Hepatitis C Virus: Biological and Clinical Consequences of Genetic Heterogeneity

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Hepatitis C Virus infection accounts for the majority of post-transfusion and sporadic hepatitis. In Western Europe, anti-HCV is detected in 0.4–1.5% of healthy blood donors. There is a high frequency of progressive chronic hepatitis, ranging from 50 to 80%, which leads to cirrhosis in 20–50% of patients after 10–20 years. Viremic patients with minimal biochemical abnormalities may have chronic liver disease histologically. There is growing evidence that virological features of HCV are associated with different clinical manifestations and response to therapy. The RNA genome consists of a 5' and 3' Untranslated Region, a structural domain encoding the core and envelope proteins, and a non-structural domain. Different HCV isolates show a high sequence heterogeneity, which has led to the classification of currently six genotypes and several subtypes. There is a marked difference in the geographic distribution of HCV genotypes, with types 1, 2 and 3a being most frequently found in western countries. In The Netherlands, subtype 1b accounts for approximately 60% of all cases of chronic HCV. Serologic diagnosis based on recombinant C-100 antigens (first generation immunoassays) only reliably detected type 1, due to the heterogeneity of the NS4 region; inclusion of more conserved proteins c22 and c33 (second generation assays) has largely improved sensitivity of anti-HCV testing. Genotype 1b is associated with more severe liver disease and with lower response rates for antiviral therapy, compared with types 2 and 3. Quasispecies nature and escape mutants may enable viral persistence and the development of chronic liver disease. As cross-reactivity between genotypes is unlikely, prevention of HCV disease may be dependent on the development of multivalent vaccines.

Key words: Antiviral treatment; chronic HCV disease; disease severity; genomic heterogeneity; genotypes

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CLINICAL ASPECTS OF HCV INFECTION AND DISEASE

Hepatitis C Virus (HCV) is a major cause of morbidity and mortality worldwide and accounts for the vast majority of post-transfusion hepatitis and sporadic or community acquired hepatitis (1–4). In western Europe, antibodies against HCV are detected in 0.4–1.5% of healthy blood donors, with higher levels for southern Europe (5, 6). In southern Africa, the middle East, south- and south-east Asia, significantly higher prevalence rates of up to 20–30% have been reported (7–9). Epidemiological data imply that worldwide a larger number of people are infected with HCV than with HBV (10). Annually, more than 1 million new infections are reported.

Acute HCV infection is usually mild or asymptomatic, but there is a remarkably high tendency to develop viral persistence and chronic hepatitis in 50–80% of infected individuals (11, 12). Chronic HCV hepatitis is often associated with absent or mild clinical manifestations; symptoms are present in only 50% of patients; they are usually non-specific and include fatigue, which is the predominant feature,

general malaise, nausea and dull abdominal pain (13). Biochemical analysis may show persistently normal or typically fluctuating alanine aminotransferase (ALT) levels. In spite of this rather quiet clinical picture, the inflammatory progress in the liver is often progressive and will evolve to cirrhosis in 20–50% of the patients after 10–20 years (14, 15), which is associated with a high risk for complications due to portal hypertension and deteriorating liver function, and hepatocellular carcinoma (HCC) (16). In one study of post-transfusion hepatitis with a follow-up of 20–25 years, 66–71% of patients had bridging fibrosis or cirrhosis (17). At the time when chronic HCV hepatitis becomes symptomatic, cirrhosis will often already be diagnosed on a liver biopsy. In immune-compromised patients, e.g. patients with malignant haematological disorders, hypogammaglobulinaemia, HIV infection, HCV hepatitis has a severe and rapidly progressive course and a poor response to antiviral treatment. Unfortunately, this group of patients has a high rate of HCV positivity due to polytransfusion of blood and blood products or intravenous drug use (18, 19).

