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Review

The long and winding road leading to the identification of the hepatitis C virus☆

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This review describes work conducted largely in my laboratory at the Chiron Corporation between 1982 and 1989 that led to the identification of the hepatitis C virus (HCV). Key colleagues included Dr. Qui-Lim Choo in my laboratory and Dr. George Kuo also of Chiron as well as my collaborator Dr. Daniel Bradley at the CDC who provided many biological samples from the NANBH chimpanzee model. Numerous molecular approaches were explored including the screening of tens of millions of bacterial cDNA clones derived from these materials. While this early genomics approach resulted in the identification of many host gene activities associated with NANBH, no genes of proven infectious etiology could be identified. A separate avenue of our research led to the molecular characterization of the complete hepatitis delta viral genome but unfortunately, this could not be used as a molecular handle for HCV. Largely following input from Dr. Kuo, I initiated a blind cDNA immunoscreening approach involving the large-scale screening of bacterial proteomic cDNA libraries derived from NANBH-infectious chimpanzee materials (prior to the development of PCR technology) using sera from NANBH patients as a presumptive source of viral antibodies. Eventually, this novel approach to identifying agents of infectious etiology led to the isolation of a single small cDNA clone that was proven to be derived from the HCV genome using various molecular and serological criteria. This discovery has facilitated the development of effective diagnostics, blood screening tests and the elucidation of promising drug and vaccine targets to control this global pathogen.

***Keywords*: Hepatitis C virus discovery; HCV history; Identification of HCV; Non-A, Non-B hepatitis NANBH**

1. Introduction

The field of viral hepatitis was initiated in the 1950s and 60s with the distinction of so-called "infectious" and "serum" hepatitis [1], later proven to be due to infection by the hepatitis A virus (HAV) by Dr. Stephen Feinstone and colleagues at the NIH [2] and by the hepatitis B virus (HBV) by Dr. Baruch Blumberg and colleagues [3], respectively. With the advent of serological tests to detect infection by HAV and HBV in the mid-1970s, it was surprising to find that most cases of parenterally-transmitted hepatitis were not in fact due to either virus [4] hence heralding the term, Non-A, Non-B hepatitis (NANBH). Dr. Harvey Alter and other groups then pioneered the use of the chimpanzee as a reliable model for serial passage of NANBH infection from human materials [5,6]. The use of this model provided data indicating the existence of multiple NANBH agents, one of which was shown to cause characteristic membranous tubules within the cytoplasm of infected chimpanzee hepatocytes (the so-called tubule-forming agent (TFA; Ref. [7])). Dr. Bradley's and Dr. Purcell's laboratories then went on to show that the TFA could be inactivated by organic solvents consistent with it being a lipid-enveloped agent [8,9] and

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Abbreviations: HCV, hepatitis C virus; HAV, hepatitis A virus; HBV, hepatitis B virus; PCR, polymerase chain reaction; RT, reverse transcriptase; NANBH, Non-A, Non-B hepatitis; cDNA, complementary DNA; TFA, tubule-forming agent.

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following the demonstration that it could be filtered through a 80 nM pore-sized filter, Dr. Bradley suggested in the mid-1980s that this could be a small enveloped virus related to either the flaviviridae, togaviridae or to the hepatitis delta agent or alternatively, that it could be a novel type of enveloped virus [10]. This hypothesis was later further refined by Dr. Purcell's laboratory that showed that the TFA could pass through a 50 nM filter [11].

Concurrently, the work of Drs. Harvey Alter, Dienstag, Hoofnagle, Seeff and others demonstrated the insidious development of NANBH disease in humans with up to ~20% of infected patients slowly progressing to liver cirrhosis typically over the course of many years [12,13]. Dr. Harvey Alter [14] and separately, the large TTVS consortium [15], also showed that the incidence of chronic NANBH was up to 10% following blood transfusion in the USA. Additional work from Dr. Miriam Alter of the CDC also demonstrated the frequent acquisition of NANBH within the community, unlinked with blood transfusion [16].

Despite this progress, it proved impossible to definitively characterize the NANBH agents despite well over a decade of intensive research by many laboratories throughout the world [17]. This then was the research arena that I entered following my emigration from the UK in 1982, where I had characterized the human interferon-beta gene [18,19], to set up a laboratory at a newly-formed biotechnology company called Chiron.

2. Molecular approaches: 1982–1988

For many years, my laboratory focused on trying to identify the NANBH genome by cloning nucleic acids derived from infected human and chimpanzee materials and then identifying viral clones by showing their specific hybridization to radioactive cDNA probes derived from infected but not uninfected liver or blood samples. Numerous chimpanzee samples were obtained from my collaborator Dr. Daniel Bradley of the CDC and some human samples were provided by Dr. Tatsuo Miyamura of the Japanese NIH (who worked with me in 1983). By improving the sensitivity of this +/− hybridization method and screening many tens of millions of clones using a cumulative total of ~250 mCi of P^{32}-radiolabelled cDNA, we were able to identify many host-derived cDNAs specific to infected samples but none could be demonstrated to be derived from an etiologic agent of NANBH.

Given that various studies had suggested a relationship between the NANBH agents and a variety of known viruses such as the flaviviruses, togaviruses, hepadna viruses, picornaviruses and other agents, we used hybridization probes derived from these viruses in repeated attempts to identify a signal derived from a NANBH genome or specific mRNA. Our inability to do so was interpreted as either being due to limiting amounts of the NANBH agents in our samples or that they were novel viruses lacking substantial sequence identity with the known viruses. In 1977, the hepatitis delta antigen had been discovered by Dr. Mario Rizzetto and shown to be present in the nuclei of some HBV carriers [20]. This work led to the detection of an uncharacterized RNA molecule associated with infectious HDV samples from which a small cDNA sequence had been identified [21,22]. Because work from Dr. Purcell's laboratory had shown that HDV infection of chimpanzees led to the appearance of cytoplasmic tubules in hepatocytes similar to those observed following infection with the NANBH TFA [23], my laboratory in collaboration with that of Dr. John Gerin went on to fully characterize the HDV genome and to show that it was a circular covalently-closed, single-stranded RNA molecule that extensively base-paired intramolecularly to form a highly stable double-stranded rod-like structure, highly reminiscent of plant-like viroids and virusoids ([24]; Fig. 1). Evidence that it really was the true genome of HDV was derived by my colleagues Drs. Kang-Sheng Wang, Amy Weiner and Qui-Lim Choo who showed that it did indeed encode the delta antigen [24,25]. Unfortunately, using the full HDV genome as a molecular probe for NANBH, Dr. Weiner was unable to observe specific hybridization even under conditions of low stringency [26].

