

## CHAPTER II

### REVIEW AND RELATED LITERATURES

#### Hepatitis C virus

Hepatitis C was first recognized as a distinct form of liver disease in the mid-1970s with the advent of diagnostic tests for hepatitis A and B virus infections<sup>(23-24)</sup>. The etiologic agent of hepatitis C was proposed to be a small, enveloped virus based on demonstrations of its transmissibility to chimpanzees<sup>(25-26)</sup>, small size (<80 nm)<sup>(27)</sup> and sensitivity to chloroform<sup>(28)</sup>. The genome of hepatitis C virus (HCV) was first cloned in 1989 by screening a  $\lambda$ gt11 cDNA expression library, derived from the plasma of a persistently infected chimpanzee, with hepatitis C virus patient serum<sup>(29)</sup>. Hybridization and nuclease digestion experiments indicated that the HCV genome consists of a single-stranded, positive sense, RNA molecule<sup>(29)</sup>.

Analyses of the cloned sequences revealed that HCV is related to members of the family *Flaviviridae*<sup>(30)</sup>. All of viruses in this family have a small enveloped virions and positive-sense RNA genomes that are translated as single, long polyproteins, with the structural proteins grouped together in the NH<sub>2</sub>-terminal portion, followed by the nonstructural proteins. Their polyproteins are processed into individual viral proteins by a combination of host and viral proteases, including host signalase and a viral serine protease located in nonstructural protein 3 (NS3). Amino acid similarity among these viruses is limited to the serine protease and nucleotide triphosphatase (NTPase) domains of NS3 and the NS5/5B polymerase domain<sup>(30)</sup>. Together, these similarities have since led to the classification of HCV in a separate genus (*Hepacivirus*) of the family *Flaviviridae*, which includes two other genera, *Flavivirus* and *Pestivirus*<sup>(31)</sup>.

These viruses have an enveloped particle harbouring a plus-strand RNA that has a length of 9600 nucleotides. The genome carries a single long open reading frame (ORF) encoding a polyprotein that is proteolytically cleaved into a set of distinct products (Figure 1)<sup>(32-33)</sup>. The HCV polyprotein is cleaved co- and post-translationally by cellular and viral proteinases into 10 different products, with the structural proteins

located in the amino-terminal one-third and the nonstructural replicative proteins in the remainder. The detail of each product is indicated below <sup>(5)</sup>.

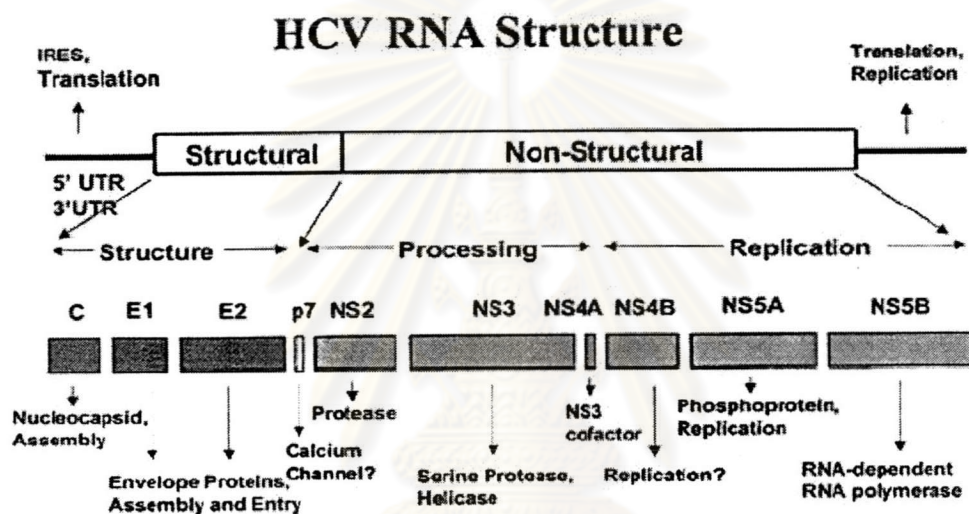


Figure 1 Structure of the RNA genome of hepatitis C virus. The functional regions of the genome are shown beneath the cartoon of the organization of the viral RNA. The 10 polypeptides that are cleaved from the polyprotein are shown, as well as their suspected functions. IRES, internal ribosomal entry site; UTR, untranslated region; C, nucleocapsid core; E1, envelope protein 1; E2, envelope protein 2; NS, non-structural. <sup>(33)</sup>

## 5' Untranslated region (5'UTR)

An obvious characteristic of HCV is the presence of a long untranslated region (UTR) at the 5' end of the genome and detailed molecular analysis indicates that polyprotein synthesis is initiated at nucleotide 342<sup>(34)</sup>. The highly conserved 5' UTR is 341-344 nucleotides long. Several stem-loop structures in this region contribute to an internal ribosome-binding site (IRES) that mediates the CAP-independent translation of the viral RNA. Because of the high degree of sequence conservation and the crucial role plays in the translation of the viral polyprotein, the 5' UTR has become a target for the development of nucleic acid-based antiviral agents, such as antisense oligonucleotides and ribozymes<sup>(35)</sup>.

In fact, this region is the most conserved region of the whole genome, a characteristic which has allowed it to be used as a diagnostic marker for HCV by RT-PCR<sup>(36)</sup>.

## Virus-encoded proteins

### Structural proteins

#### 1. Core protein

The protein located at the amino terminus of the polyprotein is highly basic in nature and is considered likely to be the viral capsid protein. It is released from the viral polyprotein by nascent proteolytic cleavage at amino acid 191 by host proteases. The full-length protein, known as P21, has been identified by both *in vitro* and *in vivo* expression but a second species (P19) generated by a secondary cleavage at amino acid 173 is the major product observed following expression in mammalian cells. Both P21 and P19 are located in the endoplasmic reticulum (ER) membrane and the conversion of P21 to P19 is presumably mediated by membrane-associated cellular enzymes. A third collinear species of core which can also be detected in expression studies is approximately 151 amino acids long (P16) and appears to be localized in the nucleus and more specifically in the nucleolus. Apart from its highly basic nature, the core protein also processes a number of distinct hydrophobic regions (residues 121-151, 170-191) which are involved in the association of P21 and P19 with the ER. It is likely that the carboxy-terminal hydrophobic region (170-191) plays a critical role in the translocation of the viral structural glycoproteins into the ER where the enzymes

responsible for peptide processing and glycosylation are located. The biological functions of the core found in the nucleus, if this also occurs in the natural virus replication, are still unclear. Several studies have reported the suppression, by core protein in transcription of several host genes as well as interference in expression of co-infecting genomes of hepatitis B and human immunodeficiency viruses. In addition core can specifically suppress apoptotic cell death in artificial systems and also specifically interact with the cytoplasmic tail of the lymphotoxin- $\beta$  receptor (LTR), a member of the tumour necrosis factor family. Since LTR is known to be involved in apoptotic signalling this strongly suggests that core protein may have an immunomodulatory function and play a critical role in the establishment of persistence and in disease pathogenesis <sup>(37)</sup>.

## 2. Enveloped glycoproteins, E1 and E2

The major viral structural proteins are the glycoproteins E1 and E2, which are released from the viral polyprotein by the action of host-cell signal peptidases. Analysis of the amino termini of both E1 (gp35) and E2 (gp70) indicates that they are cleaved at amino acids 383 and 746 respectively. Both proteins are heavily glycosylated with 5/6 and 11 *N*-linked glycosylation sites respectively and E2 is sometimes found extended at its carboxy terminus to include a smaller protein known as p7 as observed after expression in eukaryotic system. Several studies have shown convincing evidence that E1 and E2 form complexes which are mainly non-covalently associated but in some lysates disulphide-linked complexes have also been reported. The efficient folding and assembly of the heterodimeric complexes has been shown to occur within the ER and is dependent on an initial prolonged association with the chaperone calnexin. These complexes then slowly mature into non-calnexin bound complexes which may represent pre-budding native heterodimers <sup>(37)</sup>. The E1-E2 heterodimer is believed to represent the functional subunit embedded in the lipid envelope of the virion <sup>(35)</sup>.