Interferon alpha (IFN) is the only antiviral drug with proven efficacy registered for treatment of chronic HCV

Fig. 1. Schematic representation of the HCV genome with the encoded structural and non-structural proteins.

disease so far. Several clinical trials have shown that the immediate response rate, defined by ALT normalization and/or a negative serum HCV-RNA test, is about 40–50% with a dosage of 3×3.10^6 U IFN for 6 months. However, as up to 50% of these initial responders will relapse within 6 months of follow-up, the long-term response rate is only 20–30% (14, 20, 21). IFN therapy is expensive, not without side effects, and requires subcutaneous injections by the patient for a long period of time; consequently, several investigators have tried to identify host and virus associated factors that are predictive of disease progression and of outcome of therapy, in order to establish criteria that allow identification of patient groups that would profit most from IFN treatment.

GENOMIC STRUCTURE OF HCV

HCV is a positive-stranded, enveloped RNA virus of ca. 9.4 kb. According to its genomic structure, it has been classified as a genus into the group of the Flaviviridae, together with the flaviviruses and the pestiviruses (22). The HCV genome consists of a 5' and 3' non-coding region, a structural region coding for the putative nucleocapsid and envelope proteins and a non-structural region (Fig. 1). The genome has one Open Reading Frame, coding for one polypeptide precursor of 3010 amino acids that is co- and post-translationally split into several structural and non-structural proteins (23).

The 5' untranslated region (UTR) is highly conserved in all HCV isolates with a sequence variability of ca. 6%. It is considered to be important for initiation of translation and binding to ribosomes, and the putative stem-like structure must be maintained to function properly as an internal ribosomal entry site (24). The structural domain contains the core (C) and envelope (E1 and E2/NS1) regions respectively. The envelope region of the HCV prototype has two hypervariable regions (HVR 1 and 2), with a sequence variability of more than 50% (25). The C-terminal region encodes the non-structural proteins NS2, NS3, NS4, and NS5.

The functions of the HCV non-structural proteins are partly elucidated. NS3 encodes a serine proteinase which is responsible for the cleavage of the polyprotein into NS4a, NS4b, NS5a and NS5b (26–29). A second proteinase located in the NS2 region is probably responsible for cleavage at the NS2/NS3 site (26, 30). The NS5 region plays a role in viral replication; the C-terminal part NS5b contains the consensus polymerase sequences. The functions of NS4a, NS4b and NS5a are unknown, but the proteins all contain immunogenic epitopes.

GENOMIC VARIATION

Genotypes and subtypes

HCV is characterized by a high degree of nucleotide sequence variability. Overall heterogeneity of the viral genome is as much as 30–35% between different genotypes, but is unevenly distributed; there are highly conserved regions, such as the 5' UTR, and hypervariable regions (HVR) in the envelope region with a sequence variability of up to 51% (8, 25). Pairwise comparison has shown that there are peak values for the prevalence of degrees of sequence variability, with most values lying between 30 and 35%, 20 and 25% and 5 and 10%; this supports the rationale for present hierarchical classification into several genotypes, subtypes and isolates (8).

As different laboratories performing sequencing of the HCV genome used their own classification system as they discovered new genotypes, a rather confusing situation is now found in the literature. Early classification systems are restricted to the pattern of genotype prevalence in a specific geographic area. Simmonds et al. proposed a classification system according to the degree of sequence variability and based on phylogenetic analysis, with different genotypes having a heterogeneity of 25 to 35% and subtypes 15 to 25%; genotypes are numbered chronologically, according to the order of description, whereas subtypes are defined alphabetically (31). This method of grouping allows integration of all

Table I. Classification of Hepatitis C Virus genotypes

Simmonds	Chiron	Okamoto	Enomoto
1a	I	I	K-PT
1b	II	II	K-1
1c	nc*	nc	nc
2a	III	III	K-2a
2b	III	IV	K-2b
2c	III	nc	nc
3a	IV	V	nc
3b	IV	VI	nc
4	nc	nc	nc
5a	V	nc	nc
6a	nc	nc	nc

* nc = not classified.

identified genotypes and subtypes into one system that can be adapted as new types are discovered on the basis of nucleotide sequence divergence, without the need for reassessment. By definition then, the virus isolated by Choo et al. at Chiron ('prototype HCV') is called HCV 1a. The classification systems of Okamoto/Mori, Enomoto and Chiron are also commonly used (23, 32), making 'translation' necessary for comparison of results (Table I). In this paper the nomenclature proposed by Simmonds et al. will be used, unless otherwise specified.