Next, Dr. Weiner developed highly sensitive silver staining techniques for visualizing electrophoretically-separated nucleic acids in order to identify high molecular weight RNA and DNA viral genomes that may be present in NANBH-infected chimpanzee materials. Again, no specific signals were identified. Concurrently, Dr. Weiner and other colleagues spent a great deal of time trying to propagate the NANBH agents in various tissue culture systems looking for cytolysis or some other form of cytopathogenic effect possibly induced by successful replication of the virus. In addition, we employed the electron microscope to attempt the visualization of any viral progeny. Despite some false leads due to contaminating endogenous adenoviruses, no evidence for successful cell culturing of NANBH could be obtained (we now know that the *in vitro* propagation of HCV is extraordinarily difficult and has only been achieved recently by Dr. Wakita's laboratory using one specific HCV strain derived from a Japanese patient [27]).

A very exciting report from another laboratory concluded that patients clinically-diagnosed with chronic NANBH exhibited reverse transcriptase (RT) activity [28]. This stimulated us and many other laboratories to investigate this claim closely but after extensive analyses, the general conclusion was that no specific RT activity was associated with NANBH sera. Similarly, a report for the *in vitro* propagation of NANBH-associated spumaviruses was shown to be due to

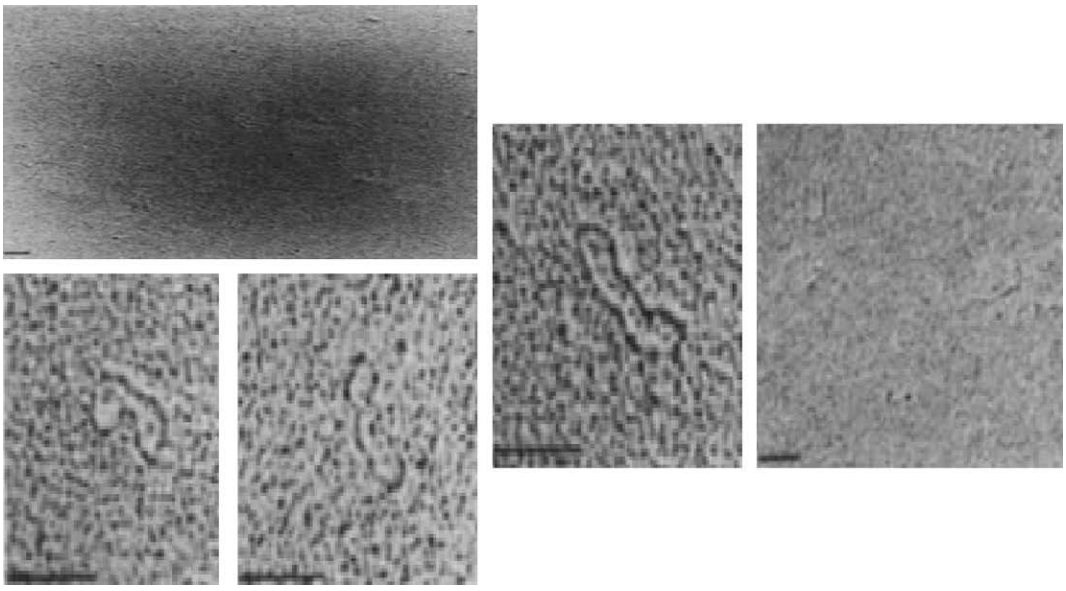

Fig. 1. The circular nature of the HDV genome [24]. Reproduced with permission.

contaminating endogenous viruses which were not specific to NANBH [29].

An elegant report described the isolation of human monoclonal antibodies derived from NANBH patients that could bind specifically to NANBH liver sections and not to control, uninfected liver tissue. It appeared that for the first time, antibodies specific to the NANBH agents themselves might have been isolated by Dr. Yohko Shimizu and colleagues [30]. This stimulated us to do a large amount of work to obtain such antibodies and to better define their reactivity. One method that we used was immunoscreening of expression cDNA libraries. We cloned cDNA derived from poly-A^+ mRNA obtained from many different liver samples from Dr. Bradley's NANBH-infected chimpanzees into the bacteriophage expression vector lambda gt11 and then screened the resulting proteomic library with Dr. Shimizu's antibody. As quite often the case with such immunoscreening experiments, no specific binding could be observed. Subsequently however, Dr. Shimizu was able to purify the reactive antigen from infectious materials and using sequencing analyses showed that it was in fact a host-derived protein and not a NANBH viral antigen [31].

3. The blind cDNA immunoscreening approach

The isolation of cDNA clones via immunoscreening of expression plasmid cDNA libraries using monospecific antisera was first developed in the late 1970s [32] and then further advanced with the development of bacteriophage expression vectors such as lambda-gt11 [33]. The latter facilitated the large-scale screening of cDNA libraries due to the use of high plaque densities. While such approaches can be very effective, it was known that success depended greatly on the quality of the antibody used and the immunoreactivity profile of the encoded protein and that this method was not always successful even when a well-characterized, monospecific polyclonal antisera or monoclonal antibody was available. In the case of NANBH, no viral-specific antibody had been demonstrated in patient sera despite numerous investigations by many laboratories [17]. Aligned with the knowledge that NANBH appeared to develop chronic, persistent disease at a very high rate, possibly indicating a poor immune response to the etiologic agent(s) (as in the case of chronic HBV infection), I had considered the use of this approach early on in the program in 1983 and 1984 but had thought it too risky.

