV-negative during the follow-up period. These suggest to be the most convincing is the sequence of loss of HCV RNA with HCV core antigen staining in a single case. Although in anti-HBs appeared to have been present at an antibody titre in the mid ranges of 1:100, the core antigen was also stained in the anti-HBs-positive patient. In a patient with two different serological markers, antibody to anti-HBs was not detected in the absence of anti-HCV or the absence of both markers, antibody to coreAg was also positive in the same patient. In patients with evidence of anti-HCV, anti-HBc (1:1,000) was detected in a patient with a titre of 1:1,000 and a negative anti-HBC titre. In the absence of both markers, antibody to anti-HBs was also not detected.

These data suggest that HCV infection could potentially represent another HCV antigen and leads to significantly increased HBsAg levels. The clinical relevance of this problem is also discussed (see also 2, 3), in which HCV-infected individuals with sustained HCV-RNA negative elevation of HCV-RNA on a viral load showed significantly increased levels of anti-HBs and HBc. Therefore, using a decline in anti-HBs titre in HCV-RNA positive for selecting for appropriate HCV therapy among HIV-infected individuals is discussed.

Acknowledgements

The authors thank Lucy Padiya for editorial review and Volodya Lahondor for statistical analyses. This work was supported by Health Research and Development Council (20-029/10), and was done as part of the Amsterdam Cohort Studies on HIV and AIDS, a collaboration between the Academic Medical Center, the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, and the Municipal Health Service Amsterdam. The Netherland.

References

General discussion

Infectivity and HCV detection assays

Since its discovery in 1989, numerous questions have been raised about HCV, of which many remain unanswered. HCV infects the liver, replicates primarily in hepatocytes, and is responsible for the onset of liver disease, which is usually characterized by liver enzyme abnormalities. HCV infection can be diagnosed by indirect antibody assays or by the direct molecular detection of HCV RNA. HCV antibody assays have been most widely used, because the level of circulating HCV antigens in serum is too low to be detected by conventional enzyme immunoassays. One disadvantage of using antibody detection for diagnosis is that it does not discriminate between ongoing and resolved HCV infections, but it remains the “gold standard” for the identification of HCV infection. The presence of antibodies to HCV almost always indicates infectivity, particularly in haemodialysis patients, haemophiliacs, and patients with chronic HCV. The third-generation screening assay (EIA 3.0; Abbott Laboratories, Chicago, IL) contains NS4 antigens, reconfigured Core and NS3 antigens, plus an additional NS5 antigen not present in the EIA 2.0 test. Positive EIA results usually are confirmed by RIBA (Chiron Corp., Emeryville, CA), to help resolve false-positive EIA test results. A positive RIBA result requires reactivity in at least two band positions. However, a second disadvantage of antibody detection is that assays usually are negative in the acute phase of HCV infection. HCV needs a certain incubation period -the seronegative window phase- and may be detected by direct molecular assays during this period. The mean time to seroconversion in transfusion recipients has been shortened to 8 weeks using third-generation assays, but testing in high-prevalence populations indicates that not all HCV RNA-positive patients are identified with EIA screening tests. Molecular amplification technologies are therefore an increasingly important tool for confirming the diagnosis of HCV by qualitative PCR-methods, as was described in chapter 2. During the acute phase of infection, detection of HCV RNA is the only method that can determine viraemia, since no antibodies to HCV are detectable. We studied the seronegative window-phases and the presence of HCV RNA in 19 seroconverting injecting drug users in an attempt to identify the proportion of serologically undiscovered HCV carriers. We identified a substantial proportion of silent HCV carriers, in whom antibody-responses were absent for more than one year prior to seroconversion, independent of HIV-status, as seen earlier in high-risk groups. One single explanation for these prolonged window-phases is elusive, but several will be discussed. The most obvious reason may be the sensitivity of the antibody test for the diagnosis of HCV infection. Since this was the problem in predecessor antibody tests, it cannot be ruled out that future serological assays with enhanced performance in terms of sensitivity (including more epitopes) would shorten the seronegative window-phase. However, recognition of antigens by helper T-lymphocytes (T_H cells) normally leads, depending on the lymphokine repertoire, to increased number and responsiveness of B-cells. It might be that a certain threshold of antigenic-mass is needed for sufficient exposure to T_H cells, since our silent carriers had low levels of HCV RNA before seroconversion. The fact that HCV RNA was found in serum at such low levels suggests that HCV replication is downregulated while the virus persists in the liver or other putative extrahepatic reservoirs in a quiescent state. Allowing the virus to “hide” from the host, this state may protect its environment by inducing an indolent disease that does not destroy its target-cells. Loss of antibodies, or seroreversion, accompanied by viral clearance, as observed in untreated immunocompetent humans and chimpanzees and described by us in chapter 4 may, also explain the prolonged window-phases in some injecting drug users. The lifestyle of injecting drug users may also be an important factor for delayed or lacking antibody responses. The frequent injection of drugs contributes not only to reexposure and reinfections with HCV but also to higher levels of an-
ergy, which is reflected by lower anti-CD3 responses \(^1\). In conclusion, independent of HIV infection, the immune-system appears to be sometimes unable to mount a sufficient antibody response to HCV infection. The virus seems to be able to “hide”, and the antibody response is absent or at least delayed. Therefore, direct detection of the virus by RT-PCR should be used in addition to serological assays, to further shorten the seronegative window-phase in HCV-infected individuals. This approach may resolve the problem of silent carriers in low- and high-risk groups.