E2 represents the most variable region of the HCV genome. Comparison of the HCV sequences has shown 2 hypervariable regions within E2, called HVR1 (amino acids 390-410) and HVR2 (amino acids 474-480) <sup>(35)</sup>. The HVR1 region has been suggested to be particularly important in HCV neutralization because of its extreme variability and the fact that this variability was not observed in a patient with agammaglobulinemia even over a period of 2-5 years <sup>(37)</sup>. Mutations in HVR1 occur

during the course of HCV infection in individual case. The high mutation rate associated with this region is believed to be the result of selective pressure by the host immune system. It is currently believed that E2 may elicit production of virus-neutralizing antibodies, resulting in immune selection of HCV genes with escape mutations in HVR1. The development of HCV-neutralizing antibodies is thus possible, but the extremely high degree of variability tolerated by the virus in the HVRs which apparently contains one or more epitopes that are crucial for neutralization enables the selection and immune escape of viruses that may initially represent only a small fraction of the viral population. Interestingly, a cellular protein that can bind E2 has recently been identified<sup>(38)</sup>. This protein is CD81, a member of the tetraspanin family. CD81 is expressed on the surface of several cell types, including lymphocytes and hepatocytes, and is currently believed to be a co-receptor. Antibodies that neutralize infection by HCV appear to do so by preventing E2 binding to CD81<sup>(35)</sup>. In addition, E2 was shown to interact with the IFN-induced double-stranded RNA-activated protein kinase PKR. Upon induction by IFN- $\alpha$ , this enzyme reduces protein synthesis via phosphorylation of translation initiation factor eIF2- $\alpha$ , but in cells containing E2, PKR is inhibited, allowing continuation of translation in the presence of IFN<sup>(5)</sup>.

## **Non-structural proteins**

### **1. NS2**

The NS2 protein is a hydrophobic 23 kDa transmembrane protein with its carboxy terminus translocated into the lumen of the ER while its amino terminus lies in the cytosol<sup>(37)</sup>. It is released from the polyprotein precursor by two proteolytic cleavages. The N-terminus of this protein is separated from the E2/p7 polypeptide by a cleavage thought to be mediated by host signal peptidases, whereas cleavage at the NS2/NS3 junction is mediated by a virus-encoded proteinase composed of NS2 and the serine proteinase domain of NS3, the so-called NS2/3 proteinase. The catalytic activity of NS3 serine proteinase is not required for NS2/NS3 cleavage. The HCV NS2-NS3 proteinase was originally thought to be a metalloproteinase based on the observation that its enzymatic activity was stimulated by zinc and inhibited by metal chelators such as EDTA. Site-directed mutagenesis studies have shown that amino acids His-952 and Cys-993 are important for proteolytic activity<sup>(35)</sup>.

## 2. NS3

The NS3 protein has been shown in numerous studies to be a protein of approximately 70 kDa in size and to possess several diverse biochemical functions. The NS3 protease was entirely responsible for proteolytic processing of the whole downstream region of the viral polyprotein and the protease activity resided completely in the amino-terminal one-third of the NS3 molecule. (Figure 1) The NS3 protease mediates proteolysis at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions to release the mature NS3, NS4A, NS4B, NS5A and NS5B proteins. It was observed that cleavage of NS3 from NS4A was a spontaneous rapid autocatalytic event which could only be mediated *in cis* whereas all other cleavages could be carried out *in trans*. Furthermore, efficient cleavage at the *trans* sites was dependent on the presence of the NS4A protein itself as a cofactor for NS3<sup>(37)</sup>.

Analysis of the remainder of the sequence of the NS3 protein also revealed the presence of other motifs characteristic of NTPase and RNA helicase enzymatic functions. The purified enzyme was shown to actively bind RNA substrates with a minimal RNA binding size of between 7 and 20 nucleotides and to unwind both RNA/RNA, RNA/DNA and DNA/DNA heteroduplexes.

Despite the fact that there are three known catalytic activities in the NS3 protein there is no evidence to suggest that the two domains are separated by proteolysis *in vivo*. This could mean that there is a functional interdependence between the helicase and protease functions. Since these enzymes are likely to be components of a complex replication structure, it is reasonable to assume that the interplay between the various domains of NS3, as well as other viral proteins, may be critical in regulating virus replication.

Apart from the reported biochemical functions of the NS3 protein, there are also a number of intriguing reports on the possible role of NS3 in disease development. The NS3 protein is able to specifically interact with the catalytic subunit of protein kinase A (PKA). PKA is involved in intracellular signal transduction processes and so interference by NS3 in these pathways would be expected to have a dramatic effect on normal cellular functions and would be closely associated with pathogenic mechanisms<sup>(37)</sup>.

### 3. NS4

The NS4 region of the polyprotein comprises two proteins, namely NS4A and NS4B. Both of these are released from the viral polyprotein by the NS3 serine protease by *cis* cleavage at the NS3/NS4A and *trans* cleavage at the NS4A/NS4B and NS4B/NS5A junctions. NS4A is a small protein, approximately 8 kDa in size, and appears to have diverse functions such as anchorage of replication complexes and as a cofactor for the NS3 protease. Currently, there is no ascribed function for the NS4B protein but it is likely that it plays an integral role within HCV replication complexes<sup>(37)</sup>.

### 4. NS5

The NS5 region of polyprotein is composed of two major proteins, NS5A (p56 or p58) and NS5B (p65), which are released as mature products by the action of the NS3 protease in conjunction with NS4A<sup>(37)</sup>.

Both forms of NS5A are phosphorylated at serine residues and phosphorylation occurs after the mature NS5A protein is released from the polyprotein. Basal phosphorylation has been shown to occur in two regions (2,200-2,250 and the carboxy terminus of NS5A) and is independent of the presence of any other viral proteins. In contrast, hyperphosphorylation is extremely dependent on the presence of serine residues 2,197, 2,201 and 2,204 (p58) and is enhanced by the presence of NS4A as a cofactor<sup>(37)</sup>.

Although the function of NS5A in viral replication is unknown, several pieces of evidence suggest that NS5A might be involved in the resistance of HCV to IFN- $\alpha$ . Comparison of full-length sequences of IFN- $\alpha$ -sensitive and IFN- $\alpha$ -resistant HCV isolates revealed clusters of amino acid differences in the NS5A region between amino acids 2,209 and 2,248, suggesting a correlation between the response to IFN- $\alpha$  treatment and mutations in a discrete region of NS5A. This region was thereafter called the "interferon sensitivity determining region" or ISDR<sup>(35)</sup>. Subsequent analysis indicated that the likely mechanism by which this occurred was through a direct interaction of NS5A with the IFN-induced protein kinase, PKR, a mediator of IFN-induced antiviral resistance. Activated by viral double-stranded RNA, PKR phosphorylates the  $\alpha$ -subunit of eukaryotic translation initiation factor 2, resulting in inhibition of protein synthesis and death of the infected cell. According to this study, HCV NS5A blocks the kinase activity

via protein-protein interactions by interfering with PKR dimerization. Disrupting the interaction between PKR and NS5A may provide a way to increase the responsiveness of HCV to treatment with IFN- $\alpha$  <sup>(35)</sup>.

The sequence of NS5B protein is highly conserved, not only between different strains of HCV but also in pestiviruses, flaviviruses and even in other RNA viruses. In particular, the amino acid motif G-D-D is totally conserved in HCV, flaviviruses, poliovirus and tobacco mosaic virus. This motif is a characteristic of all known RNA-dependent RNA polymerases and so the function of NS5B in HCV has been speculated to be the viral polymerase. As yet it is unclear whether NS5B alone can perform this role or whether it is merely a critical component of a multimolecular replication complex <sup>(37)</sup>.

### 3' Untranslated region (3' UTR)

Three different regions can be recognized within the 3'UTR of the HCV genome. The first is a variable stretch of 28-42 nucleotides in length that is poorly conserved. This region is in turn followed by a polypyrimidine stretch consisting primarily of a poly (U) with interspersed C nucleotides. The third portion of the 3'UTR, also designated X tail, is a well-conserved 98 nucleotide sequence which probably forms stem-and-loop structure. The actual function of the X tail has not yet been determined, but this genetic element has been demonstrated to be absolutely necessary for viral replication. In the case of other RNA viruses, conserved terminal sequences are often involved in crucial processes like genome packaging and/or initiation of RNA synthesis by the virus replication complex. These processes are normally mediated by the interaction of these RNA elements with viral and/or cellular proteins. The HCV X tail has been shown to interact with the ubiquitous polypyrimidine tract-binding protein (PTB). The role of PTB in the viral replication cycle has not yet been determined <sup>(35)</sup>.