In 1985 however, at which time we were actively working on the seemingly NANBH-specific antibodies obtained by Dr. Shimizu, I discussed the sensitivities of screening for such antibodies with Dr. Kuo who had a laboratory next to mine at Chiron and who was conducting research on human factor 8, as well as contributing to the development of a yeast-derived HBV vaccine at Chiron. The topic of conversation revolved around Dr. Shimizu's immunofluorescence assay methods for identifying such antibodies using target NANBH antigen present in thin microscopic sections of NANBH-infected liver biopsies. Since Dr. Bradley had by this time determined the approximate NANBH infectivity titer of several of his chimpanzee liver samples, it was possible to calculate the approximate average amount of NANBH-specific antigen present per hepatocyte, an amount which Dr. Kuo considered below the detectable limit for such methods. It was Dr. Kuo who then highly recommended immunoscreening bacterial expression cDNA libraries using NANBH patient sera to overcome this limitation

and hence to isolate HCV since theoretically, the greater yield of NANBH antigen per bacterial cell as compared with that present in infected hepatocytes should no longer limit the detection of circulating antibodies specific for the etiologic agents of NANBH (provided of course that such antibodies existed with appropriate affinity and concentration). After further discussions with Dr. Bradley, who also independently suggested this method as a possible approach to isolating HCV and armed now with a possible rationale for the failure of numerous groups to detect circulating antibodies to the NANBH viruses within patient sera (deriving from input from Dr. Kuo), I decided to attempt to apply these methods to the identification of HCV cDNA clones, in parallel with the many other approaches ongoing in my laboratory.

Firstly, Dr. Kuo compared the sensitivities of various detection methods for screening lambda gt11 cDNA clones and concluded that the use of I^{125}-radiolabelled mouse anti-human Ig as a detector antibody was optimal. Next, I made a lambda gt11 cDNA library from poly-A^+ mRNA purified from biopsy samples obtained from four different NANBH-infected chimpanzees provided by Dr. Bradley and Dr. Choo demonstrated that he could detect a rare host mRNA within this library using a characterized monospecific antibody. He then screened this library with a variety of sera samples from many different NANBH-infected chimpanzees and humans, including apparent convalescent individuals who might be expected to exhibit a stronger humoral immune response to try to identify HCV clones but succeeded only in isolating yet more NANBH-associated genes but none that could be proven to be derived from an actual NANBH viral genome itself.

Around this time, Drs. Wang and Choo in my laboratory were involved in characterizing the HDV genome with a view to using it as a possible hybridization probe for HCV, work that included the characterization of the gene encoding the delta antigen. Dr. Weiner had shown that an open-reading-frame in the anti-genome strand actually encoded the delta antigen and then Dr. Wang proceeded to map the B cell epitopes within this antigen. To do this, we decided to clone very small cDNA fragments (encoding small peptides) from infectious HDV plasma into lambda gt11 and then screen with human sera derived from hepatitis delta patients. This experiment worked tremendously well enabling the comprehensive and fine mapping of the delta antigen. By this time, I had received a large quantity of chimpanzee plasma from Dr. Bradley that he had shown possessed a NANBH TFA infectivity titer of at least 10^6 chimpanzee infectious doses per ml [34]. As such, this was equivalent to the best titers obtained earlier by other groups in the field (Ref. [35] for example). Bolstered with the success of the delta antigen mapping study and a knowledge of the relative infectious titers of the HDV and NANBH plasmas, I was encouraged to extend the blind cDNA immunoscreening approach to Dr. Bradley's NANBH chimpanzee plasma. When Dr. Choo screened a lambda gt11 cDNA library that I had prepared from this material, none of the positive clones could be deemed to be derived from the HCV genome. A second lambda gt11 library that I generated using random primers of reverse transcriptase from both RNA and DNA present in the ultra-centrifuged pellet of this chimpanzee plasma suffered from major technical problems due to oily, viscous contaminants in the extract. Uncertain whether to discard this problematic library and to start again, we decided eventually to persevere with it which was then screened by Dr. Choo. This time, we decided to use serum from a NANBH patient exhibiting unusually high serum ALT levels as a putative source of antibodies to the NANBH agent(s). A high ALT is indicative of very active liver disease and we thought it possible that this might be accompanied with higher antibody titers, although we knew at that time that in the case of HBV infection for example, anti-HBsAg antibodies are associated with convalescence rather than active disease (we now know that chronic HCV patients generally do have higher anti-HCV antibody titers than convalescent individuals).

When Dr. Choo screened this library, for which we had but small hope of success due to the technical problems encountered in its production, he identified around six positive clones. Surprisingly, some of these turned out to be derived from the MS2 bacteriophage RNA that I had added to the plasma extract as a carrier for the minute amounts of endogenous nucleic acids present. This was a good illustration of the power of this approach to identify genes that encoded immunoreactive, pathogen-derived antigens (in this case a "virus" of bacteria). Other clones were shown to be derived from host genes encoding proteins apparently eliciting an autoimmune response. Finally, just one small clone remained (containing about 150 base-pairs and termed 5-1-1 by Dr. Choo) for which eventually, we were able to show was truly derived from the genome of HCV (Fig. 2). Ironically, all subsequent cDNA libraries that I made from this same chimpanzee plasma failed to contain HCV sequences illustrating the difficulties of cloning tiny amounts of nucleic acid prior to the widespread availability of PCR techniques.

4. Proof that clone 5-1-1 was derived from the HCV genome

First of all, clone 5-1-1 (and overlapping clones identified from the same cDNA library by routine cross-hybridization techniques) was shown to be incapable of hybridising to genomic DNA extracted from control, uninfected humans and chimpanzees. Secondly, the clones did hybridise to poly-A^+ RNA found only in