Determining the genotype can be performed by sequencing the whole genome, which is laborious and not practicable for routine or clinical purposes, or by selecting one or a limited number of particular nucleotide sequences from a relatively small part of the genome, e.g. 5' UTR, C, E1, NS3, NS5 (33, 34). As for the 5' UTR region, there are few consistent differences between subtypes within one genotype, so subtypes cannot generally be differentiated when genotyping is performed on this part of the genome (35). The Simmonds classification system is based on the analysis of a 222 base fragment of the NS5 gene amplified by polymerase chain reaction (PCR). This practice is based on the assumption that no recombination of viruses occurs, e.g. a virus with a nucleotide sequence corresponding to one genotype in its structural region and to another genotype in its non-structural domain. As such recombinant viruses have not been described, determining the genotype by selecting one part of the genome is accepted practice. However, as coinfection with more than 1 genotype occurs in 1–2% of infected cases (32), the possibility that such recombined viruses exist has not yet been ruled out.

Quasispecies nature of HCV

Viral RNA replication has high error rates, and consequently RNA viruses are characterized by a high mutation rate of about 10^{-3} – 10^{-2} substitutions/site/year, which is about 1 million times the substitution rate in eukaryotic cells (36). As a consequence, serum of an infected individual will contain not only one invariable viral genetic sequence, but in fact a spectrum of circulating HCV genomes within one

subtype with a variable degree of nucleotide and amino acid substitutions (37, 38). This phenomenon has been described as quasispecies nature, and offers RNA viruses several possible adaptation advantages (38): mutants that escape neutralizing antibodies and cytotoxic T lymphocytes may be selected (immunogenic drift), enabling establishment of persistent viral infection and leading to vaccine failure; it may render resistance to antiviral agents and may be associated with changes over time in cell tropism and virulence.

These two aspects of genetic heterogeneity, the high degree of sequence variability found between different isolates, leading to classification into genotypes and subtypes, and the quasispecies nature of HCV will be discussed in the context of current knowledge of their biological and clinical significance. The possibility that different genotypes may indeed be associated with different behaviour of the virus and thus with different corresponding clinical manifestations seems obvious, as a degree of heterogeneity in the pestiviruses, which are probably the most closely related viruses to HCV, similar to that between HCV genotypes corresponds with infection of different hosts, different methods of transmission and tissue specificity, and thus different types of diseases (8). All HCV genotypes described so far, however, cause chronic liver disease; there is to date no evidence for non-pathogenic or non-hepatotropic HCV variants. Unfortunately, there is still not an adequate cell culture system for HCV, allowing the in vitro study of viral replication, cytotoxicity, resistance to antiviral treatment and the impact of genetic heterogeneity on these viral properties.

BIOLOGICAL AND CLINICAL CONSEQUENCES OF HCV GENOMIC VARIABILITY

Geographic distribution of HCV genotypes and subtypes

Epidemiological data available today support the assumption that most HCV genotypes and subtypes have a worldwide distribution, but there are striking differences in relative prevalence in different geographic areas. Genotypes/subtypes 1a, 1b, 2a, 2b and 3a account for the majority of cases in Western Europe and North America (35, 39), with type 1 being responsible for roughly 55% of cases, and types 2 and 3 each for 20% (40). In the United States, type 1a ('prototype HCV') is the most prevalent genotype; 60% of HCV positive subjects in The Netherlands are infected with type 1b. In samples from French patients, 34.5% had type I (Okamoto, Simmonds: 1a), and 48.7% type II (Simmonds: 1b) (41). Genotype 1b is also the most prevalent variant in Southern and Eastern Europe; in one French–Italian study, 62% of patients had HCV 1b (42); in Hungary genotypes 2 and 3 were almost absent and nearly all infections could be attributed to type 1 (35). In the Middle East and Central Africa, countries with high HCV prevalence rates, genotype 4 is found in a large percentage of the infected population (8). In Egypt, subtype 4a has been identified and many more subtypes of this