### HCV Heterogeneity

The rapidity of viral replication and the lack of error proofing of the viral polymerase probably account for the fact that the HCV RNA genome mutates frequently. As a result, HCV circulates in serum not as a single species but as a population of quasispecies with individual viral genomes differing by 1% to 5% in nucleotide sequence <sup>(39-40)</sup>. The quasispecies diversity of HCV may contribute to the development



of chronicity during infection and may contribute to immune escape because the envelope protein changes rapidly in response to immune pressure.

Six major genotypes (1 to 6) and more than 50 subtypes (e.g., 1a, 1b, 2a, 2b . .) of HCV have been described (Table 1)<sup>(33)</sup>. Different isolates of HCV among the same subtype differ by 5% to 15%, among subtypes by 10% to 30%, and among genotypes by as much as 30% to 50% in nucleotide sequence. The different HCV genotypes have marked geographic variation in their relative frequencies. Genotype 1a is the prototype genotype and is common in the United States and Northern Europe. Genotype 1b has a worldwide distribution and is often found to be the most common genotype. Genotypes 2a and 2b are also distributed worldwide, representing 10% to 30% of HCV types, and particularly common in Japan and Northern Italy. Genotype 3 is most frequent in the Indian subcontinent; this genotype have been recently introduced into the United States and Europe, perhaps as a result of the spread of injection drug use in the 1960s and 1970s. Genotype 4 is the most common genotype in Africa and the Middle East and it is extremely diverse. Genotypes 5 and 6 are found in some geographic areas i.e. genotype 5 in South Africa and genotype 6 in Hong Kong and Southeast Asia.

There is not much different in clinical manifestation and clinical outcome or severity of illness by the HCV genotypes<sup>(40-41)</sup>. Infection with any genotype can lead to cirrhosis, end-stage liver disease, and hepatocellular carcinoma, and the frequency of these complications appears to be similar.

**Table 1** Heterogeneity of Hepatitis C Virus<sup>(32)</sup>

Term	Nomenclature	Degree of Nucleotide Sequence Variation (Range)
Quasispecies		1% to 5%
Isolate		5% to 15%
Subtype	a,b,c, . . .	15% to 30%
Genotype	1 through 6	30% to 50%

NOTE. Genotypes are often referred to as both genotype and subtype e.g. genotype 1a,1b.

Hepatitis C has a variable course and outcome (Figure 2)<sup>(33)</sup>. The acute illness can be severe and prolonged but is rarely fulminant. Between 55 % and 85 % of patients with acute HCV infection develop chronic infection. Chronic hepatitis C is often silent and may be nonprogressive and mild. Alternatively, it can be slowly progressive, or associated with marked extrahepatic manifestations, or rapidly progressive leading to cirrhosis and hepatocellular carcinoma.

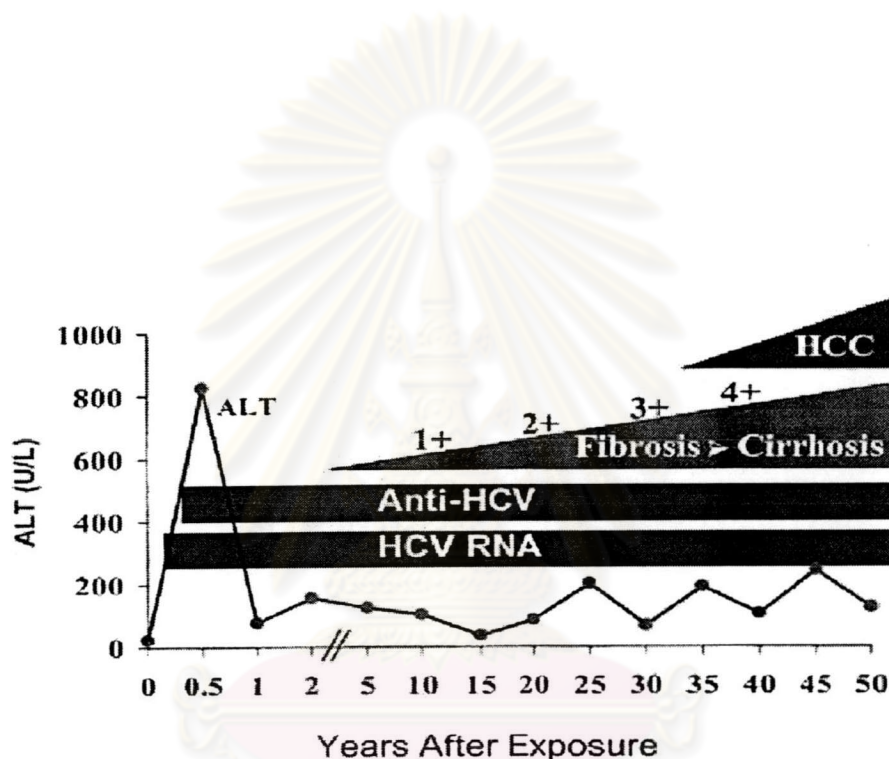


Figure 2 The natural history of chronic hepatitis C over 50 years. During chronic infection, a progressive worsening of fibrosis to cirrhosis (from none to 4+) can occur. Hepatocellular carcinoma typically appears after decades of infection and usually in a patient with an underlying cirrhosis. ALT, alanine aminotransferase; HCV, hepatitis C virus; anti-HCV, antibody to HCV; HCC, hepatocellular carcinoma<sup>(32)</sup>.

## HCV genotyping methods

### 1. RFLP

In 1995, Davidson et al.<sup>(12)</sup> employed 5' NCR fragments which were submitted to *Hae* III-*Rsa* I and *Mva* I-*Hinf* I restriction digests for detection of genotypes 1 to 6. Additional *Bst*U I or *Scr*F I cleavage may allow subtype discrimination.

Although type 6a and all of the previously described major genotypes (type 2 to 5) show conserved nucleotide differences from type 1 in the 5' NCR that allow them to be differentiated by restriction endonucleases or by type-specific probes in INNO-LiPA, many (but not all) of the novel variants from Vietnam and Thailand have sequences in the amplified region used for genotyping that are identical to those of type 1a or 1b.

The core gene encodes a putative nucleocapsid protein, which is the most conserved protein in the HCV genome. Nevertheless, it is considerably more variable than the 5' NCR and contains sufficient sequence information to identify all known subtypes and major genotypes, including those in the type 6 group that are misclassified by using the 5' NCR. In 1996, Mellor et al.<sup>(13)</sup> have developed a genotyping assay in which sequences in the core region are amplified and cleaved by restriction enzymes: *Ava* I and *Sma* I that reliably differentiate genotype 1 variants from type 6 variants.

Until now, many groups of researchers tried to use different enzymes to digest PCR products from different regions of HCV genome for genotyping. In 1999, Buoro et al.<sup>(14)</sup> demonstrated that amplified PCR product covered both 5'NCR and core regions digested with 3 enzymes: *Acc* I, *Mbo* I and *Bst*N I could allow discrimination of all subtypes or types.

### 2. Reverse hybridization techniques

The line Probe Assay (INNO-LiPA assay)

The INNO-LiPA HCV II employs the distinction of 5'NCR polymorphisms specific for the different HCV genotypes. Up to 21 probes are attached to a nitrocellulose membrane. Labelled PCR products obtained from 5'NCR will only hybridize to a probe (or line) perfectly matching the sequence of the isolate, allowing stringent discrimination at the subtype level. Such a high specificity can be obtained by using very stringent hybridization conditions, consequently, a 100% correlation with 5'NCR sequences is

achieved. With certain isolates up to 60% of the entire 5'NCR can be deduced from single hybridization. After an isothermal stringent wash, the presence of probe/amplicon-biotin hybrids is revealed by an alkaline phosphatase labelled streptavidin conjugate. The captured enzyme converts NBT and BCIP into a purple colour, which uncovers the sequence of part of the 5'NCR of the isolate studied. The second generation test allows discrimination of type 1 to 6 and nearly all subtypes.