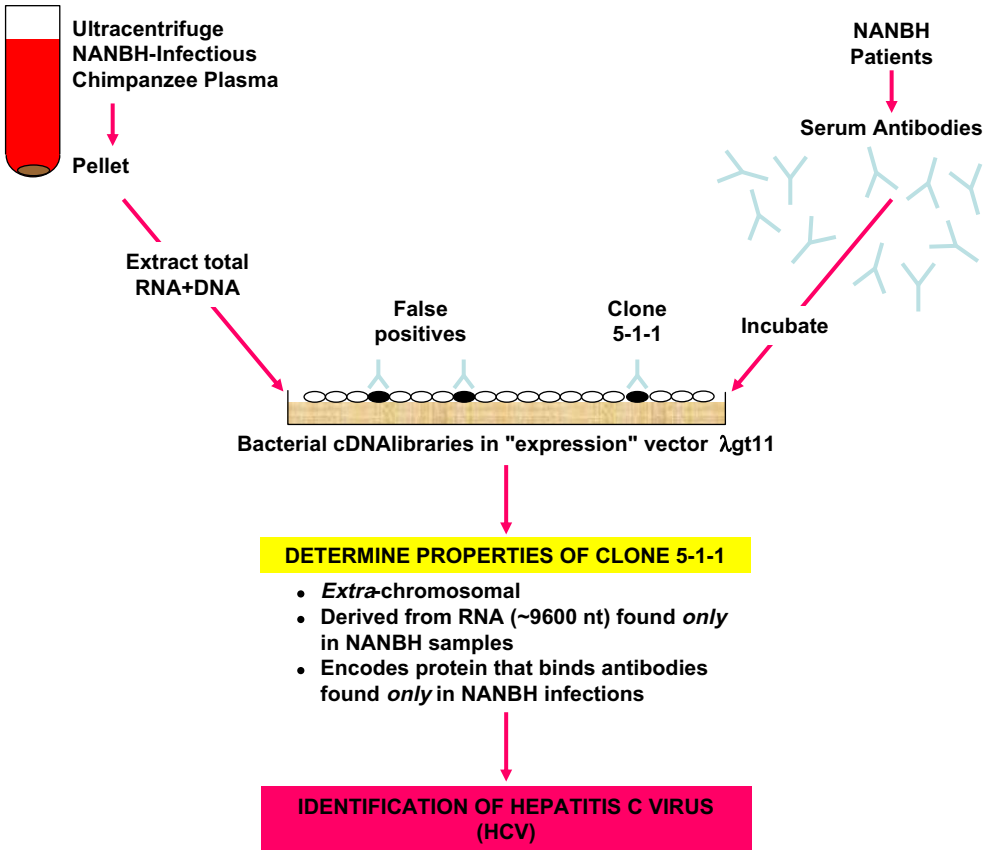

Fig. 2. Schematic of the successful molecular isolation and identification of HCV [36]. Reproduced with permission.

NANBH-infected chimpanzees and not in uninfected control animals. Thirdly, this RNA was found to be large (~10,000 nucleotides in length), single-stranded and to be positive-stranded (i.e. encoded protein). Next, when expressed in bacteria or yeast, the RNA encoded an antigen that bound to circulating antibodies found only in parenterally-transmitted, NANBH-infected chimpanzees and not in HAV- or HBV-infected animals (animal samples provided by Dr. Bradley). Importantly, these NANBH-infected animals seroconverted to anti-5-1-1 after acute NANBH infection (example shown in Fig. 3) and similar seroconversion was observed in a few human transfusion recipients tested (provided by Dr. Gary Tegtmeier) who then went on to develop post-transfusion NANBH. Moreover, when we checked for the presence of anti-5-1-1 in a small collection of chronic NANBH patients (obtained from Dr. Gary Gitnick), we found such antibodies in the majority whereas control, uninfected humans were negative. Finally, the sequence of clone 5-1-1 and overlapping clones displayed distant but significant sequence identity with the positive-stranded flaviviruses (such as dengue and yellow fever viruses). Taken together, this constituted firm evidence that after nearly 6 years of incessant failure, we had finally identified a major etiological agent of parenteral NANBH which we now termed the hepatitis C virus [36]. The testing algorithm for clone 5-1-1 spanned 6 months approximately meaning that the "eureka moment" was in fact a very gradual and extended one.

5. Developments since the identification of HCV

With the ready availability of large amounts of recombinant HCV antigen (encoded by 5-1-1 and overlapping clones), we were now able to produce the first EIA tests for NANBH-specific antibody which quickly showed that HCV was the major cause of parenterally-transmitted NANBH around the world and that these tests could detect and screen out the majority of infectious blood donors from the blood supply. A blinded panel of infectious and control blood samples prepared by Dr. Harvey Alter had hitherto not been successfully decoded by any putative NANBH test until Dr. Kuo assayed this panel with our tests [37,38].

Resuming the collaboration with Dr. Tatuso Miyamura's group in Japan, it quickly became clear that Japanese strains of HCV had significant sequence divergence from our USA-derived strain [39]. We now know that there is considerable sequence diversity among the so-called hepaciviruses with at least six major genotypes identified and hundreds of different sub-types [40]. The

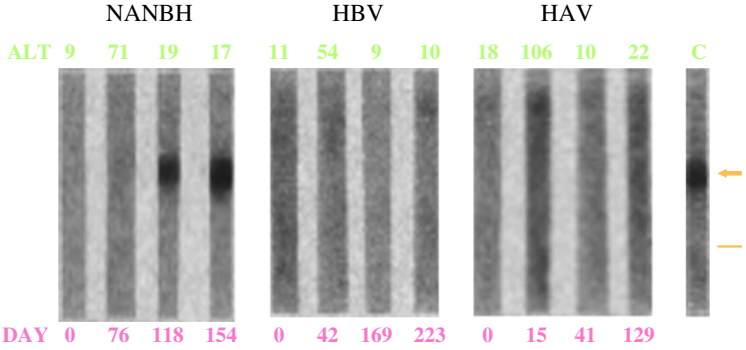

Fig. 3. Seroconversion to anti-5-1-1 observed after acute NANBH infection of chimpanzees [36]. Reproduced with permission.

hepaciviruses were later classified as their own genus within the *flaviviridae* family and are very distant relatives of the flavivirus genus and the pestivirus genus [41]. The realization of the diversity of HCV has important clinical implications since while 80–90% of patients infected with genotypes 2 and 3 are curable with standard-of-care therapy (comprising a combination of pegylated interferon-alpha plus ribavirin), only around 40–50% of genotype 1 patients are cured by such treatment [42]. Unfortunately, genotype 1 is the most common genotype around the world [43] and we still do not know the reason for its incomplete treatment response. This remains an urgent research priority for the future.

