Detection and characterization of the hepatitis C virus

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Detectie en karakterisatie van het Hepatitis C virus

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Contents

List of abbreviations

aa	amino acids
ALT	alanine aminotransferase
AST	aspartate aminotransferase
bp	basepairs
BVDV	Bovine Viral Diarrhoea virus
DNA	deoxyribonucleic acid
bDNA	branched DNA
cDNA	complementary DNA
dpi	days post infection
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbent assay
g-GT	gamma-glutamyltranspeptidase
h	hour
HAV	hepatitis A virus
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDV	hepatitis D virus
HEV	hepatitis E virus
HIV	human immunodeficiency virus
HoChV	Hog Cholera virus
HVR	hypervariable region
IRES	internal ribosomal entry site
LIA	line immuno assay
LiPA	line probe assay
min	minute
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
NANBH	non-A, non-B hepatitis
NS	non-structural
nt	nucleotides
NTP	nucleoside triphosphate
ORF	open reading frame
PBS	phosphate buffered saline
RCPA	RNA capture PCR assay
RIBA	recombinant immunoblot assay
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction.
TMACl	Tetramethylammoniumchloride
UTR	untranslated region

Nucleotide codes (IUPAC-IUB)

A	adinine
C	cytosine
G	guanine
T	thymine
Y	C or T
R	G or A
M	A or C
K	G or T
S	G or S
W	A or T
H	A, C, or T
B	G, T, or C
V	G, C, or A
D	G, A, or T
N	G, A, T, or C

"De beschrijving van het onderzoek heeft, in de meeste gevallen, slechts in de verte iets te maken met de wijze waarop het zich in werkelijkheid heeft afgespeeld.
Er zijn bloed, zweet en tranen geïnvesteerd. Van dat alles is in het eindresultaat, het gepubliceerde artikel, niets terug te vinden. Het is zorgvuldig gezuiverd van iedere zweem zweetlucht, van iedere menselijke emotie."

Prof. Dr. Ir. A. van den Beukel, in: De dingen hebben hun geheim, gedachten over natuurkunde, mens en God. Ten Have, Baarn

"The true objective is to take the chaos as given and learn to thrive on it."

Peters T. (1987), in: Thriving on chaos, handbook for a management revolution. Pan Books, London.

voor Corine, Ellen en Rik

CHAPTER 1

The biology of the Hepatitis C virus

a review of the literature

A condensed version of this chapter has been accepted for publication in the Journal of Medical Virology as a review article.

Introduction

The term hepatitis literally means 'inflammation of the liver'. Hepatitis can be caused by toxic substances, metabolic disorders or viral infections. Most clinical hepatitis cases have a viral etiology. Viral hepatitis appears to be an ancient disease (Deinhardt, 1991) and has been recognized as infectious since centuries. Evaluation of hepatitis outbreaks led to the hypothesis that more than one type of viral hepatitis existed. Initially, two different forms of viral infectious agents were identified (Krugman et al., 1962, 1967). Hepatitis A virus (HAV) is orally transmitted and has a short incubation period. The hepatitis B virus (HBV) is transmitted parenterally and has a long incubation period. HAV contains a single stranded RNA genome and belongs to the Picornaviridae, whereas the Hepatitis B virus (HBV) contains a partially double stranded DNA genome and is classified as a Hepadnavirus (Tiollais et al., 1985). In the early 1980s, an RNA virus designated as hepatitis Delta (Rizetto, 1983) was recognized. This defective hepatotropic virus requires helper functions provided by HBV or another Hepadnavirus (Wang et al., 1986).

After the discovery of hepatitis A and B viruses as etiological agents for viral hepatitis, sensitive serologic assays were developed to diagnose the presence of these viruses. However, after excluding hepatitis B surface antigen positive donor blood, posttransfusion hepatitis remained a significant problem. Post-transfusion hepatitis is rarely caused by hepatitis A virus (Wick et al., 1985). Therefore, another form of hepatitis was postulated in the early 1970s, which could not be explained by hepatitis A or B virus infections, and was provisionally named 'Non-A, Non-B hepatitis' (NANBH, Prince et al., 1974, Alter et al., 1975, Feinstone et al., 1975). The average incidence rate among transfused recipients was 7% in the United States (Hollinger, 1990). Due to lack of an identification method for NANBH, diagnosis was based on exclusion of HAV and HBV as well as all other possible viral causes, like hepatitis Delta virus, Epstein-Barr virus and Cytomegalovirus. The observed incubation period of NANBH was intermediate between HAV and HBV. Although most of the patients with NANBH remain anicteric and asymptomatic, a significant number of cases progresses towards chronicity (Koretz et al., 1976) and liver cirrhosis (Realdi et al., 1982).