**HCV RNA levels and genotypes in relation to disease**

Quantitative measurements of circulating virus during the course of infection and following antiviral therapy has led to major insights into the natural history of HCV and its response to interferon therapy. Whether, HCV RNA levels in serum and plasma and the various genotypes are related to disease progression is still a matter of debate. Elevation of ALT levels merely defines existing liver disease and damage of parenchymal liver cells; it correlates poorly with chronic HCV infection, as was found in several studies \(^3,4\). However, HCV RNA levels may fluctuate dramatically, and they sometimes show a temporal relationship to fluctuation in serum ALT. In such cases, a rise in HCV RNA immediately precedes the rise in ALT \(^5\), suggesting, that a burst of viral replication may cause liver damage, either by direct cytotoxicity and immune complex formation, or indirectly, by mediation of cytotoxic T-cell responses. Typically, the temporal relation between HCV RNA level and ALT level is often absent, because HCV may persists at high levels for long periods, forming HCV-antibody complexes without causing significant hepatocellular inflammation. We studied the levels of ALT in relation to HCV RNA levels and genotypes at different stages of HCV infection among 19 seroconverting injecting drug users, as described in chapter 3 of this thesis \(^6\). Mean ALT levels were above the upper limit of the normal range of 37 U/liter in the first six months after HCV seroconversion, but they were below the upper limit of the normal range after a mean follow-up of five years, with no relationship during the early or chronic phase of infection to HCV RNA levels or genotype. Other studies of chronically HCV-infected patients, have shown HCV RNA levels and ALT values not to be associated with the progression or presence of liver fibrosis, cirrhosis, or hepatocellular carcinoma \(^7,8\). Therefore, ALT may be used as a diagnostic marker only during the acute phase of HCV infection, whereas viral properties, such as HCV RNA levels and genotypes, may finally turn out to be poor prognostic disease markers during HCV infection.

