Springer Semin Immunopathol (1997) 19: 5–26

Springer Seminars in Immunopathology
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The quasispecies of hepatitis C virus and the host immune response

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Introduction

Infection with hepatitis C virus (HCV) represents an important public health problem worldwide, because it is a major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) [110]. Approximately 1% of the world population is infected with HCV, although geographic variation exists and further epidemiological studies are needed to draw a conclusive epidemiological map of HCV infection. The most striking and alarming feature of HCV infection is its high rate of progression to chronicity. Chronic hepatitis C develops in more than 80% of the acutely infected individuals and about 20–35% of them develop cirrhosis during the course of the disease [1, 2, 19, 25]. Currently, HCV-related end-stage liver disease is the leading indication of orthotopic liver transplantation worldwide [141].

Prospective studies of post-transfusion non-A, non-B hepatitis have been fundamental in unravelling the natural history of HCV infection [122]. Despite its inevitable progression, the course of chronic hepatitis C is usually indolent and subclinical. The average time to the clinical presentation of chronic hepatitis is about 10 years, to cirrhosis 20 years and to the development of HCC 30 years [64, 133]. In some patients, however, the course of chronic HCV infection may be rapidly progressive, leading to liver-related death within 5 years after infection ([1] and H.J. Alter, unpublished data). The overall rate of liver-related mortality varied markedly in different prospective studies, with a prevalence ranging from 1.6 to 15% [122]. A controlled clinical study conducted by Seeff et al. [111] in elderly subjects who underwent open-heart surgery failed to demonstrate an increase in the mortality rate after a mean follow-up of 18 years for patients who had developed post-transfusion hepatitis C compared to HCV-uninfected control subjects. By contrast, Tong et al. [133] showed a mortal-

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ity rate of 14.5% over a mean follow-up of 3.9 years in 131 patients with chronic hepatitis who had received blood transfusions a mean of 22 years before inclusion into the study. The mean age of the patients at the time of blood transfusion was 35 years. Although the reasons for such discrepancies are still unknown, it is likely that the different age of acquisition of HCV infection, and therefore the different life expectancy between the populations analyzed, played an important role.

Prospective studies have also suggested that the frequency of chronic sequelae of hepatitis C, in particular cirrhosis and HCC, varies according to the geographic area, being more frequently identified in Japan compared to Western countries [122]. Interestingly, a similar trend has been reported for HCV-associated fulminant hepatic failure (FHF). All the studies conducted in Western countries, with the exception of one [136], documented that HCV-associated FHF is a rare event [32, 36, 71, 79, 108, 129, 142, 143]. By contrast, studies conducted in Japan [90, 144] and Taiwan [18] have provided evidence of HCV infection in 40–60% of the patients with FHF. Whether these discrepancies reflect geographic differences in the epidemiology of HCV infection or in the pathogenicity of the prevalent viral strains is not known.

Chronic hepatitis C is associated with continuous viral replication in vivo, documented in some cases for more than 20 years [28]. This suggests that, in most patients, the immune response fails to mediate resolution of the infection, in spite of a vigorous humoral and cellular immune response directed against both structural and nonstructural viral proteins [14]. Further concerns emerged from the reanalysis of a series of cross-challenge studies in chimpanzees, which demonstrated a lack of protective immunity against reinfection with either homologous or heterologous HCV strains [29, 100]. A convalescent chimpanzee could be reinfected several times with different HCV strains, each time showing histopathological evidence of acute hepatitis, despite minimal elevations of serum alanine aminotransferase (ALT) levels [29]. Moreover, the risk of developing chronic HCV infection did not decrease after reinfection, and was similar to that observed following primary infection. In line with the above, evidence that HCV may cause more than one episode of acute hepatitis C in the same individual was obtained in multiply transfused β-thalassemic children [72]. The second episode of hepatitis was clinically indistinguishable from the first and, as seen in the chimpanzee model, was associated with the development of chronic hepatitis. Sequence analysis demonstrated that the second episode of hepatitis was due to reinfection with a different HCV strain rather than to reactivation of the original strain. Another line of evidence suggesting that HCV is unable to induce a protective immune response in the host is the occurrence of superinfection with heterologous HCV strains, documented both in humans and in chimpanzees chronically infected with HCV [58, 97].

The mechanisms whereby HCV induces persistent infection in the vast majority of the infected subjects, or which may cause more than one episode of acute hepatitis in the same individual, are still largely unknown. Furthermore, it is not clear why most patients infected with HCV have a very mild and stable liver disease, while some develop a severe and rapidly progressive disease [1]. As suggested for other viral agents that establish persistent infection in their host, several different mechanisms may account for such a high rate of chronicity. These include host factors, such as the inability of the host to mount a protective immune response, and viral factors, such as the remarkable degree of genetic heterogeneity of the virus. Clearly, the mechanism of persistence of HCV is not related to viral integration into the host genome, as a DNA replicative intermediate has never been demonstrated in the life

cycle of this virus. Over the past few years, evidence has accumulated indicating that HCV infection elicits a strong humoral and cellular immune response in the host [14], which nevertheless is unable to eradicate the virus or prevent reinfection. These observations are in agreement with the hypothesis that viral factors play a key role in the mechanisms of viral persistence.