Identification of the complete sequence of the HCV genome allowed the genetic organization to be elucidated [44,45] and the current knowledge of this is summarized in Fig. 4. It immediately became apparent to us from sequence comparisons with other RNA viruses that HCV encodes a conserved serine-like protease domain, a helicase domain and a RNA polymerase domain [44]. Later work from our and other laboratories confirmed the existence of these enzyme activities which have been the foci of intensive drug development and clinical testing work for the last 1–2 decades [45–50]. It is likely that patients will have the benefit of new drugs targeting some of these enzymes within the next few years. A key development to facilitate drug testing and screening as well as basic research was the isolation of human hepatoma cell-lines that could replicate part or all of the HCV genome by the laboratory of Dr. Bartenschlager [51] and shortly afterwards, by that of Dr. Rice [52]. Many years later, Dr. Wakita and colleagues became the first group to produce infectious virus in cell culture [27].

Various laboratories have elegantly demonstrated the existence of an additional protease within NS2 [47,53] which is required for viral production [54], the formation of a critical ion channel by p7 [55,56] that is also needed for the production of virus [54,57], a key role for NS5a in HCV virion assembly and production [58] and an interaction between NS4b and the viral RNA genome [59]. A growing understanding of the role of a variety of host proteins in viral replication has also emerged [60] some of which are now anti-viral targets (for example cyclophilin which is involved in replicating the viral RNA genome [61,62]). The requirement for at least four different host proteins (CD81 [63,64], scavenger receptor B1 [65], Claudin-1 [66] and occludin [67]) for entry of HCV into the hepatocyte has also been elucidated. These and other properties of the virus are opening up many new therapeutic avenues for the estimated 170 million HCV carriers world-wide. In addition, a potentially important role for the engagement of CD81 by HCV has been recognized in extra-hepatic manifestations of HCV-associated diseases such as non-Hodgkins B cell lymphoma and essential mixed cryoglobulinemia [68,69].

Prospects for vaccinating against HCV were discouraging initially with reports of re-infection in humans and chimpanzees [70,71]. However, we and others have now provided evidence for the existence of natural immunity to HCV which although not as robust as in the case of HAV and HBV, signifies none-the-less that a partially protective HCV vaccine is feasible [72,73]. Indeed, in the chimpanzee model we have shown that using adjuvanted recombinant envelope glycoproteins gpE1 and gpE2, it is possible to protect the majority (~80%) of vaccinated chimpanzees against the development of chronic infection following experimental challenge with either homologous or heterologous 1a strains (note that 1a is the most common sub-type within the USA [74,75]). Further encouragement for this approach has been provided by animal studies demonstrating that this vaccine can cross-neutralize HIV and lentiviral pseudotypes displaying HCV envelope glycoproteins gpE1 and gpE2 derived from multiple HCV genotypes [76]. Also, a T cell DNA vaccine that elicits T helper and cytotoxic T cell responses to 1b NS3, 4 and 5 proteins appears able to protect vaccinated chimpanzees against experimental challenge with a heterologous 1a HCV strain [77]. It remains very important to translate this work in the future into an affordable and global HCV vaccine since in the USA alone, there are an estimated 25,000 new HCV infections every year, a level which must be orders of magnitude higher in the

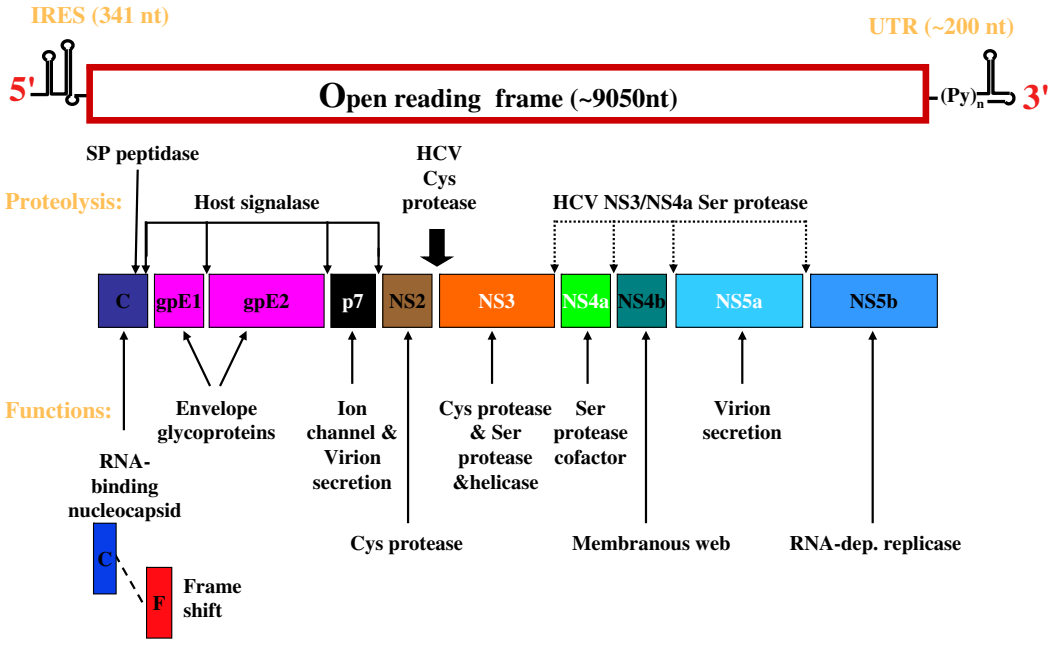

Fig. 4. The organization and function of the HCV genome and encoded proteins [88]. Reproduced with permission.

developing world [78]. Given that up to 20% of HCV infections may eventually lead to liver cirrhosis with associated risks of hepatocellular carcinoma and end-stage liver disease, this remains a most urgent goal [12–15,79].