Non-A, non-B hepatitis could be subdivided into parenterally transmitted (PT-NANBH, predominantly caused by blood transfusion) and enterically transmitted (ET-NANBH) forms. The majority of cases of ET-NANBH are associated with the hepatitis E virus provisionally classified as a Calicivirus (Reyes et al., 1990).

Initial characterization of PT-NANB

Many attempts were made to identify the causative agent of PT-NANBH. Multiple reports of 'specific' antibody systems appeared and several virus-like particles were visualized by electron microscopy (Spichtin, 1985), but these findings were not confirmed. Other unproven reports claimed that PT-NANBH may be caused by a serologically silent HBV variant (Wands, 1982), or a retrovirus (Seto et al., 1984, Itoh et al., 1986).

After the recognition of PT-NANBH, an animal model was developed. It was possible to infect chimpanzees experimentally by infectious human sera (Hollinger et al., 1978, Alter et al, 1978, Tabor et al., 1978), or contaminated factor IX concentrates (Wyke et al., 1979) and antihemophilic (factor VIII) materials (Bradley et al., 1979). Extensive in vivo studies in chimpanzees were performed by Bradley and co-workers at the Centers for Disease Control (CDC), mainly with a contaminated Factor VIII preparation (Bradley et al., 1979). As in humans, alanine aminotransferase (ALT) elevations were observed after an incubation period of approximately 7 weeks and the infectious agent could be transmitted to other chimpanzees by serum. Some animals showed persistent and fluctuating ALT levels indicative of continuing liver dysfunction, and suggesting the development of chronic PT-NANB infections (Bradley et al., 1981).

Different types of ultrastructural alterations have been consistently observed in hepatocytes infected by the chloroform-sensitive PT-NANBH. The most prominent alteration is the formation of tubular structures in the cytoplasm. Although these observations were used as a pseudodiagnostic tool, the ultrastructural alterations are not specific for PT-NANB and have been

also observed in several kinds of cells infected with RNA viruses (Bradley, 1991a, de Vos et al., 1983, McCaul et al., 1985).

There are several indications for the existence of more than one agent causing PT-NANBH: multiple attacks of hepatitis in one patient (Mosley et al., 1977), the occurrence of a long and a short incubation period, different ALT patterns (Pastore et al., 1985), the results of cross-challenge studies in experimentally infected chimpanzees (Bradley et al., 1980, Yoshizawa et al., 1981, Tabor et al., 1984), and different ultrastructural observations (Shimizu et al., 1979). However, interpretation of these findings was often controversial (Brotman et al., 1985). The existence of two distinct agents could be most clearly demonstrated by their different sensitivity to chloroform. The infectivity of the most predominant PT-NANBH causing agent could be destroyed by chloroform extraction, whereas the other agent was resistant. Chloroform sensitivity suggested the presence of an envelope containing lipid (Bradley et al., 1983). It was also possible to purify infectious particles by ultracentrifugation through sucrose gradients (Bradley et al., 1985). Filtration studies of infectious plasma indicated that the diameter of the agent was between 30 and 60 nm (He et al., 1987). These experimental data led to the hypothesis that the causative agent was a small enveloped virus, and might be a togavirus (Bradley et al., 1985). Togaviruses are small enveloped viruses, sensitive to chloroform, containing a single stranded, positive sense RNA genome.

Molecular biological characterization of Hepatitis C virus

After the initial physico-chemical characterization of the putative viral agent, molecular biological tools were applied to identify its genome. The prerequisite for this was the availability of a well defined sample, with an infectious titer of at least 10^6 viral particles per ml. Dilution of infectious materials revealed that the infectious titer is often less than 10^4 Chimpanzee Infectious Dose (CID)/ml (Bradley, 1984). Sufficiently high-titered chimpanzee plasma was obtained by pooling

several samples obtained at the periodic ALT peaks (Bradley, 1991b). This material was used for further cloning experiments by Houghton and co-workers.