In this chapter, we will focus our attention on the viral antigenic variation, which is a hallmark of several RNA viruses, associated with a low degree of fidelity of their nucleic acid polymerase [48]. Specifically, we will discuss the quasispecies nature of HCV, which is defined as a population of closely related variants simultaneously present in the same patient [81]. The generation of a quasispecies may lead to persistence by immune escape mechanisms and thereby represent the winning strategy of the virus in the delicate and continuously changing balance with the host immune system [21]. In this perspective, the study of the viral quasispecies may have critical implications for our understanding of the pathogenesis of HCV-related liver disease and for devising effective preventive and therapeutic strategies against HCV.

Genetic heterogeneity of HCV

HCV was recently classified within a third genus (provisionally designated *hepacivirus*) of the *Flaviviridae* family [11, 13]. Its genomic organization is similar to that of the two other genera of the pestiviruses and the flaviviruses [52, 103] consisting of a single-stranded positive-sense RNA genome with one long open reading frame (ORF) bracketed by 5′- and 3′-noncoding (NC) regions (see figure in Introduction) [16]. The polyproteins encoded by HCV, pestiviruses, and flaviviruses have hydrophobic amino acids distributed in a similar pattern [16, 60, 123]. The structural proteins, core (C), envelope 1 (El) and 2 (E2), and the putative structural proteins (E2-p7 and p7) of HCV are encoded by the 5′ region of the ORF; the nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) are encoded by the 3′ region of the ORF [52, 103]. In general, organization and processing of the polyprotein of HCV were most similar to those of the pestiviruses [103]. Amino acid sequence similarities between HCV and corresponding colinear regions of flaviviruses and pestiviruses exist in the serine protease and RNA helicase regions of the NS3 protein, as well as in the RNA-dependent RNA polymerase region of the NS5B protein [16, 84]. Furthermore, colinear regions with similarity were found in the 5′-NC sequences of HCV and pestiviruses [7, 44]. Thus, overall, HCV is most closely related to pestiviruses. It should be mentioned that several new viruses, genetically most closely related to HCV, were recently recovered from monkeys or humans [5, 6, 12, 13, 76, 77, 80, 88, 118, 119].

Variation was found throughout the HCV genome [11]. The most highly conserved sequences were identified in the 5′- and 3′-NC regions. The 5′-NC region contains sequences that are conserved among all HCV isolates [7, 120]. Whereas the proximal 3′-NC region varies in nucleotide sequence, as well as in length, it is followed in most isolates by a poly(U) tract [52] and 98 highly conserved nucleotides 3′ of the poly(U) tract [66, 125, 126]. The conserved NC sequences are most likely important for viral replication or gene expression.

Overall, the predicted capsid protein is the most highly conserved among the HCV proteins [9, 96], followed by conserved sequences in the NS3 and NS5B proteins [96]. The predicted envelope proteins (E1 and E2) contain conserved structural elements

[53, 96] but overall these proteins are highly variable [8, 96]. A short domain at the amino-terminal end of the E2 protein is so diverse among different HCV isolates that it was named the hypervariable region 1 (HVR1) [46, 137].

HCV genotypes

Worldwide, HCV exhibits extensive genetic variation resulting in multiple distinct genotypes of distantly related sequences [6, 10, 11, 13, 85, 94, 115]. The genomic sequences of the most distantly related HCV isolates varied by as much as 35%, which is equivalent to the differences observed among different serotypes of other human RNA viruses, such as other *Flaviviridae* (i.e., dengue viruses). In addition, sequences from different HCV isolates formed clusters of more closely related sequences. It was proposed that the most different HCV isolates be classified as major genetic groups or types (genotypes 1, 2, 3, etc) and that more closely related isolates be classified as subgroups or subtypes (a, b, c, etc.) [15, 115, 116].

The concept of 6 major genetic groups of HCV (genotypes 1–6) was the result of analysis of the genetic variation of HCV isolates collected worldwide. Analysis of the sequences of the entire core and E1 genes suggested the existence of 6 types with 14 subtypes of HCV [8, 9]. Analysis of partial NS5B sequences suggested the same 6 types with a total of 11 subtypes [116]. Overall, the genotypes identified in these studies represented 15 subtypes [11, 115]. Multiple additional subtypes within these six major genetic groups were identified subsequently [11, 83, 85, 115]. In general, sequence analysis of separate gene regions or fragments thereof resulted in a classification equivalent to that obtained by analysis of the entire genome that was determined for several subtypes of genotypes 1–3 [6, 11, 83, 115]. However, the classification of recently identified isolates from Southeast Asia is controversial [13]. These isolates were originally designated as additional genotypes 7–11 [11, 85, 130–132]. It has been argued that the isolates designated as genotype 10 should instead be classified as a more divergent subtype of type 3 and isolates designated as genotypes 7, 8, 9, and 11, should instead be classified as more divergent subtypes of type 6 [83]. The discovery of these latter isolates demonstrates that the evolution of HCV is more complex than what is reflected by the originally proposed two-tiered hierarchical classification of HCV.

The existence of multiple genotypes of HCV might reflect the long-term evolution of viruses in discrete geographical areas [11, 115]. In one geographical area, for example Zaire [8, 9], many subtypes of the same genotype (genotype 4) were found. This distribution, observed also for other genotypes in other countries, might represent endemic spread of HCV over centuries [83]. Conversely, the finding of the predominance of a limited number of specific subtypes within a given population, for example, genotypes 1b, 2a and 2b in Japan, might represent introduction of such genotypes within the last 30–40 years, followed by epidemic spread [83]. Finally, recent studies indicate that changes in the genotype distribution of HCV can occur within a specific geographic area, due to consecutive epidemics with different genotypes. In France, an epidemic of HCV, genotype 1b, caused by transfusion of unscreened blood products, was followed by another epidemic among intravenous drug addicts with genotypes 1a and 3a [4, 98, 99]. These genotype changes are likely to reflect differences in the population groups at risk for HCV infection during the two epidemics.