### 3. Sequencing

In the absence of biological or true neutralization assays for HCV, classification of HCV is currently based upon nucleotide sequence comparisons. In 1992, Chan et al<sup>(42)</sup> have carried out phylogenetic analyses of nucleotide sequences amplified in the region of the genome encoding the core protein and parts of the NS3 and NS5 proteins. Although different degrees of variability were found in various parts of the genome, analysis of each produced trees topologically identical to those obtained upon analysis of complete genome sequences. Simmonds et al<sup>(15)</sup> have carried out phylogenetic analysis of a 222 bp fragment of NS5 between nucleotide positions 7,975 and 8,196 and found that this region could be used as the representative sequence of whole genome for phylogenetic analysis.

### Viral Replication

HCV nonstructural proteins and viral RNA have been detected in livers of infected patients or experimentally inoculated chimpanzees, confirming that the liver is a site of HCV replication. The dynamics of HCV replication can be deduced from the rapid rates of viral production and emergence of mutants. A careful analysis of viral dynamics during antiviral treatment of patients with IFN- $\alpha$  revealed a virion half-life of 3-5 hr and a clearance and production rate of  $10^{12}$  particles per day. Assuming that 10% of hepatocytes of a liver are infected and that a liver contains  $2 \times 10^{11}$  hepatocytes, this would correspond to a virion production rate of 50 particles per hepatocyte per day. Another feature of HCV replication is the rapid generation of viral variants. Even within a patient, HCV does not exist as a single entity but rather as a swarm of microvariants of a predominant 'master sequence', a phenomenon that has been referred to as quasispecies. The production of such a large number of variants is primarily due to the

high error rate of the viral RNA-dependent RNA polymerase (RdRp) that, based on analogies with RdRps of other plus-strand RNA viruses, is expected to be in the range of  $10^{-4}$ . This high error rate is reflected by the high mutation rate observed in patients or experimentally inoculated chimpanzees. Using comparative sequence analyses of HCV genomes isolated over intervals of 8 or 13 years a mutation rate of  $1.44 \times 10^{-3}$  or  $1.92 \times 10^{-3}$  base substitutions per site per year was found, respectively <sup>(5)</sup>.

### Attachment and entry

The first step in a virus life cycle is the attachment of the infectious particle to the host cell, for which a specific interaction between a receptor on the cell surface and a viral attachment protein on the surface of the particle is required.

Suramin, a polyanionic compound similar to heparin, was found to block HCV binding to human hepatoma cells at a concentration similar to that reported to be effective against dengue virus, suggesting an interaction of HCV with glycosaminoglycans on the cell surface <sup>(43-44)</sup>. Recently, CD81, a transmembrane protein of the tetraspanin family, was identified as a putative HCV receptor based on its strong interaction with E2 as well as with virus particles *in vitro* <sup>(38)</sup>. Interestingly, the HCV E2 protein, after binding to CD81, induced aggregation of lymphoid cells and inhibited the proliferation of a B cell line <sup>(45)</sup>. This might have important consequences for the pathogenesis of HCV. Since glycosaminoglycans and CD81 are expressed by a wide variety of cells, additional specific factors must determine the liver tropism of HCV <sup>(46)</sup>.

Apart from this route, HCV as well as other members of the *Flaviviridae* family may enter the cell by binding to low-density lipoprotein (LDL) receptors <sup>(47)</sup>. The observation of an association between HCV and lipoproteins may be critical for the virus replication cycle because LDL and VLDL are taken up by hepatocytes through the LDL receptor, which may provide a unique and efficient mode of viral entry <sup>(37)</sup>.

While the nature of the HCV receptor is not currently known, the major envelope glycoprotein E2 is thought to be responsible for initiating virus attachment to the host cell because E2-specific antisera can block binding to cells. The role of E1 is less clear but the presence of a stretch of hydrophobic amino acids tentatively called the E1 fusion peptide, displaying similarities to the fusion peptides of paramyxovirus and flavivirus suggests that E1 is involved in membrane fusion <sup>(45)</sup>.

## Polyprotein translation and processing

Once inside the cytoplasm the genomic RNA is directly translated. Therefore, translation of the viral RNA is not mediated by a cap-dependent mechanism with ribosomes scanning along the RNA up to the first initiator AUG codon, but rather by an IRES. This RNA element residing approximately between nucleotides 40 and 355 forms four highly structured domains. It was also shown that the HCV IRES binds specifically to the 40S ribosome subunit and does not require any additional translation factors. Directed by the IRES, the polyprotein is translated at the rough endoplasmic reticulum (ER) and cleaved co- and post- translationally by host cell signalases and two viral proteinases.

## RNA replication

The individual steps underlying RNA replication are largely unknown. It is obvious that the NS5B RdRp is the key player catalyzing the synthesis of minus- and plus-strand RNA. While *in vitro* NS5B is able to copy even a complete full-length HCV genome, it is very likely that *in vivo* additional viral or cellular factors are required. Possible viral factor candidates are the NS3 helicase, by unwinding stable structures in the RNA template and facilitating replication, or the phosphoprotein NS5A. Although the replication function of this protein could not be studied thus far, by analogy to other RNA viruses, it is tempting to speculate that NS5A is involved in regulation of RNA replication.

In addition to viral proteins, cellular components are probably also involved in RNA synthesis. One candidate is PTB, found to specifically interact with sequences at the 3' NTR. Another candidate is glyceraldehyde-3-phosphate dehydrogenase, binding to the poly(U)-sequence in the 3' NTR. Finally, cellular proteins provisionally called p87 and p130 were identified by UV-cross linking experiments with the X-tail sequence, but the nature of these proteins remains to be determined<sup>(48)</sup>.

## Virion assembly and release

In the absence of systems allowing the production of biochemical amounts of virus particles, the assembly of HCV cannot be studied in detail.

Particle formation may be initiated by core protein interacting with the RNA genome. Although *in vitro* core protein binds to RNA without detectable specificity, recent evidence indicates a preferential intracellular binding to RNA sequences in the 5'

half of the HCV genome. Such binding may not only accomplish a selective packaging of the plus-stranded genome but also appears to repress translation from the IRES, suggesting a potential mechanism to switch from translation/replication to assembly. Whether the core protein forms a distinct nucleocapsid structure or a rather non-structured ribonucleoprotein complex with the RNA genome is not known. Certainly, core protein interacts with itself and the sequences required for this interaction have been mapped to the amino-terminal 115 residues. Within this region a tryptophan-rich primary interaction domain was identified between residues 82 and 102 that is masked in the full-length core protein and revealed only under certain experimental conditions.

A feature typical of the HCV E proteins is their retention in the ER compartment when expressed with various heterologous systems in cell culture. The retention is achieved by signals in the transmembrane domains of E1 and E2 and it has been shown to be a true retention in the ER. This observation suggests that viral nucleocapsids acquire their envelope by budding through ER membranes. In this case the virus may be exported via the constitutive secretory pathway. In agreement with this assumption, complex N-linked glycans were found on the surface of partially purified virus particles, suggesting virus transit through the Golgi. However, since HCV particles tend to associate with cellular compartments, it remains to be determined whether these glycans are present on the E proteins or on cellular proteins associated with HCV particles <sup>(5)</sup>.

### **HCV Markers and Their Kinetics**

Four virological markers of HCV infection can be used in management of infected patients, namely HCV genotype, HCV RNA, HCV core antigen, and anti-HCV antibodies. The kinetics of HCV markers during spontaneously resolving acute and chronic hepatitis C are shown in Figure 3A and B, respectively <sup>(49)</sup>.

#### **HCV Genotype**

The HCV genotype is an intrinsic characteristic of the transmitted HCV strain(s) and does not change during the course of the infection. HCV genotypes form 6 clades or types (numbered 1 to 6) and are themselves subdivided into a large number of subclades or subtypes identified by lower-case letters (1a, 1b, 1c, etc) <sup>(4)</sup>. Phylogenetic

analysis can distinguish HCV types, subtypes, and isolates on the basis of average sequence divergence rates of approximately 30%, 20%, and 10%, respectively <sup>(4)</sup>.