The working hypothesis, based on all physico-chemical data obtained previously, was that the etiologic agent was a small, enveloped virus, with a sedimentation coefficient that permitted concentration by ultracentrifugation, and presumably contained an RNA genome. From the high-titer plasma pool the viral particles were pelleted by centrifugation and RNA was purified. RNA was converted into c(omplementary) DNA by random priming (anticipating that the viral RNA has no poly (A) tail) and cloned into a bacteriophage lambda expression library.

By screening the expression library, using serum of chronically infected patients as a source of virus-specific antibodies, one clone, designated as 5-1-1, was isolated. Analysis of this clone showed that it was not host-derived, expressed a virus-specific antigen and hybridized specifically to RNA extracted from infectious plasma and liver (Choo et al., 1989). Clone 5-1-1 was used as a probe to isolate overlapping clones and a larger clone, C100-3, was constructed and expressed in yeast for the development of antibody detection assays (Kuo et al., 1989). This first generation antibody assay allowed identification of most PT-NANB cases. The newly identified agent was designated hepatitis C virus (HCV). The entire nucleotide sequence of the virus was rapidly determined (Houghton et al., 1990) via isolation of overlapping clones and revealed the presence of an RNA genome of approximately 9400 nucleotides.

Genomic organization of Hepatitis C virus

The RNA genome of hepatitis C virus, shown in figure 1, contains a large translational open reading frame (ORF) which spans almost the entire genome and encodes a polyprotein of at least 3010 amino acids (Choo et al., 1991a). At the 5' terminus an untranslated region of 341 nt is present. A 3' untranslated region of variable length with either a poly(U) (Chen et al., 1992, Tanaka et al., 1992, Kato et al., 1990a, Takamizawa et al., 1990, Okamoto et al., 1991, 1992a) or a poly(A) tail (Han et al., 1991) tail has been reported.