It is still unclear whether the various genotypes of HCV have different pathogenicity related to differences in transmission rate or infectivity, replicative capacity and the occurrence and severity of associated liver diseases and extrahepatic manifestations. Importantly, no major biological differences were observed among the various HCV genotypes and infection with HCV of any of these genotypes can lead to chronic liver diseases [11, 115]. Thus, all currently recognized genotypes seem to be hepatotropic and pathogenic. Studies of the genotype distribution in hemophiliacs compared with the distribution of genotypes in the blood products administered to these patients provided indirect evidence against a difference in the infectivity of various genotypes [56]. The level of viremia, which might reflect the replicative capacity of the virus, was not significantly different among the common genotypes of HCV in multivariate analysis of blood donors from around the world [121] and of patients with chronic liver disease from the U.S. [75]. Furthermore, following liver transplantation, no significant difference was observed in the level of viremia in patients infected with different genotypes [37, 40].

Conversely, in many but not all studies of Japanese patients with chronic liver disease, the level of viremia was significantly higher in patients infected with genotype 1b than in patients infected with genotypes 2a or 2b [11, 13]. Similarly, the data on a potential differential role of HCV genotypes on associated liver disease were inconclusive [4, 11, 115]. Evidence for more severe liver disease in patients infected with genotype 1b comes from studies of patients with chronic liver disease [92] and HCC [114, 127], as well as from studies of de novo infection in liver transplant patients [37, 40, 41]. However, this evidence for the differential pathogenicity of genotype 1b was not confirmed in studies of other patients with chronic liver disease [75, 117] and HCC [146]. In general, control of confounding factors and the limitations in the laboratory techniques used to determine the HCV genotype and the level of viremia contribute to the difficulties in obtaining definitive results in the above studies [11].

A number of studies, including those with multivariate analyzes, have found that the genotype, as well as the pretreatment level of viremia, are important predictive factors for the outcome of interferon [IFN] treatment in HCV-infected patients [11, 115]. In particular, genotype 1b predicted a poorer response to IFN therapy than infection with other genotypes, including genotypes 2a, 2b and 3a.

Quasispecies nature of HCV

The replication of RNA viruses is an error-prone process, primarily because the viral RNA polymerase lacks a proof-reading 3′–5′ exonuclease [48]. As a consequence of this low fidelity of the viral replication machinery, RNA viruses are characterized by a high mutation rate. Such mutation rates are often 1,000- to 1,000,000-fold greater than the mutation rates of DNA viruses [48]. For some RNA viruses, the mutation rate appears to be near the maximum that can be tolerated without catastrophic loss of genetic information [49]. For HCV, similar to other RNA viruses, the rate of nucleotide misincorporation by the viral RNA polymerase has been calculated to be approximately 10^3 to 10^4 base substitutions per genome site per year [93, 95]. As a consequence of this high genetic variability, HCV is never present in vivo as a homogeneous population of identical RNA genomes, but rather as a mixture of divergent, albeit closely related genomes, exhibiting a distribution that follows the model referred to as a quasispecies [81].

The quasispecies encompasses a "master" genome, which is quantitatively predominant, and a multitude of minor genomes, representing variable proportions of the total population. It is assumed that, at any given moment during the natural history of the infection, the quasispecies distribution represents the best fitting population that has established a status of equilibrium with the host [21]. The "master" genome predominance is most likely due to a superior replicative capacity in the host milieu at that specific moment. It is the quasispecies swarm in its complex, not just a single predominant genomic species, that constitutes the actual target for the immune system [22]. Thus, the selective pressure of the host results in a precise, albeit transient, homeostatic quasispecies distribution.

The genetic variability of RNA viruses may have important biological implications, particularly for their persistence in vivo by escape mechanisms, for the generation of drug resistance and for vaccine failure or potential reversion to virulence of live attenuated vaccines [21, 101]. The likelihood that genetic heterogeneity could result in a phenotypic change is dependent on several parameters, including the average mutation rate per infectious genome, the magnitude of the viral population and the number of mutations which is required for a phenotypic change to occur [21].

Striking examples have been reported of mutations at a single genomic site, which resulted in important alterations of the viral phenotype. Jameson et al. [55] detected point mutations of poliovirus Sabin type-1 vaccine strain in antigenic variants appearing in normal infants after vaccination. A specific nucleotide change in Sabin type-3 poliovirus vaccine strain was associated with an increase in neurovirulence [26]. In influenza virus, a single amino acid substitution in hemagglutinin H3 determined a change in receptor-binding specificity [104]. Similarly, Di Marzo Veronese et al. [20] described a single amino acid substitution within the V3 domain of the major envelope glycoprotein, gpl12O, of HIV-1, which resulted in a conformational alteration of the V3 loop, with loss of a neutralization epitope recognized by a monoclonal antibody. Furthermore, specific amino acid substitutions within the reverse transcriptase of HIV-1 and Feline immunodeficiency virus have been described, which determine resistance to the drug 3′-azido-3′-deoxythymidine [73, 102].