**Relationship of structural and nonstructural proteins with HCV viraemia**

In chapter 4 the correlation, between possible viral clearance and detectable antibody responses to core, NS3, NS4, and NS5 were described. Chronic or persistent HCV infection, as measured by direct molecular techniques, is usually estimated to develop in the range of 60-80% of persons infected with HCV \(^9,10\). In virtually all persistently infected individuals, there are antibody levels to core, E1/2, NS3, NS4, and NS5 proteins, suggesting that these antibodies have a minimal role in viral clearance and immunity. Given the high frequency of persistence, the main issue is why the majority of patients are persistently infected and whether any patients completely clear the virus. The mechanism of viral persistence probably resides in the ability of HCV to mutate rapidly under immune pressure and to exist as related but immunologically distinct variants, with most mutations occurring in the hypervariable region (HVR) of the envelope. It was recently demonstrated that chimpanzees vaccinated with recombinant E1 and E2 envelope proteins were protected from homologous HCV challenge due to the presence of antibodies capable of inhibiting the binding of E2, suggesting that E2 may be responsible for binding of HCV to target cells \(^11\). Moreover, in another study it was shown that binding of E2 to CD81, a putative receptor, was prevented by neutralizing antibodies directed to E2 \(^12\).
Therefore, escape of the virus from antibodies directed to the envelope proteins of HCV probably coincides with the persistence of the virus. Chimpanzee studies have shown that despite the presence of antibodies against nonstructural and structural HCV proteins (including E1/E2), homologous and heterologous rechallenge result in multiple episodes of acute hepatitis. This finding suggests that genotype-specific antibody responses do not always provide protective immunity, but the exact mechanisms of viral selection still remain unclear. In our study of 13 patients, putative HCV clearance from blood was found in an unexpectedly high percentage (5 of 13 or 38.5%) among HCV-infected immunocompetent individuals. In 4 of these individuals, viral clearance from blood with gradual loss of antibodies to various HCV proteins, independent of HCV genotype, was observed, within approximately 1 year after HCV seroconversion, whereas 1 of these individuals cleared the virus from blood with complete seroreversion. Persistent antibody responses to core, NS3, NS4, and NS5 were indicative of persistent viraemia, and no evidence was obtained for protection against new infections. In contrast, viral clearance was associated with a significant decrease of antibodies to NS3 and NS5, as compared to individuals with persistent viraemia. A significant decrease of antibodies to NS3 and NS5 could be used in addition to HCV RNA, as diagnostic markers of chronic HCV infection and might be an alternative for monitoring the efficacy of HCV therapy.

**HCV genotypes as a pitfall for antibody-assays**

The analysis of antibody responses with respect to HCV genotypes revealed a difference between genotype 1 and other genotypes, as described in chapter 4. In individuals infected with HCV genotype 1, the median antibody responses to all four proteins were higher than in individuals infected with other genotypes and median antibody responses to core and NS4 were significantly higher. Since serological screening-assays are based on HCV genotype 1, these differences in antibody responses could be an artifact of the assays. Alignments of amino acids in these regions, as used in the RIBA 3.0 assay, revealed genotype-specific sequences in those coding for core and NS4, especially in the 5-1-1p peptide. Although the core region of HCV is the most conserved region used in the RIBA assay, even antibody titers to core were observed to be influenced by antigenic variation. In individuals infected with HCV genotype 1, significantly higher median antibody responses to both core and NS4 were found, as compared to those infected with other genotypes, suggesting a significant impact of NS4 and core epitope variability on core and NS4 seroreactivity. Several other authors have reported comparable results, proposing that the RIBA assays are suboptimal for screening and confirmation of infections with HCV genotypes other than genotype 1. These findings show that genotype affects the sensitivity and specificity of serological screening-assays, which therefore may underestimate the total prevalence of HVC-infected individuals.

Comparable findings were observed using a RIBA serotyping SIA assay based on seroreactivity to core and NS4, as described in chapter 5. Several methods have been described for HCV genotyping, but they are complex and require proper handling and storage of specimens. Serotyping may be a rapid and cost-effective alternative for routine use and for screening large populations for the determination of HCV genotypes, but it is dependent on antibody responses to antigens implemented in the assay, and the sensitivity is limited by the immunocompetence of the infected host. HCV serotypes are determined primarily on the basis of reactivity to NS4 serotype-specific HCV antigens, which distinguish between types 1, 2, and 3. In the absence of NS4 reactivity, core reactivity is used to determine serotypes. However, cross-reactivity between type 1 and 3 HCV antibody to the core peptide makes it impossible to distinguish between these two types. In general, we found that samples from HIV-negative individuals containing genotype 1a showed a higher sensitivity, specificity, and concordance in the serotyping assay, whereas samples containing genotype 3a were more often cross-reactive.
and untypeable. The prevalence of genotypes other than genotype 1 could thus be underesti­
mated if determined by serotyping. Although PCR remains the gold standard because of its
higher sensitivity and specificity, the RIBA HCV serotyping SIA assay could be useful in
screening large numbers of immunocompetent HCV-infected individuals and providing epide­
miological data, but improvements in its specificity are recommended.