### HCV RNA

The presence of HCV RNA in peripheral blood is a reliable marker of active HCV replication, which takes place principally in the liver. HCV RNA is detectable in serum within 1 to 2 weeks after infection. It generally increases to reach a peak, before disappearing when the infection resolves spontaneously. In contrast, in most patients progressing to chronic infection, the decrease in HCV RNA gradually slows then stabilizes; occasionally, however, HCV RNA may become undetectable for a few days or weeks before reappearing and reaching a plateau. HCV RNA levels are stable over time in patients with chronic infection <sup>(50)</sup>. Indeed, viral kinetics are at a steady state, i.e., peripheral release of viral particles is counterbalanced by their constant peripheral degradation, while *de novo* infection of hepatocytes is counterbalanced by infected hepatocytes' death by apoptosis <sup>(51)</sup>. During this steady state, the estimated mean half-life of HCV virions in the general circulation is of the order of 3 hours, and virus turnover (production/clearance) is of the order of  $10^{12}$  virions a day <sup>(51)</sup>. The HCV RNA level may increase slightly after several years of chronic infection.

The HCV RNA level is not affected by the severity of liver disease, except in patients with end-stage liver disease, who generally have low or even undetectable HCV RNA levels <sup>(52-53)</sup>. The decrease in viral level with end-stage liver disease is probably due to hepatocyte depletion and extensive fibrosis rather than to changes in the virus itself, as HCV recurrence after liver transplantation is generally associated with high HCV RNA levels, which is facilitated by immunosuppressive treatment <sup>(52-53)</sup>.

### HCV Core Antigen

The icosahedral HCV capsid is formed by polymerization of HCV core protein, a 21-kd structural phosphoprotein composed of the first 191 amino acids of the viral polyprotein <sup>(54)</sup>. It has recently been shown that total HCV core antigen levels correlate with HCV RNA levels <sup>(55)</sup>. During the pre-seroconversion period, core antigen is detected on average 1 to 2 days later than HCV RNA with current assays <sup>(56-57)</sup>. Thereafter, core antigen kinetics run closely parallel to HCV RNA kinetics <sup>(55)</sup>. The HCV core antigen titer can thus also be used as a marker of HCV replication.



## Anti-HCV Antibodies

The “serologic window” between HCV infection and the detectability of specific antibodies varies from patient to patient. With current assays, seroconversion occurs on average at 7 to 8 weeks after onset of infection<sup>(58-60)</sup>. Anti-HCV is detectable in 50% to 70% of patients at the onset of clinical symptoms and later in the remaining patients<sup>(61)</sup>. In patients with spontaneously resolving infection, anti-HCV may persist throughout life, or decrease slightly while remaining detectable, or gradually disappear after several years<sup>(62)</sup>. Anti-HCV persists indefinitely in patients who develop chronic infection, although antibodies may become undetectable (with current assays) in hemodialysis patients or in cases of profound immunodepression. Apparent seroreversions and/or seroconversions can occur in immunodepressed patients, in whom the chronic nature of the infection is confirmed by the persistence of HCV RNA.

There are no robust cell culture systems for propagation of HCV, nor are there simple small animal models of the infection, so that the replicative cycle of the virus has largely been deduced from that of other flaviviruses. Hepatitis C virus replicates in the cytoplasm of hepatocytes where it is not directly cytopathic. Persistent infection appears to rely on rapid production of virus and continuous cell-to-cell spread, along with a lack of vigorous T-cell immune response to HCV antigens. The rate of viral production in hepatitis C is often quite high, in the range of  $10^{10}$  to  $10^{12}$  virions per day. There is also rapid turnover of virus, at least in the serum, the predicted half-life being 2 to 3 hours<sup>(51)</sup>.

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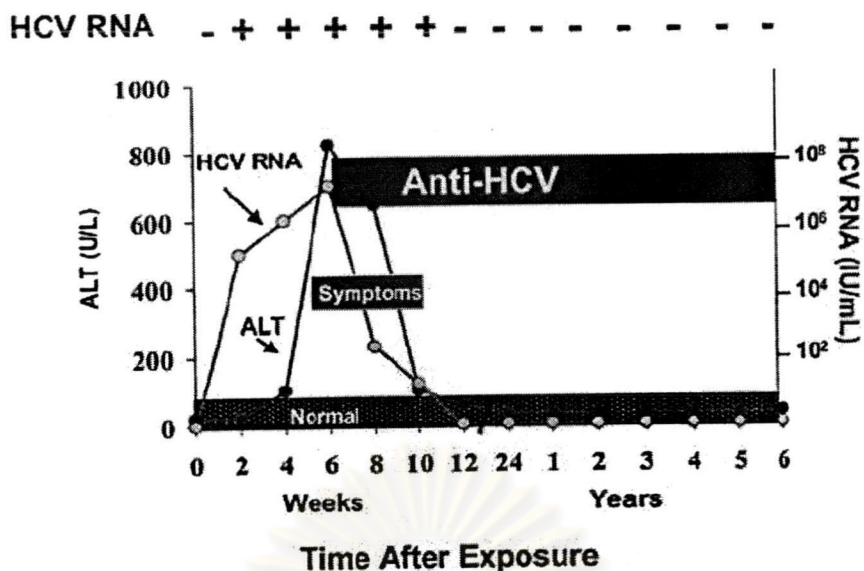


Figure 3 A Course of acute, resolving hepatitis C. Serial results of quantitative tests for HCV RNA are shown in IU per ml by the lightly shaded circles and results of quantitative tests for HCV RNA as either positive (+) or negative (-). Serial results of ALT levels are shown with darkly shaded circles. The normal or undetectable range of values is displayed by the stippled area<sup>(33)</sup>.

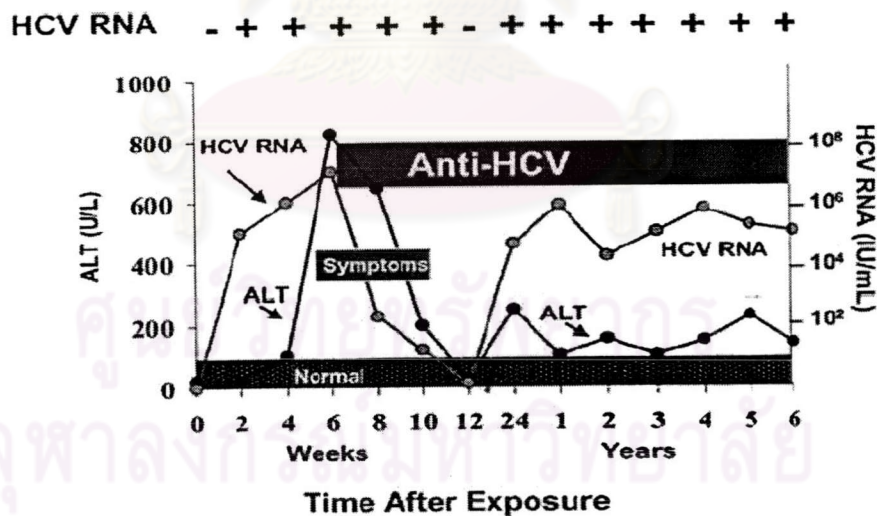


Figure 3 B Course of acute hepatitis C that evolves into chronic infection. Serial results of quantitative tests for HCV RNA are shown in IU per ml by the lightly shaded circles and results of quantitative tests for HCV RNA as either positive (+) or negative (-). Serial results of ALT levels are shown with darkly shaded circles. The normal or undetectable range of values is displayed by the stippled area<sup>(33)</sup>.

## Immunity against HCV infection

### 1. Humoral immune response to HCV

HCV specific antibodies are generally detectable in the serum between 7 and 31 weeks after infection<sup>(63)</sup>. This response appears to be rather late when compared with humoral response against other viruses and to the cellular immune response to HCV<sup>(64)</sup>.

Antibody production is critical for neutralization of free viral particles and for interference with virus entry into host cells. Protection by antibodies is most important before invasion of the host cells. Afterwards, antibodies can help limit cell-to-cell spread of viral particles. The main target of neutralizing activity is the HVR1 region of the E2 protein and its variability probably reflects the selective pressure imposed by the antibody response. The most likely interpretation is that occurrence of viral mutations within the epitopes recognized by neutralizing antibodies can abrogate antibody recognition of the new variant virus. In this way the new variant would be protected from neutralization while previous isolates would be cleared by the antibody response. The final result would be selection of the variant. Repetitive steps of clearance and selection could contribute to viral persistence and cause accumulation of mutations within immunologically relevant regions. The high heterogeneity of the HVR1 region in the viral quasispecies at this stage of infection could explain why these neutralizing antibodies are associated with persistent viremia rather than viral clearance. Although immunodominant epitopes have been identified within core, NS3 and NS4 regions, definite information is lacking on the pathogenetic role of the antibody response against these regions.