As seen with other RNA viruses, the pattern of nucleotide sequence variation is not consistent over the entire HCV genome [11]. The genes encoding the two envelope proteins [E1, E2] are the most variable, but the extent of variation is not constant in all the regions of the envelope proteins. As mentioned above, the greatest sequence variation has been detected at the amino terminus of the E2 gene. This region contains a domain of approximately 30 amino acids, defined as HVR1, which mutates rapidly in infected patients [46, 137]. The fact that the HVR1 shows the highest degree of variability has been instrumental both for identification of individual viral strains and for investigation of the HCV quasispecies.

The evidence of a quasispecies distribution of the HCV genomes in an infected individual was obtained with studies in which HCV was either molecularly or biologically cloned. The first description that HCV circulates in vivo as a quasispecies was provided by Martell et al in 1992 [81], who analyzed the distribution of viral mutants, in an individual coinfected with HCV and HIV, at four sequential time points over a period of 27 months. Sequence analysis of 20–27 molecular clones generated from polymerase chain reaction (PCR)-amplified products of cDNA corresponding to fragments of the 5′-NC region and NS3, demonstrated that about half of the circulating RNA molecules were identical (master sequence), while the remainder consisted of a spectrum of mutants differing from each other at one to four nucleotide sites. The

substitutions included silent mutations, missense mutations and in-frame stop codons. Subsequently, several studies have expanded these observations and confirmed that HCV circulates in vivo as a complex population of closely related viral variants [45, 61, 89, 124].

Important evidence of the quasispecies nature of HCV was obtained from studies of experimental transmission in the chimpanzee model. Several studies have used for animal inoculation the same well-characterized plasma, obtained from a patient (H) during the early acute phase of post-transfusion NANB hepatitis (H77) [30]. The inoculum, obtained before the appearance of antibody seroconversion, contained $10^{6.5}$ 50% chimpanzee infectious doses (CID_{50}) of HCV per ml, as shown by in vivo titration in chimpanzees [35]. Comparative sequence analysis was performed by direct sequencing between the virus used for inoculation and the viruses recovered from the chimpanzees. The analysis, based on the sequence of a portion of the E1 and E2 genes, including the HVR1, demonstrated that none of the sequences recovered from the chimpanzees after the viral challenge was identical to the sequence of the HCV strain used for inoculation. The nucleotide sequences differed from each other at 2–21 sites. Most of the nucleotide changes were confined to the HVR1 and were associated with nonsynonymous amino acid substitutions. Moreover, the virus recovered from chimpanzees given the same dose of challenge virus differed considerably, indicating that the degree of heterogeneity was not solely affected by viral dose. The degree of heterogeneity within the HVR1 of HCVs recovered from chimpanzees that received the same virus inoculum clearly demonstrated the presence of a quasispecies in the original H77 inoculum.

To better investigate the degree of HCV quasispecies within the H77 inoculum, an extensive analysis of the viral genome population was undertaken by cDNA cloning of amplified products spanning part of the E1 and E2 genes [34]. For this purpose, a total of 104 molecular clones derived from the H77 inoculum was sequenced. This analysis demonstrated that at least 19 different viral strains were simultaneously present within the inoculum. The most predominant sequence (master) was represented by 70 out of 104 clones (67%). The remaining 34 were a multitude of different variants, represented by 6, 5, 4, 2 or 1 clone, respectively. Four variants were represented by 2 clones each and 11 by a single clone. Because the inoculum was derived from an infected patient, these data demonstrate that a large number of variants is simultaneously present in an infected individual.

In another report, a human plasma containing a complex HCV quasispecies was both inoculated into a chimpanzee and used to infect human lymphoid cell lines in vitro. Interestingly, only two of the seven variants present in the inoculum were able to replicate both in vivo and in vitro, suggesting a selective transmission of HCV quasispecies [47].

HCV quasispecies and natural history of HCV infection

Over the past few years, there has been a growing interest in the study of the HCV quasispecies and of its relation to the clinical course of HCV-associated disease. Several authors have investigated whether the degree of viral heterogeneity is related to the stage of HCV infection. In a study by Honda et al. [50], the HCV quasispecies was examined in 28 patients with liver disease of varying severity, including patients with acute hepatitis, chronic persistent hepatitis, chronic active hepatitis and cirrhosis,

with and without HCC. The nucleotide sequences of the core-E1 region of the HCV genome were used to calculate the genetic diversity. The magnitude of intrapatient variation increased significantly and progressively from patients with acute hepatitis to those with cirrhosis, suggesting that the degree of HCV quasispecies correlated with the progression of liver disease. In a subsequent study, however, the quasispecies complexity of the HVR1 did not correlate with the stage of chronic HCV infection [91], but this lack of correlation was not confirmed by other studies. Koizumi et al. [65] showed, by multivariate analysis, that viral diversity was independently related to the progression of liver disease and was not correlated with the duration of infection.

By contrast, Gonzalez-Peralta et al. [42] demonstrated that an increase in the quasispecies heterogeneity correlated with the duration of HCV infection. A higher degree of HCV quasispecies was observed in a chronically infected patient with acute exacerbation, compared to a patient who did not manifest acute exacerbation during the course of the disease [59]. The effect of multiple exposures to HCV on degree of viral quasispecies was investigated in hemophiliacs with a history of multiple transfusions, compared to patients with post-transfusion hepatitis C resulting from a single HCV inoculation. Analysis of the HVR1 sequences showed no significant difference in the number of variants, although the average number of nucleotide substitutions per variant was significantly higher in hemophiliac patients [134]. When the degree of HCV quasispecies in the HVR1 was analyzed simultaneously in serum and peripheral blood mononuclear cells of 11 patients followed sequentially, identical changes were observed in 4 patients, no changes in 5, and different changes between the two compartments in the remaining 2 patients [39]. The study of HCV quasispecies has also been extended to the neoplastic tissue of patients with HCC. Analysis of the HCV core region in noncancerous and cancerous lesions from 7 patients with HCC documented a larger number of HCV variants in cancerous than in non-cancerous lesions [51].