genotype are expected as sequencing data of other African countries become available. Interestingly, in a report from the United Kingdom, 37 of 80 patients were infected with type 1, 10 with type 2 and 8 with type 3. Twenty-three patients harboured type 4, and 90% of these patients were from Middle East countries (43). These data suggest a significant geographic clustering of genotype 4. In South Africa, genotype 5a has been isolated; although type 5 has so far been almost exclusively described in persons from Africa, it was also found in a small number of native Canadians who had not travelled to the African continent (33). In the Far East (Japan, China, Taiwan) most cases have been attributed to genotypes 1b, 2a and 2b (13, 32, 44); in a Japanese study, 82% of anti-HCV positive healthy blood donors and 60% of patients with non-A, non-B hepatitis were infected with type 1b, whereas HCV-1a was frequently observed in haemophiliacs who had received coagulation factor concentrates imported from the USA (32). In most South-East Asian countries (Singapore, Thailand, Indonesia) HCV 1b was also the most widely distributed genotype and accounted for 58% of all isolates sequenced, with the exception of the Philippines, where type 1a predominated (45). A high prevalence of genotype 3 with at least nine subtypes was observed in Singapore, Thailand, Bangladesh and Eastern India (8). In a relatively small geographic area of the Asian continent, Hong Kong and Macao, genotype 6a has been isolated, and a homologous isolate of this genotype has until now only been found in Vietnam (8, 46).

In a recent report from Vietnam, 34 of 83 (41%) HCV positive commercial blood donors had viral isolates that were not classifiable into the six major groups described above. These isolates were provisionally designated as a 7th, 8th and 9th genotype (46). Probably even more HCV isolates with significant sequence divergence can be expected as more data become available from all over the world.

Development of chronic infection

Infection with hepatitis C virus infection will progress to chronicity in the majority of cases (11, 47). Continuous viral replication suggests that, in most patients, the host immune system fails to eliminate the infection.

The high mutation rate of HCV may explain the phenomenon of immunogenic drift and the development of chronic infection, biochemically characterized by fluctuating ALT levels. Two hypervariable regions (HVR1 and HVR2) have been identified in the envelope region. Distribution of the quasispecies nature is most pronounced in the hypervariable regions of the putative E2 domain (25, 48–51). Rapid sequence variations of the HVRs during the natural course of chronic HCV infection are thought to play an important role in escape from the host's biological defence system (52, 53). There is evidence that the protein containing the HVR1 region has several sequence-specific antigenic B-cell epitopes that induce production of antibodies restricted to the specific viral variants that elicited them, and which are supposedly

neutralizing. However, as HVR1 is a major site of HCV genetic drift, the amino acid sequence is changing at a high rate, leading to selection of escape mutants. These mutants will not or with a much lower affinity bind to the pre-existing antibodies (9, 52) and are thus thought to form the basis of the establishment of persistent infection and recurrent flare-ups of the disease. In a Japanese study plasma samples from 12 chronically infected patients were taken with a time interval of 1 year, during which none of the patients received antiviral treatment. Significant sequence variation was observed during this relatively short period; there appeared to be an important difference in the rate of nucleotide and amino acid sequence variation of the HVR between four patients with flare-ups of their ALT levels and eight patients with quiescent courses (1.54 to 2.24×10^{-1} /genome site/year versus 0.13 to 1.21×10^{-1} /site/year respectively) (37).

Diagnosis of HCV infection

Serologic diagnosis. All currently available assays use antigens derived from HCV type 1 sequences and rely on cross-reacting antibodies to detect infection with other HCV genotypes.

First-generation immunoassays used recombinant NS4 antigens (5.1.1 and C 100-3 protein) from prototype HCV isolated at Chiron, HCV 1a. This part of the NS4 gene has a sequence heterogeneity between different genotypes of more than 30%. As a consequence, the antigens bound with a high affinity to serum antibodies against HCV genotype 1, but with much less affinity against corresponding antibodies elicited by other genotypes, leading to false negative results in patients or healthy blood donors infected with non-type 1 HCV.