As a result of the lack of an *in vitro* system supporting HCV replication, it has been impossible to develop an *in vitro* assay for the presence of neutralizing antibodies. A "neutralization of binding assay" ("NOB assay")<sup>(65)</sup> is promising, but because it only measures inhibition of binding of recombinant E2 protein to target cells, it does not necessarily reflect the neutralization of infectious HCV *in vivo*.

Antibodies may also be involved in immunopathological aspects of chronic HCV infection. For example, chronic HCV infection is associated with type II mixed cryoglobulinemia, which can be associated with glomerulonephritis, cutaneous vasculitis, and arthritis<sup>(66)</sup>. Immune complexes consisting of HCV and anti-HCV

antibodies are concentrated within the cryoprecipitate. It has also been suggested that immune complexes may be involved in viral persistence via putative immunosuppressive effects on Fc-receptor-bearing cells<sup>(67)</sup>.

## 2. Cell-mediated immune response to HCV

Recent studies suggest that the human leukocyte antigen (HLA) class II restricted Th cell response is particularly important for the outcome of acute HCV infection<sup>(68-73)</sup>. Specifically, patients with acute self-limited hepatitis C have been demonstrated to mount an early, vigorous, and multispecific T-helper response that is readily detectable in the peripheral blood. If, in contrast, this immune response is weak, less efficient, or not maintained for a sufficient length of time, patients proceed to persistent infection and chronic hepatitis<sup>(73)</sup>. Thus, the intensity, epitope specificity, and cytokine profile of the T-cell response during the early stages of HCV infection seem to be predictive for the outcome of HCV infection.

To address this issue, studies of the HLA class II restricted T cell response to HCV antigens have been sequentially carried out with recombinant proteins from the early stages of infection in large groups of patients with acute hepatitis C, showing that T cell reactivity to structural and non-structural HCV antigens is significantly stronger in patients who succeed in normalizing ALT than in those who develop a chronic infection. In acute hepatitis C, the CD4-mediated response is multispecific and immunodominant epitopes have been recently identified by studying the fine specificity of HCV-reactive polyclonal T cell lines derived from PBMC stimulation with recombinant HCV proteins. Of particular interest with respect to the design of preventive or immunotherapeutic vaccines is the NS3 sequence 1,248-1,261, which is highly promiscuous and located within a highly conserved HCV region.

Not only the strength but also the quality of the T cell response is different in patients who recover compared to those who develop chronic HCV infection, because recovery is associated with a prevalent  $T_H1$  profile of peripheral blood HCV-specific T cells, whereas  $T_H2$  and  $T_H0$  patterns are prevalent among circulating HCV-specific T cells of patients with chronic evolution. Since the effect of  $T_H1$  cytokines is crucial for protection against intracellular pathogens, including viruses, whereas the preferential

production of  $T_H2$  cytokines has been reported to favor persistence of these microbial agents and severe evolution of associated diseases, both the vigor and quality of HCV-specific T cell responses may play an important role in the pathogenesis of HCV persistence<sup>(16)</sup>.

Due to the large number of chronically infected patients, extensive studies on the immunopathology of chronic hepatitis C have been performed. Collectively, these data imply that the immune response mediates chronic liver cell injury if it is not able to clear the infection.

Similar to the inability of the cellular immune response to control HIV, the apparent resistance of HCV to immunological control in an area of great interest and importance. Research has been hampered by the fact that the acute stage of HCV infection is usually clinically inapparent and also because efficient *in vitro* models of infection do not exist. To date, these reports suggest that the HCV-specific immune response exerts some control over virus replication but is unable to resolve or to terminate persistent infection and chronic hepatitis in most cases.

## 2.1 The role of CD4-positive T-helper cells

CD4<sup>+</sup> helper T cells recognize short antigenic peptides derived from proteolytic cleavage of exogenous antigen in the antigen-binding groove of HLA class II molecules at the surface of antigen-presenting cells such as dendritic cells, macrophages and B cells<sup>(74)</sup>. The function of CD4<sup>+</sup> T cells is principally regulatory by secreting cytokines that modulate the activity of antigen-specific B cells and CD8<sup>+</sup> T cells<sup>(75)</sup>. The CD4<sup>+</sup> T cell response to HCV is detected usually by measuring the antigen-specific proliferative responses of peripheral blood mononuclear cells to recombinant HCV antigens in patients with chronic and acute hepatitis C<sup>(68,70-71,75-76)</sup>. A hierarchy of T cell responsiveness to HCV proteins has been defined in these patients, with core, NS3, and NS4 being most immunogenic<sup>(76)</sup>. Interestingly, several studies have consistently found a significant association between a strong and maintained HCV-specific CD4<sup>+</sup> T-cell response and viral clearance in acute hepatitis C<sup>(70-71,78-79)</sup>.

Multiple peptides that are recognized by  $T_H$  cells and include immunodominant epitopes have already been identified in HCV. The NS3 protein is of special importance since a CD4-positive T cell response against its protease and helicase domain is much

stronger and more frequently found in patients who resolve acute hepatitis C than in patients who develop persistent infection. Furthermore, a strong NS3-specific T cell response seems to be necessary for viral clearance. NS3-specific T<sub>H</sub> cells recognize a short immunodominant region at amino acid position 1,248-1,261 with the putative minimal epitope at amino acid position 1,251-1,259. This epitope promiscuously binds to ten common HLA class II alleles with a high binding affinity and is recognized in the context of five different HLA alleles. Moreover, its sequence is completely conserved within HCV 1a, 1b, 1c, 2a and 2b genotypes. Thus, they are ideal candidates for preventive or therapeutic T-cell-based vaccines.

After recovery from acute hepatitis C, the NS3 specific Th cell response can be maintained in the peripheral blood for years, suggesting the existence of a strong immunological memory. Whether NS3-specific T cells eradicate the HCV completely, or they control and restrict HCV replication to very low levels at immunoprivileged sites, is not known. The latter mechanism has been reported to occur after clearance of the HBV and allows periodic *de novo* induction of virus-specific T cells to keep the virus in persistent subclinical latency.

Because T<sub>H</sub> cells that mediate this HCV-specific immune response frequently display a T<sub>H</sub> 1 (IFN- $\gamma$  as the predominant) or T<sub>H</sub> 0 (IL-2 as a predominant) cytokine profile to support differentiation and proliferation of CD8<sup>+</sup> T cells, it is not surprising that early, vigorous, and multispecific CTL responses have also been associated with a self-limited outcome of HCV infection<sup>(80)</sup>. The activation of T<sub>H</sub>2 responses seems to be involved in the development of chronic hepatitis C.

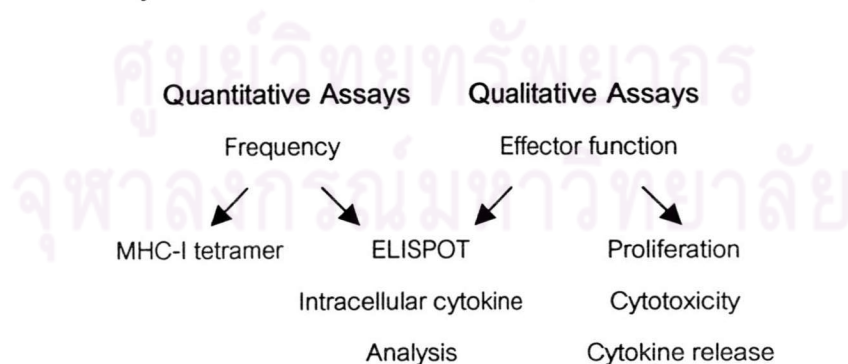
## 2.2 The role of CD8-positive T-cytotoxic cells

HLA class I-restricted HCV-specific CD8<sup>+</sup> CTL were detected in the peripheral blood and the intrahepatic lymphocytic infiltrate in patients with chronic hepatitis C<sup>(81-90)</sup>. A similar CTL response has been demonstrated in the liver of acutely infected chimpanzees, and the response can last indefinitely, demonstrating that the virus can persist in the presence of these CTL<sup>(91)</sup>. The CTL identified thus far in infected patients are able to recognize both conserved and variable regions of all of the HCV proteins in the context of several different HLA molecules. Moreover, the response is often polyclonal and multispecific in chronically infected patients:

Since repetitive *in vitro* stimulation with HCV-derived peptides is required to expand CTLs from the peripheral blood, the frequency of progenitor CTLs in the circulation is assumed to be rather low. Indeed, the frequency of CTL precursors targeted against individual epitopes has been determined to be in the range of 1 in  $10^6$  to 1 in  $10^5$  PBMCs, which is lower than the frequency of CTLs targeting against HIV (5 in  $10^4$  PBMCs). In contrast, HCV-specific CTLs are present at a higher frequency in the liver, because non-specific stimulation is sufficient to expand HCV-specific CTLs from liver biopsies. The relatively low and weak HCV-specific CTL response is not due to generalized immunosuppression since the CTL response against influenza virus and Epstein-Barr virus is normal in HCV-infected patients <sup>(81-83,89-70)</sup>. At the site of inflammation, the infected liver, the frequency of CD8<sup>+</sup> T cells was found to be at least 30-fold higher than in blood <sup>(92)</sup>. In contrast, it is necessary to expand the peripheral blood CTL precursor population with HCV-derived peptides to demonstrate a CTL response to HCV <sup>(83-87,93-94)</sup>.

Results obtained by several groups show an inverse correlation between levels of antiviral CTL activity and viral load, suggesting that the virus is controllable, at least to some extent, by CTL <sup>(93,95-96)</sup>. The virus-specific immune response may not only target virally infected cells, but also uninfected bystander hepatocytes via release of soluble pro-apoptotic mediators such as Fas-ligand and soluble tumor necrosis factor  $\alpha$  <sup>(97)</sup>.

### Methods to analyze the cellular immune response <sup>(98)</sup>



Numerous studies have characterized the functional properties of HCV-specific, peripheral blood derived T helper lymphocytes of patients during acute HCV infection (Table 2) and of chronically infected and recovered patients (Table 3). In all studies,

recovery from acute infection was clearly associated with a multispecific CD4 T cell response targeted against structural and non-structural HCV proteins. This response has to be maintained to achieve long-term control of the virus<sup>(73)</sup>.

**Table 2** Studies investigating circulating HCV-specific MHC class II-restricted T cells in patients with acute HCV infection<sup>(98)</sup>

Author	Reference	Method		Antigens		Patient number
		Proliferation	Cytokine production	Structural HCV proteins	Non-structural HCV proteins	
Diepolder <i>et al.</i>	Lancet 1995 ; 346 : 1006 <sup>(69)</sup>	+	-	+	+	14
Missale <i>et al.</i>	JCI 1996 ; 98 : 706 <sup>(71)</sup>	+	-	+	+	21
Tsai <i>et al.</i>	Hepatology 1997; 25 : 449 <sup>(78)</sup>	+	+	+	+	17
Diepolder <i>et al.</i>	J Virol 1997 ; 71 : 6011 <sup>(70)</sup>	+	+	-	+	5
Gerlach <i>et al.</i>	Gastroenterology 1999 ; 177 : 933 <sup>(73)</sup>	+	+	+	+	38
Lamonaca <i>et al.</i>	Hepatology 1999 ; 30 : 1088 <sup>(72)</sup>	+	-	+	+	22
Lechner <i>et al.</i>	J Exp Med 2000 ; 191 : 1499 <sup>(99)</sup>	-	+	+	+	3

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**Table 3** Studies investigating circulating HCV-specific MHC class II-restricted T cells in patients with chronic HCV infection or after recovery <sup>(98)</sup>

Author	Reference	Method		Antigens		Patient number
		Proliferation	Cytokine production	Structural HCV proteins	Non-structural HCV proteins	
Botarelli <i>et al.</i>	Gastroenterology 1993 ; 104 : 80 <sup>(100)</sup>	+	-	+	+	63
Ferrari <i>et al.</i>	Hepatology 1994 ; 19 : 286 <sup>(68)</sup>	+	-	+	+	44
Koskinas <i>et al.</i>	Gastroenterology 1994 ; 107 : 1436 <sup>(101)</sup>	+	-	+	-	27
Hoffmann <i>et al.</i>	Hepatology 1995 ; 21 : 632 <sup>(76)</sup>	+	-	+	+	43
Iwata <i>et al.</i>	Hepatology 1995 ; 22 : 1057 <sup>(102)</sup>	-	+	+	-	46
Leroux-Roels <i>et al.</i>	Hepatology 1996 ; 23 : 8 <sup>(76)</sup>	+	-	+	+	35
Lechmann <i>et al.</i>	Hepatology 1996 ; 24 : 790 <sup>(103)</sup>	+	-	+	+	39

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## Mechanisms of Immune-mediated Liver Injury

Destruction of infected hepatocytes may occur in several ways: HCV-infected hepatocytes can be killed by HCV-specific CTL clones via Fas ligand, TNF $\alpha$ , and/or perforin-based mechanisms. Fas ligand-induced apoptosis of hepatocytes is especially feasible because expression of Fas, a mediator of apoptosis<sup>(104)</sup>, is upregulated on HCV-infected hepatocytes and on uninfected bystander cells in response to inflammatory cytokines<sup>(105)</sup> and because Fas-ligand is expressed on activated liver-infiltrating T cells<sup>(106)</sup>. TNF $\alpha$  is predominantly produced by macrophage<sup>(107)</sup> but also released by<sup>(108)</sup> and expressed on the surface of CTL<sup>(109)</sup>. Finally, perforin-mediated mechanisms may contribute to lysis of antigen-presenting, Fas-, and TNF $\alpha$ -resistant cells<sup>(110)</sup>.

After exerting their effector functions, the majority of these liver-infiltrating HCV-specific T cells undergo programmed cell death. Especially, T<sub>H</sub>1 cells, which are preferentially attracted into HCV-infected livers, are sensitive to Fas-FasL-induced apoptosis, whereas T<sub>H</sub>2 effectors express high levels of FAP-1, a Fas-associated phosphatase that presumably inhibits Fas signaling.<sup>(109)</sup> The loss of lymphocytes by intrahepatic sequestration and apoptosis has been estimated to be as high as  $2 \times 10^8$  cells (i.e., 0.1% of the total body lymphocytes per day)<sup>(110)</sup>.

## Potential mechanisms for persistence

One hypothesis is that the HCV-specific immune response is quantitatively and/or qualitatively inadequate to eliminate the large number of infected cells in an organ as large as the liver. The load of viral antigen depends on HCV replication, viremia and consequently, HCV protein expression. With small doses of antigen, T cell activation and proliferation are generally suboptimal. In the case of HCV, this may be reflected by the relatively low number of HCV-specific cytotoxic T cells in the peripheral blood of chronically infected patients. Small doses of viral antigens may therefore preclude strong HCV-specific proliferative T cell responses due to limited IL-2 production.

Another hypothetical explanation for HCV persistence, sequence variation due to the quasispecies nature and the high mutation rate of HCV, has often been discussed. Amino acid changes in immunodominant epitopes may permit HCV to escape from the

antiviral immune response. Indeed, evidence for immune selection pressure has been obtained from several clinical studies.

Theoretically, HCV could also interfere with antigen processing or presentation by the hepatocyte. It may diminish its visibility to the immune system sufficiently to permit chronic low grade inflammation, but not enough for the immune response to kill all of the infected cells.

Finally, it is possible that HCV, unlike other viruses such as HBV, CMV, HIV or rotaviruses may not be susceptible to control by CTL-derived cytokines. If the virus is naturally resistant to the antiviral effects of T cell-derived cytokines, viral clearance would depend entirely on the destruction of infected hepatocytes by the immune response. Because direct CTL lysis is an inherently inefficient process, requiring direct cell-cell contact, if this is the only mechanism available to eradicate an overwhelming number of infected cells in a large organ such as the liver, one would expect the process to be incomplete, leading to the destruction of some but not all of the infected hepatocytes, i.e., chronic hepatitis <sup>(113)</sup>.