Impact of HIV-coinfection on HCV

HIV-coinfections influence significantly the reactivity to serotype-specific core and NS4 anti­
gens, most probably by immunesuppression (chapter 5). Whether HIV extends its range of
pathogenic mechanisms was described in chapter 6 and 7. Virus interactions can easily be
studied in HCV risk groups that have high prevalences of HIV. Since HCV and HIV share par­
tenteral transmission routes, coinfections are frequently found in injecting drug users. In our
19 HCV seroconverters, we studied the direct effect of HIV on HCV replication during a mean
study period of 5.4 years by comparing HCV levels among 10 individuals remaining HIV-sero­
negative during follow-up, 5 individuals seroconverting for HIV during the study period, and 4
individuals who were HIV-seropositive at the start of the study period. HCV RNA levels were
higher in the HIV-positive group than in the HIV-negative group, and the HCV RNA detected
in HIV seroconverters rose from levels that were indistinguishable from those of the HIV nega­
tive group to levels comparable to those of the HIV-positive group. In addition, when subjects
were stratified according to CD4+ cell counts, a significant difference was found in HCV RNA
levels between the HIV-negative and HIV-positive group. This independent effect could be
confirmed among the HIV seroconverters. At HIV seroconversion, HCV RNA levels were
significantly higher and remained high, whereas CD4+ cell counts were relatively stable during
the first year after HIV seroconversion but declined later during HIV infection, as described in
another study. The possible direct effect of HIV on HCV replication was not associated with
the level of HIV in blood, indicating that HIV or HIV proteins may enhance HCV replication
when a certain threshold is reached. Thus HIV may insome way directly affect HCV replication,
or HIV may possibly reduces HCV-specific immunity through impairment of the overall
immune response. The negative influence of HIV on the natural history of HCV was supported
in chapter 7 and in other studies. Loss of antibodies to HCV, or seroreversion, may largely
result from immunological disorders caused by therapy or by HIV-coinfections (chapter 5), but
it also occurs spontaneously, accompanied with viral clearance (chapter 2 and 4). Currently,
the gold standard for the detection of HCV infection is the detection of antibodies to HCV by
second- or third-generation EIA, with confirmation of at least 2 antigens by RIBA 3.0. Anti­
bodies to HCV are more accurately detected in HIV-positive individuals by EIA 3.0 than by
RIBA 3.0, as indicated by our finding that EIA 3.0 results remained positive throughout the
study period regardless of immune status and by a report from another group studying HCV-
positive and HIV-coinfected haemophiliacs. In HIV-negative individuals infected with HCV
genotype 1, antibody titers to NS3 and NS5 were significantly lower and HCV RNA levels
were significantly higher than in HIV-positive individuals infected with HCV genotype 1. Anti­
bodies to core were shown to be both less dependent on HIV or immune status and more per­
sistent than antibodies to NS3, NS4, and NS5, a finding that may be significant for the diagno­
sis of HCV infection among patients coinfected with HIV. HIV infection appears to influence
antibody responses to certain HCV antigens, leading to significantly decreased antibody titers
to NS3 and NS5 accompanied by significantly increased HCV RNA levels. Although
contradictory reports were published about the influence of HIV-coinfections on the severity
of liver disease, introduction of HIV into areas with endemic HCV infections may influ­
ence the natural history of HCV.
**Final remarks**

The pivotal questions for HCV-infected patients, as well as for researchers and clinicians, remain: who progresses to liver disease most rapidly and who should be included in clinical trials. Currently, treatment is recommended for patients with persistently elevated ALT levels, detectable HCV RNA, and findings of moderate inflammation of liver tissue. Duration and type of treatment regimen (drug and dosage) seem to have a major impact on the outcome of HCV infection. Monotherapy with interferon is less effective than the combination therapy of interferon plus ribavirin. Ribavirin is a synthetic guanosine analogue with activity against DNA and RNA viruses, but with an unknown molecular mechanism. In addition to its direct antiviral effect, it may have an immunomodulatory effect, at least in patients infected with HIV, by inhibiting Th2 cytokine production and stimulating Th1 cytokine production, resulting in strong CTL responses. The combination therapy doubles the response rate as measured by virological and biochemical parameters in patients with chronic HCV who have not previously been treated. Many other antiviral drugs may be soon available and other combination therapies may be even more effective. At present, however, clinicians should be cautious about treating HCV-infected individuals too early with currently available therapies, because such treatment may lead to the circulation of drug-resistant mutants. Giving the low pathogenicity of HCV, as compared to HIV, and the few effective anti-HCV drugs, a conservative attitude toward HCV-treatment may be warranted until more anti-HCV drugs have become available.

**References**


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