In one study including 155 HCV-RNA positive sera, anti-HCV antibodies were detected with the first generation ELISA in 93% and 79% cases of HCV genotypes I and II (Okamoto) respectively, in contrast to the low detection rates of 34% for both genotypes III and IV (54). These results correlate with the degree of divergence between the C-100-3 amino acid sequence, with 90% conservation between genotypes I and II (Okamoto), but only 75% between I and III or IV.

The detection rate with the RIBA antigen C100-3 was as low as 40% for genotypes 2 and 3, but it was between 60 and 100% for genotypes 1, 4 and 5 (35). McOmish further reports 68% false negative first-generation anti-HCV results for genotypes 2 and 3, compared with only 10% for genotype 1. Even when surrogate markers as elevated ALT are used, 12% of HCV positive donors will not be excluded because of normal ALT levels (39). This lack of cross-reactivity between genotypes provides at least one explanation for the continuing transmission of HCV by blood screened by anti-C100 assays alone.

Second-generation immunoassays include recombinant peptides c33-c and c22-3, and have reduced this diagnostic problem to a great extent (55). Protein c22-3 is an antigen

from the conserved core region, with an amino acid conservation of 90% between genotypes, compared to 75–80% conservation among the non-structural proteins (35). Therefore, it probably detects antibodies in most patients, irrespective of the genotype, although this should be interpreted with care as c22-3 is a component of screening and confirmatory assays for HCV infection, and it is not known to what extent more divergent HCV types are missed with these assays. In this context, it should be mentioned that in a report from China, anti-HCV antibodies were not detectable by second generation enzyme immunoassay in 13% of sera from HCV-RNA positive blood donors (56). Third generation immunoassays include recombinant NS5 antigens and although they may further improve immuno-diagnostic tests, they still have a major drawback of including antigens of only one genotype. Further improvement of the sensitivity of serologic assays may thus require incorporation of additional antigenic peptides corresponding to epitopes of other HCV genotypes not shared with type 1.

HCV serotypes. Different antibodies are generated due to amino acid heterogeneity between genotypes. An ELISA assay with type-specific antibodies to the antigenic epitopes of the NS4-region has recently been described and allows serologic determination of the corresponding genotype (57), although separate identification of subtypes was not possible, probably because of antigenic similarity between subtypes within one major genotype. Serotyping on envelope proteins, which have greater sequence differences, may overcome this shortcoming, and would be a simple, cheap, highly sensitive method available in any diagnostic virology laboratory (58).

Diagnosis with molecular biology techniques. As serology has its limitations and tissue culture and electron microscopy are not yet feasible, direct detection of the viral genome by PCR has become widely available for diagnosis of HCV infection. PCR enables diagnosis of acute infection during the seronegative window phase prior to the appearance of HCV antibodies. In addition, PCR permits monitoring the effects of antiviral drugs and the identification of the chronic viraemic carrier state.

PCR of the HCV RNA genome involves the production of cDNA by reverse transcription (RT), followed by the amplification with primers for well-conserved genomic sequences. A variety of RT-PCR assays for HCV RNA detection in liver and plasma samples have been described (59). Most systems use primers from the 5' UTR, since this region is the most conserved part of the HCV genome (60). The moderately conserved core region and the NS5 region are also used since variations in these domains are limited (32, 61).

The 5' UTR was shown to contain more sequence diversity than was initially estimated (62–64). The exact location of primers within the 5' UTR should thus be selected with care in order to detect all known genotypes with a single set of primers. The use of more than one primer set may even be necessary to detect each HCV genotype and subtype.

PCR can also be used as a tool for genotyping of HCV virus isolates. In fact, the few differences in the highly conserved 5' UTR are currently being used for this purpose (61, 65). Furthermore, optimizing the melting temperatures of primers increases the stringency in the system (66), and increases the ability to discriminate between the different genotypes as well.

Disease transmission

There is currently not much data available about the impact of HCV genetic variability on the mode and frequency of viral transmission. One recent study from Italy found genotypes I and V (Okamoto; Simmonds: 1a and 3a) in 7 of 9 HCV positive subjects who had acquired the infection through intravenous drugs, and iv drug users accounted for 50% of these genotypes, which were otherwise less frequent in the geographic region in which the study was performed (67). Two studies from France found the same pattern of prevailing 'non 1b', in particular 1a and 3a genotypes in drug addicts (68, 69). These preliminary data suggest a possible association between genotype and route of transmission.