Although most of the studies mentioned above suggested that the degree of viral complexity correlates with the progression of HCV-related liver disease, little is known about the HCV quasispecies during the acute phase of hepatitis and of its relation to the outcome of HCV infection. The natural history of HCV infection generally follows a typical pattern, with progression to chronicity and slow evolution toward end-stage liver disease. However, a different disease evolution occurs in a limited proportion of patients, who represent the two extremes of the clinical spectrum of HCV-related liver disease [1]. On one side, there are individuals who experience an acute self-limited hepatitis, with rapid clearance of HCV infection; on the other, the clinical course can be rapidly progressive, leading to liver-related death within 5 years after infection ([1] and H.J. Alter, unpublished data).

The mechanisms responsible for this diversity in the clinical course are at present unknown. It is uncertain whether it reflects differences in the viral characteristics or in the host immune response during the initial phase of infection, or, possibly, a combination of viral and host factors. Prospective studies of post-transfusion or community-acquired hepatitis C have failed to identify any clinical, serological or virological features that could predict the clinical outcome of HCV infection [1]. Only recently, the role of the degree of viral complexity as a pathogenetic mechanism that can influence the clinical outcome of HCV infection has been investigated [33]. Serial serum samples from prospectively followed blood recipients, obtained within the first 4 months of post-transfusion hepatitis, were analyzed for the degree of HCV quasispecies, the level of HCV replication, and the HCV genotype. A total of 12

patients were studied: 3 with fulminant hepatitis, 3 with acute resolving hepatitis, 3 with slowly progressive chronic hepatitis and 3 with rapidly progressive chronic hepatitis, leading to liver-related death within 5 years after infection. All patients, except 2 with fulminant hepatitis, were derived from the NIH prospective study of post-transfusion NANB hepatitis. For the analysis of the HCV quasispecies, PCR products of the E1/E2 genes including the HVR1 region, were cloned, and up to 10 molecular clones from each sample, from three or four time points per individual, were sequenced.

Preliminary results of this study indicate that HCV circulates in vivo as a quasispecies from the very early stage of the infection. The degree of viral quasispecies in the first available PCR-positive sample was similar in the different patient groups, regardless of the outcome of infection. There was no apparent correlation between the degree of HCV quasispecies and the level of viremia or the viral genotype. Longitudinal analysis demonstrated that the complexity of the viral quasispecies remained constantly low until death in patients with fulminant hepatitis, decreased over time in patients with acute resolving hepatitis, increased in patients with slowly progressive chronic hepatitis and increased dramatically in patients with rapidly progressive chronic hepatitis. Thus, these data indicate that the study of the HCV quasispecies during the first 4 months of infection may provide prognostic information, as a marked increase in HCV heterogeneity was accompanied by a rapid evolution of the disease.

HCV quasispecies and orthotopic liver transplantation

The study of patients who received orthotopic liver transplantation has provided a new model for understanding the role of the viral quasispecies in HCV transmission and pathogenicity. Gretch et al. [43] performed a longitudinal analysis of the viral quasispecies in five transplanted patients, all infected with the same HCV genotype (genotype 1). An average of 30 clones per sample was sequenced, with a follow-up ranging from 6 to 24 months. Before liver transplantation, the HCV quasispecies in the five subjects encompassed between one and nine distinct variants. Interestingly, a relatively homogeneous viral quasispecies emerged in all patients following transplantation. Moreover, the three patients who developed severe post-transplant recurrent hepatitis maintained the major viral variants that were already present before transplantation, whereas the two patients with asymptomatic post-transplant HCV infection exhibited only variants that were present as a minor component of the viral population before the transplant.

Similar data were obtained by Martell et al. [82] who studied the diversity of the viral sequences in samples obtained before and after liver transplantation from two patients with end-stage liver cirrhosis. In both cases, the complexity of the viral quasispecies diminished after transplantation, with conservation of the same consensus sequences that were present before transplantation. Whether this decrease in complexity of the viral quasispecies following liver transplantation is determined by the selective effect of the pre-existing immune response of the graft recepient or, alternatively, by a lack of de novo selective pressure secondary to the iatrogenic immunosuppression, remains to be established.

Evidence for a selective mechanism in the transmission of the HCV quasispecies also emerged from a study of mother-to-child transmission [139]. Analysis of ten clones obtained from both the mother and the infant at the time of birth demonstrated

the presence of nine different variants in the mother, but only of a single viral strain in the infant. Interestingly, the variant detected in the infant was highly related to, but not identical to, the nine variants identified in the mother.

The study of HCV quasispecies has also been applied to the investigation of the effects of HCV superinfection on patients with end-stage HCV-related liver diseases undergoing orthotopic liver transplantation. The first evidence of superinfection in humans was obtained by Kao et al. [58], who documented a second episode of post-transfusion acute hepatitis C in a chronic HCV carrier. By sequence analysis, they demonstrated that the superinfection was transient and that the new virus was subsequently cleared and replaced by the original strain. A similar pattern was observed in a patient who received liver transplantation for fulminant hepatitis C [31]. Again, the superinfection was a transient event, lasting for only 2 weeks after liver transplantation; then, the original strain reappeared and persisted throughout the follow-up. Both viruses belonged to genotype 1a.