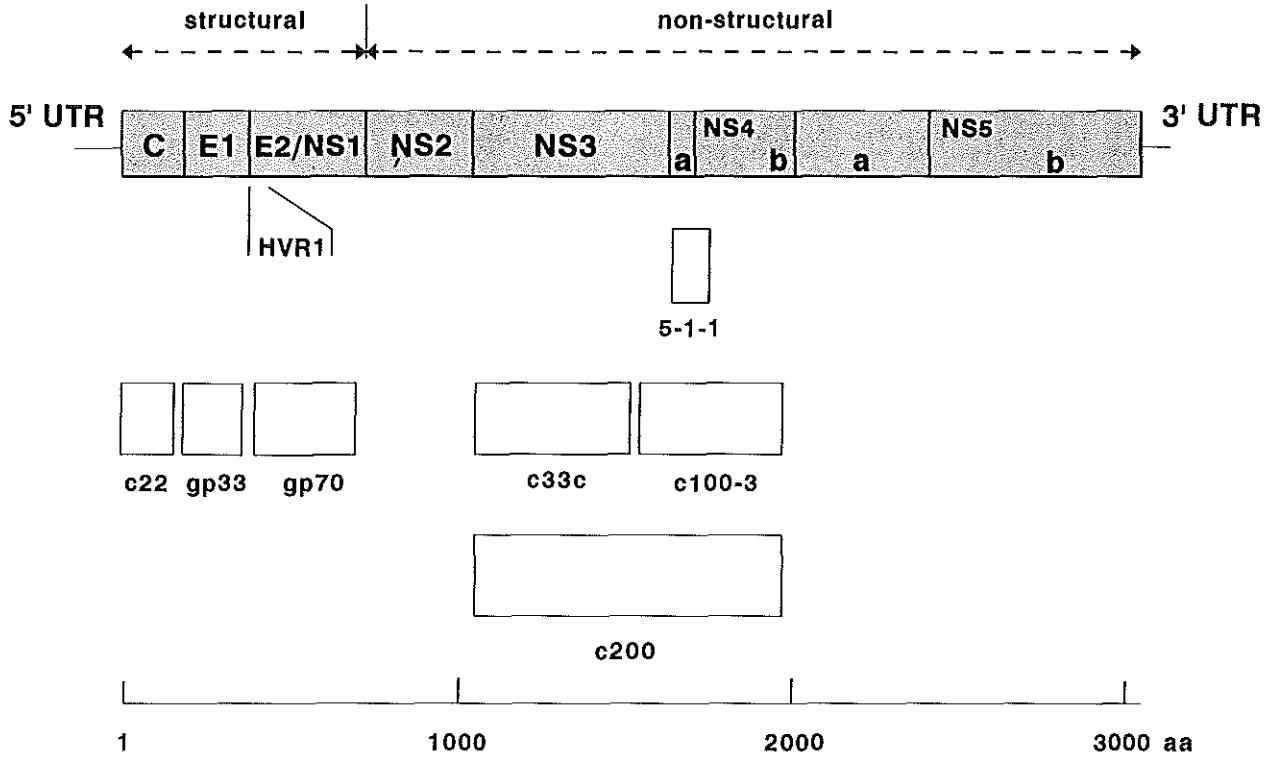

Figure 1. Outline of the HCV genome

At present several chimpanzee and human derived isolates have been completely sequenced (Choo et al., 1991a, Kato et al., 1990, Takamizawa et al., 1991, Okamoto et al., 1991, 1992a, Chen et al., 1992, Tanaka et al., 1992). There is remarkable similarity between the HCV genome and that of Flavi- and Pestiviruses. First, the sizes of the polyproteins encoded by the positive-stranded RNA genomes of HCV and the Flavi- and Pestiviruses are similar (± 3000, ± 3400 and ± 4000 amino acids (aa) respectively). Second, there are a number of small dispersed amino acid sequence homologies with the non-structural proteins 3 and 5 of the Flaviviruses (Miller and Purcell, 1990, Choo et al., 1991a). The first (aa 1230-1500) has residues in common with putative nucleoside triphosphate (NTP) binding helicases encoded by Flavi-, Pesti-, and plant Potyviruses, with HCV showing the greatest similarity with the Pestiviruses. The second region is upstream of the NTP-binding helicase and has similarity to the putative (chymo)trypsin-like serine protease from Flavi- and Pestiviruses. The third region (aa 2703-2739) contains the characteristic Gly-Asp-Asp motif, which is highly conserved among viral-encoded RNA-dependent RNA polymerase. These homologies are co-linear, i.e., occurring at the same relative position in HCV, Flavi- and Pestiviruses (Choo et al., 1991b). Third, the hydrophobicity profiles of the polyproteins from these viruses are remarkably similar (Takamizawa et al., 1991, Choo et al., 1991a), except the putative envelope coding region. The polyprotein encoded by Flaviviruses is processed into three structural proteins: the core or nucleocapsid (C), the prematrix (pre-M), the envelop (E) protein) and 7 non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). The structural proteins form the viral particle, whereas the non-structural proteins are involved in maturation and replication of the virus. Based on the similarity between the hydrophobicity profiles, a similar organization of the HCV genome has been postulated (Takamizawa et al., 1991, Choo et al., 1991a,b). The structural proteins are at the N-terminal part of the polyprotein, while the non-structural proteins originate from the C-terminal region (figure 1).

An interesting difference between the polyproteins from HCV and Flaviviruses is the relatively small size of the HCV structural protein region. In the Flaviviruses a structural membrane protein (pre-M) is located immediately downstream of the core protein, and is followed by a viral envelope protein (E). HCV seems to lack the pre-M region and encodes a much smaller

E protein. Furthermore, in the Pestiviruses the domain corresponding to non-structural protein 1 of Flaviviruses encodes the structural major envelope protein gp53-55.

HCV, Flavi- and Pestiviruses are all small enveloped viruses and therefore HCV is currently classified as a separate genus within the Flaviviridae (Heinz, 1992).

The different regions of the HCV genome

From the 5' end to the 3' end the following distinct regions are identified on the HCV genome. The location of each region is shown in figure 1 and table 1 summarizes some important characteristics of the individual proteins.