Determining the genotype is an important tool for diagnostic and epidemiological purposes. Analysing the degree of heterogeneity between isolates allows tracing of infectious sources and will shed light on the route of transmission, especially for those 40% of cases that appear to have no known risk factor for acquisition of the infection and which are currently classified as sporadic or community acquired HCV infection, and also for evaluating the frequency of sexual, intrafamilial and vertical transmission.

Severity of disease and disease progression

As HCV disease is a slowly progressive chronic hepatic inflammation with absent or mild symptoms for years or decades in most patients, it is important to identify host and virus associated factors that are predictive of disease progression and development to cirrhosis and HCC.

There is growing evidence that as far as HCV is concerned, ALT may not be a reliable parameter for evaluating the underlying liver cell injury, and may indeed be persistently normal in the presence of inflammatory activity (67). Instead, histological (degree of inflammation and fibrosis) and viral parameters (viral load) seem to be more appropriate for diagnosing chronic HCV hepatitis, for establishing criteria for antiviral therapy, and for documenting effectiveness of treatment.

In a recent study, 71 (%) of 167 anti-HCV positive blood donors with normal ALT levels were positive for HCV-RNA, and all of 95 anti-HCV positive donors with elevated ALT levels were RNA positive (67). Those with normal ALT had predominantly type III (Okamoto, Simmonds: 2a), whereas in the group with elevated ALT and in another patient group with severe HCV related liver disease genotype II (1b) was more frequently found. However, individuals infected with genotype 1b were generally older than those infected with

other genotypes, and therefore factors like the duration of infection and the age of the patient may be confounding variables. The same pattern of age distribution was reported in a study from Japan, where it was found that patients with K2 genotype (Enomoto, Simmonds: type 2) were younger and less likely to have PT-chronic hepatitis than patients infected with K1 (1b) (70). In a French study, HCV 1b accounted for 80% of infections in persons older than 40 years, but for only 47% in those under 40 (42); the presumed longer duration of the infection may in part explain the fact that advanced liver disease, chronic active hepatitis, cirrhosis and HCC are more often diagnosed in patients positive for type 1b compared to other genotypes (67, 69).

Genotype 1b thus seems to be more often associated with elevated ALT and more advanced liver disease, but epidemiological studies suggest a tendency that type 1 infection has become less frequent during the 1980s, and that other types became more prevalent during the last decade (68, 71); as a consequence, the non-type 1 HCV positive patients are likely to have a shorter duration of infection. In a study of kidney transplant patients, those infected with HCV from 1960–1975 had predominantly genotype 1b, but during the 1980s 1b was becoming less frequent and genotypes 2 and 3 prevailed (71).

Evidence that HCV 1b itself is indeed associated with more aggressive liver disease, at least as far as recurrent infection in liver graft recipients is concerned, came from a recent study of 60 anti-HCV positive patients who underwent liver transplantation (72). HCV 1b was the predominant type in cirrhotic patients requiring transplantation either for end-stage liver disease or for HCC with 41/60 (68%) of cases; actuarial rates of chronic active hepatitis 3 years after transplantation were 59% for HCV 1b cases but only 22% for the other genotypes. Although there was no statistical relation between the level of viraemia and HCV genotypes before transplantation, HCV RNA levels were significantly increased in patients who developed hepatitis after transplantation. A major advantage of these observations is that the exact duration of HCV infection of the transplant liver was known in each case.

The mechanism for increased pathogenicity of HCV 1b is not known; a possible higher replication rate, viral proteins with an enhanced cytopathic effect on liver cells, the presence of two HVR, whereas other genotypes possess only one (HVR-1) making type 1b more versatile, enabling it to escape more efficiently from host immunity have been discussed but are all hypothetical (72).