Recently, the outcome of HCV superinfection was evaluated in 14 patients with end-stage HCV-related liver disease who received liver grafts from HCV-infected donors [74]. Both liver and serum samples were analyzed in the recipients, whereas only liver samples were available from the donors. Analysis of sequential viral sequences, by direct sequencing and single-strand conformation polymorphism assay, demonstrated that the recipient strain prevailed in 6 patients, while the donor strain replaced the recipient strain in 8 patients. In five donor/recipient pairs in which one of the patients was infected by genotype 1 (either 1a or 1b) and the other by a different genotype (i.e., 2c, 3a, 5b), the former invariably became the predominant strain after transplantation. Similarly, genotype 1b consistently prevailed over genotype 1a. Thus, this study confirmed that HCV superinfection does occur in humans and provided evidence that genotype 1 (1b or 1a) may have biological advantages over the other HCV genotypes.

As previously reported [74], patients retaining their own strain after transplantation had more severe disease than those who acquired the donor strain, suggesting that, at least in some patients, viral factors might play an important role in the pathogenesis of HCV-related liver disease. These studies [31, 74] also provided evidence that the original and the superinfecting HCV strains rarely, if ever, coexist for a prolonged period of time, most likely because of the phenomenon of viral interference in vivo, which has been extensively documented in the chimpanzee model [27].

In patients undergoing liver transplantation for HCV-related chronic liver disease, recurrence of HCV infection after surgery is almost universal. Furthermore, in light of the evidence so far accumulated, both in humans and chimpanzees, the patients may not be protected against superinfection by heterologous HCV strains. Because of the general shortage of graft donors and the steadily increasing need of liver tranplantation for end-stage HCV-related liver disease worldwide, prospective studies will be important to investigate the impact of HCV superinfection on the natural history of the disease in transplanted patients.

HCV quasispecies and response to interferon therapy

The HCV quasispecies has also been intensively investigated to determine whether its complexity correlates with the response to IFN-α therapy. At the present time, IFN is the only effective treatment for chronic hepatitis C. However, only 10–20% of

the treated patients show a sustained response to IFN, with eradication of the virus. Although both the HCV genotype and the level of HCV viremia have been reported to be predictive factors for the response to IFN therapy [38], there is growing evidence that a greater degree and complexity of HCV quasispecies is associated with a lack of response to IFN therapy [57, 65, 86, 87, 145].

In most of these studies, the target sequence used to assess the genetic heterogeneity of HCV has been the HVR1 domain. In individuals infected by HCV genotype 1b, Enomoto et al. [23] recently demonstrated that IFN treatment induces a change in the composition of the HCV quasispecies, with selection of specific variants, suggesting that the sensitivity to IFN is different among the diverse strains simultaneously present in the same patients. A cluster of amino acid substitutions in the carboxy-terminal half of the NS5A region (amino acid positions 2154–2383) was found to be associated with the sensitivity to IFN of HCV genotype 1b, particularly missense mutations in a 40-amino acid fragment of the NS5A region (amino acid positions 2209–2248). By contrast, in IFN-resistant HCV, the sequence of this region was identical to that of prototype HCV genotype 1b [24]. Although these data may have important implications for predicting the response to IFN treatment in patients infected with genotype 1b, they need to be validated by more extensive clinical studies.

HCV quasispecies and host immune response

The ability of HCV to evade the immune response of an infected individual, using the strategy of genetic variation, may be an important key for the virus to survive and establish persistent infection in the host. Driven by the propensity of the viral polymerase to introduce random mutations at each replicative cycle, a large number of variants is continuously generated in vivo during the course of HCV infection. Upon this background, the selective force exerted by the humoral and cellular immunity allows the emergence of variants that are recognized less efficiently or go totally unrecognized by the host's immune surveillance.

One of the most effective clearance mechanisms enacted by the immune system against viral infections is the generation of neutralizing antibodies, which also provide the best correlate of protection in vaccinated individuals. Conclusive evidence that neutralizing antibodies are produced in patients with chronic HCV infection was obtained with experiments conducted in the chimpanzee model [30]. Nevertheless, the same studies also clearly demonstrated that the effectiveness of such neutralizing antibodies in resolving the infection is limited, because they have a restricted spectrum of activity.

The serum from a chronically infected individual (patient H) was tested for its ability to neutralize an HCV inoculum derived from the same patient and previously titrated in vivo (H77). The residual infectivity was assayed by intravenous inoculation of seronegative chimpanzees. Neutralization of HCV infection was achieved with plasma obtained from patient H 2 years after primary infection (H79), but not with plasma collected from the same patient 11 years later (H90), despite the presence in both plasmas of antibodies against structural and nonstructural HCV proteins, including the E1 and E2 envelope glycoproteins. Analysis of sequential viral isolates from patient H revealed a significant degree of genetic divergence from the original dominant strain (H77), which was already evident 2 years after primary infection. However, the sequence of HCV recovered from patient H 2 years after infection (H79)