Finally, one is left with admiration for the evolutionary process that has produced such a highly-adapted RNA virus that even in the absence of an ability to integrate into the host genome, can persist for the lifetime of the host in the majority of infections. The mechanisms underlying this persistence represent some of the most intriguing and challenging questions in the field and when completely understood, will open up yet more therapeutic avenues. Elegant work showing that HCV inhibits the NK cell response and the induction of type 1 interferon demonstrates the virus's effectiveness at inhibiting the rapid, innate immune response to HCV infection [80–82]. The ability of this highly mutable virus to evolve away from specific cellular immune responses has been elegantly documented [83] as has the existence of infectious virus even in the presence of an excess of antibody-mediated, immune-complexed virus (possibly via escape mutations and/or masking [84]). It also appears likely that direct cell: cell transfer of virus contributes to immune-evasion and viral persistence [85,86] and the ability of this hepatotropic virus to present viral antigen in the liver in such a way so as to promote immune anergy could be another potential contributor to viral persistence [87].

6. Note added in proof

A set of single nucleotide polymorphisms around and within the interferon-lambda 3 gene has just been highly correlated with treatment response (Ge et al., Nature Aug 2009).

Acknowledgements

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References

[1] Krugman S, Ward R, Giles JP. The natural history of infectious hepatitis. Am J Med 1962;32:717–728.
[2] Feinstone SM, Kapikian AZ, Purcell RH. Hepatitis A: detection by immune electron microscopy of a viruslike antigen associated with acute illness. Science 1973;182:1026–1028.

[3] Bayer ME, Blumberg BS, Werner B. Particles associated with Australia antigen in the sera of patients with leukaemia, Down's Syndrome and hepatitis. Nature 1968;218:1057–1059.
[4] Feinstone SM, Kapikian AZ, Purcell RH, Alter HJ, Holland PV. Transfusion-associated hepatitis not due to viral hepatitis type A or B. N Engl J Med 1975;292:767–770.
[5] Alter HJ, Purcell RH, Holland PV, Popper H. Transmissible agent in non-A, non-B hepatitis. Lancet 1978;1:459–463.
[6] Hollinger FB, Gitnick GL, Aach RD, Szmuness W, Mosley JW, Stevens CE, et al. Non-A, non-B hepatitis transmission in chimpanzees: a project of the transfusion-transmitted viruses study group. Intervirology 1978;10:60–68.
[7] Shimizu YK, Feinstone SM, Purcell RH, Alter HJ, London WT. Non-A, non-B hepatitis: ultrastructural evidence for two agents in experimentally infected chimpanzees. Science 1979;205:197–200.
[8] Bradley DW, Maynard JE, Popper H, Cook EH, Ebert JW, McCaustland KA, et al. Posttransfusion non-A, non-B hepatitis: physicochemical properties of two distinct agents. J Infect Dis 1983;148:254–265.
[9] Feinstone SM, Mihalik KB, Kamimura T, Alter HJ, London WT, Purcell RH. Inactivation of hepatitis B virus and non-A, non-B hepatitis by chloroform. Infect Immun 1983;41:816–821.
[10] Bradley DW. The agents of non-A, non-B viral hepatitis. J Virol Methods 1985;10:307–319.
[11] He LF, Alling D, Popkin T, Shapiro M, Alter HJ, Purcell RH. Determining the size of non-A, non-B hepatitis virus by filtration. J Infect Dis 1987;156:636–640.
[12] Dienstag JL, Alter HJ. Non-A, non-B hepatitis: evolving epidemiologic and clinical perspective. Semin Liver Dis 1986;6:67–68.
[13] Seeff LB, Dienstag JL. Transfusion-associated non-A, non-B hepatitis. Where do we go from here? Gastroenterology 1988;95:530–533.
[14] Alter HJ. The dominant role of non-A, non-B in the pathogenesis of post-transfusion hepatitis: a clinical assessment. Clin Gastroenterol 1980;9:155–170.
[15] Hollinger FB, Mosley JW, Szmuness W, Aach RD, Peters RL, Stevens C. Transfusion-transmitted viruses study: experimental evidence for two non-A, non-B, hepatitis agents. J Infect Dis 1980;142:400–407.
[16] Alter MJ, Hadler SC, Francis DP, Maynard JE. The epidemiology of non-A, non-B hepatitis in the United States. Prog Clin Biol Res 1985;182:71–719.
[17] Shih JW, Mur JI, Alter HJ. Non-A, non-B hepatitis: advances and unfulfilled expectations of the first decade. Prog Liver Dis 1986;8:433–452.
[18] Houghton M, Stewart AG, Doel SM, Emtage JS, Eaton MA, Smith JC, et al. The amino-terminal sequence of human fibroblast interferon as deduced from reverse transcripts obtained using synthetic oligonucleotide primers. Nucleic Acids Res 1980;8:1913–1931.
[19] Houghton M, Jackson IJ, Porter AG, Doel SM, Catlin GH, Barber C, et al. The absence of introns within a human fibroblast interferon gene. Nucleic Acids Res 1981;9:247–266.
[20] Rizzetto M, Canese MG, Aricò S, Crivelli O, Trepo C, Bonino F, et al. Immunofluorescence detection of new antigen-antibody system (delta/anti-delta) associated to hepatitis B virus in liver and in serum of HBsAg carriers. Gut 1977;12:997–1003.
[21] Rizzetto M, Verme G. Delta hepatitis–present status. J Hepatol 1985;1:187–193.
[22] Denniston KJ, Hoyer BH, Smedile A, Wells FV, Nelson J, Gerin JL. Cloned fragment of the hepatitis delta virus RNA genome: sequence and diagnostic application. Science 1986;232:873–875.
[23] Kamimura T, Ponzetto A, Bonino F, Feinstone SM, Gerin JL, Purcell RH. Cytoplasmic tubular structures in liver of HBsAg carrier chimpanzees infected with delta agent and comparison with cytoplasmic structures in non-A, non-B hepatitis. Hepatology 1983;3:631–637.
[24] Wang KS, Choo QL, Weiner AJ, Ou JH, Najarian RC, Thayer RM, et al. Structure, sequence and expression of the hepatitis delta (delta) viral genome. Nature 1986;323:508–514, [Erratum in: Nature 1987;5:328, 456].
[25] Weiner AJ, Choo QL, Wang KS, Govindarajan S, Redeker AG, Gerin JL, et al. A single antigenomic open reading frame of the hepatitis delta virus encodes the epitope(s) of both hepatitis delta antigen polypeptides p24 delta and p27 delta. J Virol 1988;62:594–599.
[26] Weiner AJ, Wang KS, Choo QL, Gerin JL, Bradley DW, Houghton M. Hepatitis delta (delta) cDNA clones: undetectable hybridization to nucleic acids from infectious non-A, non-B hepatitis materials and hepatitis B DNA. J Med Virol 1987;21:239–247.
[27] Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11 (7):791–796, [Epub 2005 Jun 12. Erratum in: Nat Med 2005;11:905].
[28] Seto B, Coleman Jr WG, Iwarson S, Gerety RJ. Detection of reverse transcriptase activity in association with the non-A, non-B hepatitis agent(s). Lancet 1984;8409:941–943.
[29] Prince AM, Huima T, Williams BA, Bardina L, Brotman B. Isolation of a virus from chimpanzee liver cell cultures inoculated with sera containing the agent of non-A, non-B hepatitis. Lancet 1984;8411:1071–1075.
[30] Shimizu YK, Oomura M, Abe K, Uno M, Yamada E, Ono Y, et al. Production of antibody associated with non-A, non-B hepatitis in a chimpanzee lymphoblastoid cell line established by in vitro transformation with Epstein–Barr virus. Proc Natl Acad Sci USA 1985;82:2138–2142.
[31] Maeda T, Honda Y, Hanawa M, Yamada E, Ono Y, Shikata T, et al. Production of antibodies directed against microtubular aggregates in hepatocytes of chimpanzees with non-A, non-B hepatitis. J Gen Virol 1989;70 (Pt. 6):1401–1407.
[32] Broome S, Gilbert W. Immunological screening method to detect specific translation products. Proc Natl Acad Sci USA 1978;75:2746–2749.
[33] Young RA, Davis RW. Efficient isolation of genes by using antibody probes. Proc Natl Acad Sci USA 1983;80:1194–1198.
[34] Bradley DW. Hepatitis non-A, non-B viruses become identified as hepatitis C and E viruses. Prog Med Virol 1990;37:101–135.
[35] Feinstone SM, Alter HJ, Dienes HP, Shimizu Y, Popper H, Blackmore D, et al. Non-A, non-B hepatitis in chimpanzees and marmosets. J Infect Dis 1981;144:588–598.
[36] Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood–borne non-A, non-B viral hepatitis genome. Science 1989;244:359–362.
[37] Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, et al. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 1989;244:362–364.
[38] Miyamura T, Saito I, Katayama T, Kikuchi S, Tateda A, Houghton M, et al. Detection of antibody against antigen expressed by molecularly cloned hepatitis C virus cDNA: application to diagnosis and blood screening for posttransfusion hepatitis. Proc Natl Acad Sci USA 1990;87:983–987.
[39] Kubo Y, Takeuchi K, Boonmar S, Katayama T, Choo QL, Kuo G, et al. A cDNA fragment of hepatitis C virus isolated from an implicated donor of post-transfusion non-A, non-B hepatitis in Japan. Nucleic Acids Res 1989;17:1367–1372.
[40] Simmonds P. Variability of hepatitis C virus. Hepatology 1995;21:570–583.
[41] Robertson B, Myers G, Howard C, Brettin T, Bukh J, Gaschen B, et al. Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. International Committee on Virus Taxonomy. Arch Virol 1998;143:2493–2503.