Association of HCV infection with anti-LKM-1 positive chronic hepatitis

Chronic hepatitis positive for anti-LKM-1 (liver-kidney-microsomal antibodies) has been associated with HCV infection, suggesting that HCV infection may trigger the humoral autoimmune reaction. Although genotypes 1, 2 and 3 were found in these patients, excluding the association of the

autoimmune reaction with one particular viral type, prevalence of genotype 1 in a group of 22 patients with chronic hepatitis positive for anti-LKM-1 and anti-HCV antibodies was significantly higher compared to a group of patients from the same region with chronic hepatitis C who were negative for anti-LKM-1 (77% vs 42%). These data suggest that HCV type 1 may more easily induce anti-LKM-1 antibodies, compared to genotypes 2 and 3 (73).

Response to antiviral treatment

Interferon alpha is the only registered drug with proven efficacy against chronic HCV disease, although it is not equally efficacious for all patients and long-term response rates of ca. 20% are definitely disappointing. Several investigators have reported on host and virus dependent variables that influence outcome, but a variety of different treatment regimens have been used, hampering a direct comparison of these trials, and attention has to be paid to discriminate confounding factors from truly independent factors.

In general, HCV-1b appears to be less responsive to IFN therapy than HCV-2a and HCV-3 (42, 70, 74–77). Responder rate, which in most studies included complete response defined as ALT normalization during therapy and maintained for several months of follow-up, and partial response, defined as a decrease of ALT levels to within a given upper limit were between 40 and 63% for type 1b, ca. 85% for type 2a, and 70% for type 2b.

In one study, the long-term response to IFN therapy was much worse for genotype 1b compared with genotypes 2a, 2b and 3a. Only 29% of patients with type 1, but 74% of those with types 2 and 3 still had an ALT normalization for 12 months (32). It should again be noticed, however, that in Europe genotype 1b is most commonly found in patients older than 50 years, whereas type 3a is more often found in younger individuals, meaning a shorter duration of infection and no cirrhosis (yet), factors that are known to influence response rates (75, 78). A study from Japan also reported a higher mean age in patients with HCV-K1 (Enomoto, Simmonds: 1b) compared with those infected with HCV-K2 (type 2) (70). Thus, other factors apart from intrinsic properties of a particular genotype, such as chronological differences in the epidemiology of genotypes, may interfere.

A recent report described a changing pattern of relative prevalence of HCV genotypes in haemodialyzed patients and kidney recipients (71). This group of patients has a high prevalence of anti-HCV antibodies of ca. 25%, and the time of contamination can be estimated reliably from the dialysis and blood transfusion period. Genotype 1b accounted for more than two-thirds of HCV infection in patients who underwent dialysis before 1977 but less than one-third in those haemodialyzed after 1985. In contrast, other genotypes appeared (3a, 4a, 5a) in the 1980s, with type 2a accounting for 42% of infections after 1985. Certainly, if the prevalence of type 1b has been declining during the 1980s, as seems to be the case for post-transfusional contamination, patients harbouring HCV type

1b are likely to be older and to have a longer duration of the disease, factors that influence outcome of therapy in a negative way and should be considered when studying the clinical and therapeutic implication of genetic variability. In a study from France, however, 12 of 13 patients with type 1a, but only 6/16 patients with type 1b had a response to interferon treatment, defined as normalization of ALT during treatment, and the genotype remained a predictive factor for response after adjustment for age, duration of disease and histological diagnosis (69). Few data are available so far about the effect of IFN therapy on genotypes 4, 5 and 6, except for one study reporting a poor response to IFN- α in patients infected with genotype 4 (43).

It should further be kept in mind, that different definitions for complete, good or partial response are used, and that current knowledge strongly suggests that a normalization or decrease in ALT levels are not optimal parameters for measuring the outcome of antiviral therapy in chronic HCV patients. More data about the effect of treatment on parameters of viral replication (HCV RNA) and inflammatory activity (liver biopsy) in relation to the infecting genotype are awaited.