### Treatment of HCV infection

Interferon alone or in combination with oral ribavirin is the treatment currently known to be effective for chronic hepatitis C <sup>(114-118)</sup>. When interferon is used alone, approximately 40% of patients initially respond to treatment with normalization of the serum alanine aminotransferase (ALT) level and loss of detectable HCV RNA <sup>(114-115,117)</sup>. However, most patients relapse after short courses (6 months) of therapy and sustained viral-negative response occurs in only 6-21% of treated patients <sup>(117)</sup>. While interferon treatment for 12-24 months increases the durability of the initial response, resulting in a mean sustained response rate of 35% <sup>(117)</sup>, it significantly increases the cost and inconvenience of treatment, and relapse remains common <sup>(117-119)</sup>.

Ribavirin, a guanosine nucleoside analogue with *in vitro* activity against a number of viruses <sup>(120)</sup>, does not reduce HCV RNA when used alone <sup>(123-125)</sup>. When combined with interferon, the combination significantly increases both end-of-treatment and sustained response rates compared with interferon alone <sup>(119,126-127)</sup>. This combination results in end-of-treatment HCV RNA negativity in approximately half of

patients and offers sustained virologic responses of 31-38 % overall, ranging from 16-28 % in patients with genotype 1, to 66-69 % in those with genotype 2 or 3 <sup>(118)</sup>.

Despite the high initial and sustained response rates which are now achievable with combination therapy, half of patients do not lose detectable virus with therapy <sup>(118)</sup>. Many of these individuals have partial viral responses, suggesting that the current treatment regimens are not intensive enough to totally suppress viral replication.

### HCV vaccine development

HCV is one of the important human pathogens which cause severe liver diseases. Most patients with HCV infection will develop chronic hepatitis with a risk of progression to cirrhosis or hepatocellular carcinoma <sup>(128)</sup>. Only a minority of patients can benefit from anti-viral therapy using interferon, therefore, it is important to develop an effective HCV vaccine to prevent infection or as an immunotherapeutic to treat chronic HCV infection and thus stop the progression of chronic hepatitis.

The development of HCV vaccines has focused on the envelope proteins for the production of protective antibody <sup>(129)</sup> and the core or NS5 peptides for the induction of cytotoxic T lymphocyte (CTL) <sup>(130)</sup>. The HCV genome, especially the hypervariable region (HVR) of E2, mutates at a high rate resulting in quasispecies during the long period of chronic infection. This variability may provide the mechanism for immune escape during chronic infections as well as the lack of protective immunity against reinfection by the same strains or heterotypic strains <sup>(131)</sup>. Development of an effective neutralizing vaccine against HCV is complicated by this high level of genetic variation among HCV strains.

Recently, it has been shown that intramuscular injection of recombinant plasmid DNA containing sequences in an expression format coding for a specific antigen, can induce both humoral and cellular immune responses to that antigen <sup>(132)</sup>. It is relatively easy to make plasmid constructs that express the precise protein of interest, purify the plasmids, inject animals and evaluate their immune responses.

It will be necessary in the future to demonstrate efficacy at least against viruses of the same subtype as the originating vaccine strain. Protection against other HCV subtypes and types may be obtained either by a demonstrable cross-protective activity

or by the inclusion of vaccine antigens from additional genotypes. Enduring immunity also needs to be demonstrated in vaccinees. At the T cell levels, it seems likely that HCV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses are important for resolution of acute infection and prevention of chronicity. Since HCV-associated disease is mainly a sequelae of chronic infection, an HCV vaccine would still be effective if it only stimulates recovery from acute infection. Given the known heterogeneity of HCV, it will obviously be important to vaccinate and prime CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses to many conserved viral epitopes in association with multiple MHC alleles representative of the general population. It is then to be hoped that the vaccine will be effective at priming an earlier, stronger and broader immune response in newly infected vaccinees than would occur in unvaccinated individuals, leading to viral clearance in most vaccinees. It is also to be hoped that in chronically infected HCV patients, boosting and broadening the humoral and cellular immune responses may ameliorate the course of infection and disease either alone or in combination with antiviral drugs. In conclusion, there are many questions that remain to be answered in this challenging area of HCV research and development<sup>(133)</sup>.

### **EBV based plasmid as a tool for gene transferring**

Transduction of genes into mammalian cells is an important technology in both basic biology and medicine, not only because it offers a powerful strategy for the study of functions of given genes but also because it may be practically applicable to gene therapy. A number of vector systems as well as transduction methods have been devised, including physical transfection of plasmid vectors and viral vector-mediated gene transfer<sup>(134)</sup>. Although viral vectors usually give relatively high transduction efficiency, i.e. a large proportion of the cells can be transduced, they are sometimes disadvantageous because of the possibility of generating the replication competent viruses or disturbing transcriptional regulation of cellular genes by chromosomal integration of the proviral DNA<sup>(134-135)</sup>. However, with plasmid vectors, virtually no such undesirable situation takes place, but the potential problem is that the gene transduction efficiency is usually poor<sup>(136)</sup>.

To detect the bioactivity of a desired gene product, several gene expression systems have been established. The Epstein-Barr virus (EBV) genome has yielded reagents that have been useful in vector design. The EBV-based vectors are plasmid vectors carrying the EBNA-1 gene and the oriP region from EBV as *trans* and *cis* elements for DNA replication, respectively<sup>(137-138)</sup>. Through the binding to oriP, EBNA-1 facilitates the retention and replication of the plasmid DNA, making EBV vector a very efficient expression vector<sup>(139-142)</sup>. The EBV-based vectors are actually extremely effective in transfecting genes into lympho-hematopoietic cell lines. Even by conventional (non-EBV) plasmid vectors, gene expression occurs 2-4 days after transfection. This expression, however, is faint and transient, i.e. the expression level decreases during the following several days<sup>(143)</sup>. This is due to the degradation and/or dilution of the plasmids in the cells. Stable transfectants will be generated if the plasmid DNA is accidentally integrated into the chromosomes, but this is actually a rare event, but usually  $1/10^3$ - $10^5$ <sup>(143-145)</sup>. In contrast, EBV vectors replicate in the cells through the binding of EBNA-1 to oriP, which results in the very strong expression both at transient and stable phases. In other words, once a cell expresses the marker genes, it may keep on carrying the plasmid and expressing the genes as long as the selection is continued. In the EBNA-1 expressing cells, the preexisting EBNA-1 molecules may bind to the oriP element immediately after the entry of the plasmid into the cells, facilitating its retention, nuclear localization and replication<sup>(139-142)</sup>.

In this study, we used the modified EBV plasmid (pNS). This plasmid did not have EBNA-1 gene as shown in Figure 4. It still worked well because the target expressing cells were BLCLs which stably expressed EBNA-1 protein. We also compared the efficiency for transfection and expression of pNS vector with modified VR1012 Neo+ which had strong CMV promoter to look for the most suitable vector for BLCL transfection.

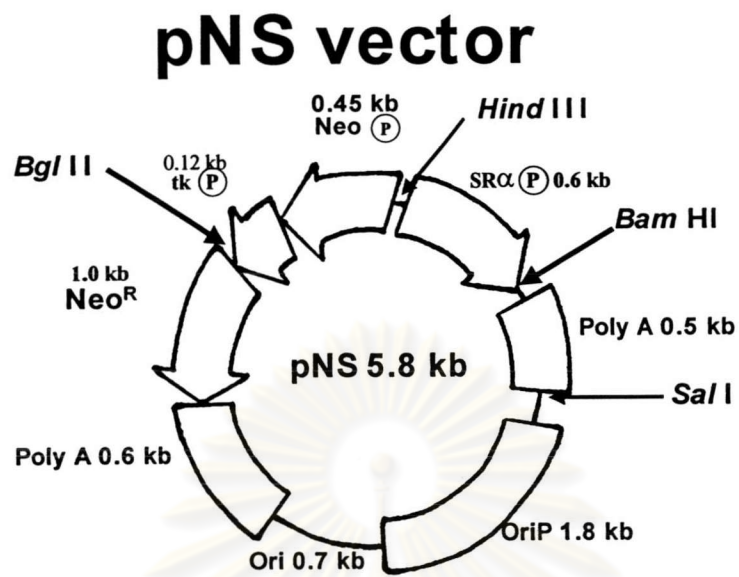


Figure 4 Schematic diagram of pNS vector used in this study. The insertion site is at *Bam*HI.

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