[42] Patel K, McHutchison JG. Initial treatment for chronic hepatitis C: current therapies and their optimal dosing and duration. Cleve Clin J Med 2004;71 (Suppl. 3):S8–S12.
[43] Bukh J, Purcell RH, Miller RH. Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc Natl Acad Sci USA 1994;91:8239–8243.
[44] Choo QL, Richman KH, Han JH, Berger K, Lee C, Dong C, et al. Genetic organization and diversity of the hepatitis C virus. Proc Natl Acad Sci USA 1991;88:2451–2455.
[45] Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA 1990;87:9524–9528.
[46] Grakoui A, McCourt DW, Wychowski C, Feinstone SM, Rice CM. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. J Virol 1993;67:2832–2843.
[47] Hijikata M, Mizushima H, Akagi T, Mori S, Kakiuchi N, Kato N, et al. Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. J Virol 1993;67:4665–4675.
[48] Eckart MR, Selby M, Masiarz F, Lee C, Berger K, Crawford K, et al. The hepatitis C virus encodes a serine protease involved in processing of the putative nonstructural proteins from the viral polyprotein precursor. Biochem Biophys Res Commun 1993;192:399–406.
[49] Suzich JA, Tamura JK, Palmer-Hill F, Warrener P, Grakoui A, Rice CM, et al. Hepatitis C virus NS3 protein polynucleotide-stimulated nucleoside triphosphatase and comparison with the related pestivirus and flavivirus enzymes. J Virol 1993;67:6152–6158.
[50] Lohmann V, Körner F, Herian U, Bartenschlager R. Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. J Virol 1997;71:8416–8428.
[51] Lohmann V, Körner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110–113.
[52] Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. Science 2000;290:1972–1974.
[53] Grakoui A, McCourt DW, Wychowski C, Feinstone SM, Rice CM. A second hepatitis C virus-encoded proteinase. Proc Natl Acad Sci USA 1993;90:10583–10587.
[54] Jones CT, Murray CL, Eastman DK, Tassello J, Rice CM. Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus. J Virol 2007;81:8374–8383.
[55] Griffin SD, Beales LP, Clarke DS, Worsfold O, Evans SD, Jaeger J, et al. The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. FEBS Lett 2003;535:34–38.
[56] Luik P, Chew C, Aittoniemi J, Chang J, Wentworth Jr P, Dwek RA, et al. The 3-dimensional structure of a hepatitis C virus p7 ion channel by electron microscopy. Proc Natl Acad Sci USA 2009;106:12567–12568.
[57] Steinmann E, Penin F, Kallis S, Patel AH, Bartenschlager R, Pietschmann T. Hepatitis C virus p7 protein is crucial for assembly and release of infectious virions. PLoS Pathog 2007;7:e103.
[58] Tellinghuisen TL, Foss KL, Treadaway J. Regulation of hepatitis C virion production via phosphorylation of the NS5A protein. PLoS Pathog. 2008;4:e1000032.
[59] Einav S, Gerber D, Bryson PD, Sklan EH, Elazar M, Maerkl SJ, et al. Discovery of a hepatitis C target and its pharmacological inhibitors by microfluidic affinity analysis. Nat Biotechnol 2008;9:1019–1027.
[60] Randall G, Panis M, Cooper JD, Tellinghuisen TL, Sukhodolets KE, Pfeffer S, et al. Cellular cofactors affecting hepatitis C virus infection and replication. Proc Natl Acad Sci USA 2007;104:12884–12889.
[61] Watashi K, Ishii N, Hijikata M, Inoue D, Murata T, Miyanari Y, et al. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. Mol Cell 2005;19:111–122.
[62] Nakagawa M, Sakamoto N, Tanabe Y, Koyama T, Itsui Y, Takeda Y, et al. Suppression of hepatitis C virus replication by cyclosporin a is mediated by blockade of cyclophilins. Gastroenterology 2005;129:1031–1041.
[63] Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, et al. Binding of hepatitis C virus to CD81. Science 1998;5390:938–941.
[64] McKeating JA, Zhang LQ, Logvinoff C, Flint M, Zhang J, Yu J, et al. Diverse hepatitis C virus glycoproteins mediate viral infection in a CD81-dependent manner. J Virol 2004;78:8496–8505.
[65] Zeisel MB, Koutsoudakis G, Schnober EK, Haberstroh A, Blum HE, Cosset FL, et al. Scavenger receptor class B type I is a key host factor for hepatitis C virus infection required for an entry step closely linked to CD81. Hepatology 2007;46:1722–1731.
[66] Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wölk B, et al. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 2007;446:801–805.
[67] Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, de Jong YP, et al. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature 2009;457:882–886.
[68] Rosa D, Saletti G, De Gregorio E, Zorat F, Comar C, D'Oro U, et al. Activation of na B lymphocytes via CD81, a pathogenetic mechanism for hepatitis C virus-associated B lymphocyte disorders. Proc Natl Acad Sci USA 2005;102:18544–185449.
[69] Quinn ER, Chan CH, Hadlock KG, Foung SK, Flint M, Levy S. The B-cell receptor of a hepatitis C virus (HCV)-associated non-Hodgkin lymphoma binds the viral E2 envelope protein, implicating HCV in lymphomagenesis. Blood 2001;98:3745–3749.
[70] Lai ME, Mazzoleni AP, Argiolu F, De Virgilis S, Balestrieri A, Purcell RH, et al. Hepatitis C virus in multiple episodes of acute hepatitis in polytransfused thalassaemic children. Lancet 1994;343:388–390.
[71] Prince AM, Brotman B, Huima T, Pascual D, Jaffery M, Inchauspé G. Immunity in hepatitis C infection. J Infect Dis 1992;165:438–443.
[72] Weiner AJ, Paliard X, Selby MJ, Medina-Selby A, Coit D, Nguyen S, et al. Intrahepatic genetic inoculation of hepatitis C virus RNA confers cross-protective immunity. J Virol 2001;75:7142–7148.
[73] Bassett SE, Guerra B, Brasky K, Miskovsky E, Houghton M, Klimpel GR, et al. Protective immune response to hepatitis C virus in chimpanzees rechallenged following clearance of primary infection. Hepatology 2001;33:1479–1487.
[74] Choo QL, Kuo G, Ralston R, Weiner A, Chien D, Van Nest G, et al. Vaccination of chimpanzees against infection by the hepatitis C virus. Proc Natl Acad Sci USA 1994;91:1294–1298.
[75] Houghton M, Abrignani S. Prospects for a vaccine against the hepatitis C virus. Nature 2005;436:961–966.
[76] Stamataki Z, Coates S, Evans MJ, Wininger M, Crawford K, Dong C, et al. Hepatitis C virus envelope glycoprotein immunization of rodents elicits cross-reactive neutralizing antibodies. Vaccine 2007:257773–257784.
[77] Folgori A, Capone S, Ruggeri L, Meola A, Sporeno E, Ercole BB, et al. A T-cell HCV vaccine eliciting effective immunity against heterologous virus challenge in chimpanzees. Nat Med 2006:12190–12197.
[78] Alter MJ. Epidemiology of hepatitis C virus infection. World J Gastroenterol 2007;13:2436–2441.
[79] Umemura T, Ichijo T, Yoshizawa K, Tanaka E, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. J Gastroenterol. 2009;44:102–107.