Pretreatment level of HCV-RNA in serum is another viral factor predictive of the response to IFN treatment. Patients with a sustained complete response to IFN had lower pretreatment viraemic levels than complete responders who relapsed after the drug was stopped and non-responders (79). Some investigators noted an association between HCV genotypes and the HCV RNA level (75, 77), with higher pretreatment RNA levels in type 1b infected individuals, compared with types 2a and 2b, whereas others demonstrated that response to treatment is also correlated to virus load, regardless of the HCV genotype or subtype, with HCV-RNA titres of less than 10^2 $\text{CID}_{50}/\text{ml}$ being an important precondition for complete viral eradication, and normalization of ALT in almost no patient with viral titres above 10^4 $\text{CID}_{50}/\text{ml}$, irrespective of the genotype or subtype (42, 44). Genotype and viral load are thus both found to be independent factors influencing response to antiviral treatment.

However, the same responses to therapy are not always found, even among patients with the same amount and subtype of HCV. The degree of HCV quasispecies complexity and the diversity of HVR1 have been found to be closely related to the responsiveness to IFN therapy (39, 40). In addition, studies from Japan indicate that various HCV quasispecies have different sensitivity to IFN and, as a consequence, IFN exerts selective pressure on HCV quasispecies (47, 48). Moreover, Grottola et al. found that repeated courses of IFN resulted in the selection of a more pathogenic genotype II (1b) (80). Appearance of a HVR variant has been shown in a case of chronic hepatitis in association with relapse after interferon therapy (81). It is not known whether the rates of mutation in untreated patients and in patients receiving IFN treatment are equivalent or whether IFN therapy accelerates the mutation rate of HVR.

Multivariate analyses have shown that genotype 1b, cirrhosis and a high pretreatment viral load are factors independently associated with a low chance of response. Infection with genotype 2 or 3, and absence of cirrhosis on the other hand are independent predictive factors for response (76). It is thus worthwhile determining the viral genotype and quantitate HCV RNA before treatment, although it is currently difficult to draw the line between patient with a good chance and those with a lower chance for response to IFN, as alternative therapeutic options are still lacking.

Prevention

A lack of effective immune response to HCV has been demonstrated in chimpanzees, where it has been repeatedly shown that experimentally infected animals can be easily re-infected with heterologous as well as homologous strains (82, 83). The mechanism responsible for the lack of protective immunity against re-infection with HCV is at present unknown. It has been postulated by Farci et al. (82) that the virus may fail to induce an effective neutralizing antibody response or that genetic variation leads to the development of escape mutants that circumvent the immune response. Experiments in which chimpanzees were infected with in vitro neutralized HCV provided evidence that HCV infection elicits a neutralizing antibody response, and that these antibodies are likely isolate specific (84). The finding that protection against challenge with a homologous strain was related to the degree of humoral immune response (9) also supports the suggestion that HCV fails to elicit protective immunity.

Little is known about the relative importance of humoral and cell-mediated immunity to HCV, and further research is necessary to establish the role of both systems in viral clearance and in protection against re-infection. A cytotoxic T-cell mediated response has been described in infected chimpanzees (85). However, virus-specific T-cell populations seem unable to eradicate HCV in most chronically infected carriers (85).

An effective vaccine should probably activate both humoral and cell-mediated immunity. Even then, the high mutation rate will remain a major burden for effective and lasting protection against infection. The identification of an immunodominant epitope is an active field of research; activation of cytotoxic T-lymphocytes may be generated by means of gene transfer, as has been successfully done for influenza A (86). Differences in antigenic epitopes between the genotypes make cross-protection unlikely, and an effective vaccine has to be multivalent, including the common genotypes in particular geographical regions. Until a vaccine becomes available, precaution and measures for preventing parenteral spread remain the only way to limit the risk for acquiring HCV infection.

In conclusion, HCV is a very heterogeneous viral genus, comprising an amazing amount of variants, causing chronic liver disease in the majority of infected persons. Other isolates

may be expected, as sequencing data from different geographic areas become available. There is growing evidence for direct clinical consequences of HCV heterogeneity, especially for disease severity, prognosis and response to treatment. Genotype 1b, which predominates in Western Europe and may also be the most frequently found genotype worldwide, is also associated with more severe liver disease, is more often associated with cirrhosis and hepatocellular carcinoma, and has the lowest response rates for interferon treatment. Immunodiagnostic and vaccine development strategies will need to include variant genotypes. A classification of the spectrum of variant viruses into an easily understandable system that is helpful for clinical and epidemiological purposes is needed.

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