had a striking similarity to that recovered from one of the chimpanzees inoculated with the original virus (H77), suggesting that the progenitor of the new strain was already present during the acute phase, 2 years earlier, rather than having emerged by sequential changes occurring in vivo over time. Indeed, different sequences of HCV were recovered from different chimpanzees that had received the same H77 inoculum (see above). A similar mechanism, i.e., the in vivo emergence of pre-existing minor variants, may also be responsible for the loss of recognition of HVR1 epitopes by the patient's antibodies, documented in longitudinal studies of chronically infected patients [62, 128, 135, 138]. These observations confirmed that HCV, like other RNA viruses, is not present in a patient as a single virus species but as a population of closely related variants. The coexistence of a mixed viral population may lead to the rapid emergence of viruses that escape neutralization by the immune system. This hypothesis has been corroborated in an in vitro system by Shimizu et al. [112], who used a human continuous cell line infected with murine leukemia virus (HPB-Ma) as a target for HCV infection. The H77 virus was neutralized by plasma obtained from patient H for the first 5 years following primary infection, but not by plasma obtained thereafter. Similarly, the virus obtained 13 years after primary infection was not neutralized by plasma obtained early in the disease course, but only by plasma obtained 1 year later.

Altogether, the data obtained both in vitro and in vivo provide evidence that HCV does elicit neutralizing antibodies. However, these antibodies are isolate-restricted and ineffective against some of the strains contained in the complex quasispecies of the inoculum. Due to its quasispecies nature, HCV may thus establish persistent infection in spite of the presence of neutralizing antibodies.

Recently, a major target of the neutralizing antibodies against HCV has been identified in the HVR1 region of the E2 envelope protein (see figure in Introduction) [34]. Several observations had already suggested that this region could be involved in HCV neutralization. The HVR1 is the most divergent region of the entire viral genome [46, 137], undergoes sequential mutations over time in vivo [45, 61, 63, 68, 70, 93, 95, 107] (suggesting that it is subjected to the selective pressure of the host), and contains linear epitopes recognized by patient antibodies [62, 78, 109, 128, 135, 138].

In a chronically infected patient, Weiner et al. [138], using synthetic peptides corresponding to sequential HVR1 sequences, have documented the temporally related appearance of isolate-restricted antibodies directed against linear epitopes of the HVR1, which underwent sequential mutations over time. Interestingly, the mutated HVR1 was no longer recognized by the antibodies present at previous time points, suggesting that the HVR1 is subjected to a selective pressure by the host immune system. The fact that the genetic variation observed within the HVR1 was sufficient to determine a lack of recognition by pre-existing antibodies strongly suggested that this region contains epitopes recognized by putative neutralizing antibodies.

In line with the above, no genetic variability in the HVR1 was documented at three time points, over a period of 2.5 years, in a patient with agammaglobulinemia [69]. The conservation of the HVR1 sequence in this patient during the chronic phase of infection supports the hypothesis that the genetic heterogeneity of the HVR1 results from the selective pressure exerted by the humoral immune response. In vitro studies conducted by Zibert et al. [147] demonstrated that human sera obtained early after HCV infection contain antibodies specific for the HVR1, which can prevent the binding of HCV to cells. Preincubation of the same sera with recombinant HVR1

fusion proteins restored in most cases the binding of HCV to cells, suggesting that the majority of the binding-neutralizing antibodies were directed against the HVR1.

More recently, another study conducted in vitro has identified virus binding-neutralizing antibodies directed against putative conserved E2 epitopes, presumably located outside the HVR1 [105]. Low titers of such antibodies were detected in patients infected with different genotypes of HCV, whereas chimpanzees vaccinated with recombinant E1 and E2 proteins had high titers, which correlated with protection from experimental HCV infection. The potential importance of the HVR1 for HCV neutralization is also underscored by its marked similarity with the V3 domain of the gp120 envelope glycoprotein of HIV-1, the causative agent of acquired immunodeficiency syndrome (AIDS) [3]. The V3 loop, which mutates rapidly in vivo under the selective pressure of the host humoral immune response, plays a critical role in the viral life cycle and is a major target of type-specific HIV-neutralizing antibodies [106].

The recent study that has demonstrated that the HVR1 is a critical target of HCV-neutralizing antibodies has also provided the first in vivo model for the emergence of neutralization escape mutants that were already present within the viral quasispecies [34]. A hyperimmune rabbit serum was raised against a synthetic HVR1 peptide, corresponding to the sequence of the predominant strain contained within the H77 inoculum, and used for in vitro neutralization of HCV, followed by testing the residual infectivity by intravenous inoculation into HCV-seronegative chimpanzees. In one of the two animals whose inoculum contained the virus mixed with the hyperimmune rabbit anti-HVR1, HCV was completely neutralized by the hyperimmune serum, suggesting that the synthetic peptide had elicited HCV-neutralizing antibodies.

By contrast, the second animal developed a typical acute hepatitis C which progressed to chronicity. The composition of the viral quasispecies recovered from this animal, however, was significantly different from that of the predominant H77 strain that was used to raise the hyperimmune rabbit anti-HVR1 serum. None of the ten molecular clones obtained from this animal was identical to the sequence of the predominant H77 strain. Comparative sequence analysis demonstrated that the strains that emerged in this chimpanzee were present as minor variants within the H77 inoculum, representing only 6% and 2%, respectively, of the original HCV quasispecies. Thus, the hyperimmune anti-HVR1 serum was able to neutralize the predominant clone, but was ineffective against the minor variants which emerged in vivo. By contrast, the predominant clone appeared in all the control animals that had received the same dose of virus mixed with either the preimmune rabbit serum or an HCV-negative human plasma.