[80] Crotta S, Stilla A, Wack A, D'Andrea A, Nuti S, D'Oro U, et al. Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. J Exp Med 2002;195:35–41.
[81] Tseng C, Klimpel G. Binding of the hepatitis C virus envelope protein E2 to CD81 inhibits natural killer functions. J Exp Med 2002;195:43–49.
[82] Loo YM, Gale Jr M. Viral regulation and evasion of the host response. Curr Top Microbiol Immunol 2007;316:295–313.
[83] Bowen DG, Walker CM. Mutational escape from CD8+ T cell immunity: HCV evolution, from chimpanzees to man. J Exp Med 2005;201:1709–1714.
[84] Hijikata M, Shimizu YK, Kato H, Iwamoto A, Shih JW, Alter HJ, et al. Equilibrium centrifugation studies of hepatitis C virus: evidence for circulating immune complexes. J Virol 1993;67:1953–1958.
[85] Timpe JM, Stamataki Z, Jennings A, Hu K, Farquhar MJ, Harris HJ, et al. Hepatitis C virus cell–cell transmission in hepatoma cells in the presence of neutralizing antibodies. Hepatology 2008;47:17–24.
[86] Witteveldt J, Evans MJ, Bitzegeio J, Koutsoudakis G, Owsianka AM, Angus AG, et al. CD81 is dispensable for hepatitis C virus cell-to-cell transmission in hepatoma cells. J Gen Virol 2009;90:48–58.
[87] Crispe N. Mechanisms of self-tolerance. Immunol Today 1988;9:329–331.
[88] Moradpour D, Penin F, Rice CM. Replication of hepatitis C virus. Nat Rev Microbiol 2007;5:453–463.