The observation that a hyperimmune rabbit serum raised against a synthetic HVR1 peptide was able to protect chimpanzees from homologous HCV infection provided direct evidence that anti-HVR1 antibodies can, in the absence of other virus-specific immune responses, prevent HCV infection. However, the failure of the anti-HVR1 antiserum to prevent the emergence of pre-existing variants suggests that the reactivity was type specific and restricted to the predominant viral strain used for immunization. These observations may have important implications for understanding the mechanism of persistence of HCV in vivo. The quasispecies nature of HCV provides a rapidly moving target that the host immune system can never usually fully control. The experiments with the hyperimmune anti-HVR1 serum clearly demonstrate that even if a predominant strain is effectively neutralized, as probably occurs in vivo following

primary infection, other variants are already present or will be generated over time, which will eventually emerge because of their ability to escape from immune control.

Evidence that the HVR1 of the E2 envelope protein is a critical neutralization domain of HCV was also obtained in vitro by Shimizu et al. [113] using the HPB-Ma human lymphoid cell line. The H77 virus was neutralized by the hyperimmune serum raised against the homologous HVR1 peptide, which failed to neutralize a genetically divergent isolate (H90) obtained from the same patient 13 years after primary infection. These authors also identified a small segment within the HVR1, spanning amino acid positions 398–410, as the epitope recognized by the hyperimmune anti-HVR1 antiserum.

As documented with neutralizing antibodies, it has been shown that escape mechanisms may be operating also in the setting of cellular immunity, as specific mutations within immunodominant epitopes have been associated with the loss of recognition by specific cytotoxic T lymphocytes (CTL) [140]. These observations suggest that the CTL response, similar to neutralizing antibodies, may have a viral isolate-restricted spectrum of activity, which would be ineffective against mutant strains present in the HCV quasispecies or emerging in vivo over time.

HCV quasispecies as a critical obstacle to the development of a broadly reactive vaccine

The bulk of the data obtained on the immune response elicited by HCV both in humans and in chimpanzees emphasizes the obstacles that need to be overcome for the induction of a broadly reactive vaccine against HCV. First, this virus represents a moving antigenic target that continuously mutates to escape from the host protective immunity. Second, a protective host response, when elicited, seems to be weak and of short duration, as demonstrated by experimental reinfection experiments with homologous HCV isolates, and restricted to individual viral strains within the replicating quasispecies population.

Consistent with these observations was the failure of both HCV antibody-positive and -negative human immunoglobulin preparations in preventing HCV infection in chimpanzees, when administered intravenously 1 h after virus challenge [67]. Serum HCV RNA was detected in each chimpanzee within a few days after inoculation, regardless of the type of immunoglobulin infused. However, the liver enzymatic peak was delayed in the animal that received the hepatitis C immunoglobulin, suggesting that post-exposure administration of hepatitis C immunoglobulin can prolong the incubation period of acute hepatitis C, but neither prevent nor delay HCV infection.

The results of an experimental vaccination attempt confirmed all the caveats that had emerged from studies of the protective immunity elicited by HCV. Choo et al. [17] vaccinated seven chimpanzees with recombinant E1 and E2 envelope glycoproteins expressed in mammalian cells using a vaccinia system. The animals were challenged with the homologous HCV (strain HCV-1). Five of the animals were completely protected from infection with a low dose (10 CID_{50}) of virus, whereas two animals had a milder disease compared to the four chimpanzees used as controls. Subsequent studies demonstrated that the vaccine was unable to protect against a heterologous HCV strain (H77), following a booster vaccination which, nevertheless, had induced a weaker antibody response than the first immunization [54]. These data are consistent with the hypothesis that the vaccine induced an isolate-restricted HCV-neutralizing immune

response of weak intensity, which was insufficient to protect against a heterologous challenge.

The finding that epitopes within the HVR1 domain can elicit protective immunity against homologous HCV infection has opened new perspectives for devising preventive strategies for the control of HCV infection. However, the complex quasispecies nature of HCV and the fact that the HVR1, which was identified as a critical neutralization domain, is the most variable region of the entire HCV genome, combined with the restriction of the neutralizing immune response, pose a major challenge for the development of a broadly reactive vaccine for the control of HCV infection.

Conclusions

Over the past few years, evidence has accumulated that HCV circulates in vivo from the early stages of infection as a complex quasispecies. The generation of the viral quasispecies is the result of an error-prone replication process, primarily due to the lack of a proof-reading exonuclease associated with the viral RNA polymerase. As a consequence of this infidelity, HCV is probably never present in vivo as a homogeneous population of identical RNA genomes, but rather as a swarm of closely related variants.

The quasispecies of HCV represents a rapidly moving target that the host immune system seems unable to control fully. The existence of a mixed viral population may lead to the rapid emergence of viruses that can escape immune recognition and neutralization. This may be the winning strategy whereby HCV can establish persistent infection in more than 85% of infected individuals. Although HCV-neutralizing antibodies have been documented in infected individuals, they are isolate restricted, ineffective against emerging viral strains and provide no protection against developing chronic infection. The HVR1 region of the viral envelope has recently been identified as a target of HCV-neutralizing antibodies. Indeed, a hyperimmune rabbit serum against HVR1 protected chimpanzees from homologous HCV infection, but not from the emergence of neutralization escape mutants.

In conclusion, the quasispecies nature of HCV may have important biological implications, particularly for the persistence of the virus in vivo, for the generation of drug resistance and for the development of broadly reactive vaccines.

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