The 5' untranslated region, translation and replication

The 5' UTR seems to comprise approximately 341 nt (Han et al., 1991, Chen et al., 1992), although many shorter sequences have been reported (Okamoto et al., 1990a, Choo et al., 1991a, Kato et al., 1990a, Takamizawa et al., 1991). It is difficult to define the precise terminus of the RNA genome as preliminary data suggested the existence of truncated RNA molecules (Han et al., 1991) *in vivo*. The 5' terminus of the major truncated RNA corresponds to position -196 (the polyprotein starting at position 1). The 5'UTR of Flaviviruses is shorter (Brinton and Dispoto, 1988, Heinz, 1992) and no significant sequence homologies with HCV exist when complete genomes are compared. However, there is striking resemblance (Takeuchi et al., 1990) between the HCV 5' UTR and the 5' terminal region of the Pestiviruses, Bovine Viral Diarrhoea virus (BVDV) and Hog Cholera virus (HoChV). There are several small co-linear regions of 8-37 nt with a homology of more than 70% between HCV, BVDV and HoChV. The overall homology is approximately 50% (Houghton et al., 1991). In addition, the 5' UTRs of these viruses are similar in length. Like poliovirus, which possesses a 5' UTR of similar size, the HCV 5' UTR contains up to five small ORFs, but it is unknown whether these are actually translated and what the function of the products might be (Yoo et al., 1992). Alignment of sequences obtained from new

genotypes of HCV (see below) indicate that these small ORF's are not conserved. Picornavirus 5' UTRs allow cap-independent translation of the polyprotein and contain a *cis*-acting internal ribosomal entry site (IRES; Pelletier and Sonenberg, 1988). The secondary structures of the 5' UTRs of HCV, BVDV, and HoChV have been determined by thermodynamic modelling and enzymatic cleavage of RNA by specific ribonucleases (Brown E.A. et al., 1992, Tsukiyama-Kohara et al., 1992). These studies revealed the presence of a large conserved stem-loop structure in the proximal part of the 5' UTR, which serves as a putative IRES (figure 2).

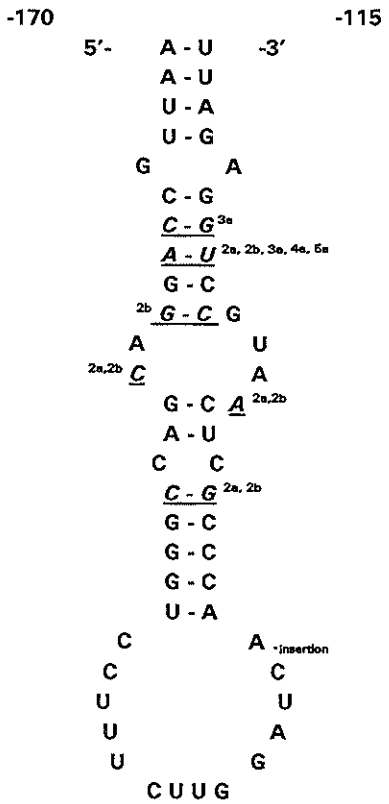

Figure 2. Predicted secundary structure of nucleotides -170 to -115 in the 5' untranslated region. This structure is conserved among all genotypes of HCV. Positions with type-specific covariants are marked with corresponding (sub)type indications.

The secondary structure of the Flavivirus 5' UTR seems to be different, although a distal stem-loop structure is present (Brinton and Dispoto, 1988). Studies using mono- and dicistronic RNA constructs revealed important functional features of the HCV 5'UTR (Wang et al., 1993), although other reports are contradictory (Yoo et al., 1992). Based on current knowledge it is most likely that the translation mechanism of the HCV genome is similar to that of Picornaviruses (Jang et al., 1989). Polyprotein translation is cap-independent and initiated at an IRES within the 5' UTR, proximal to the initiator AUG codon of the polyprotein. The initiation of translation seems inhibited by the presence of a 5' terminal 27-nt sequence capable of forming a stable hairpin structure (Han et al., 1991). No hairpin structures have been described for the 5' terminus of Pestiviruses, but are highly conserved in Picornaviruses.

The replication mechanism of HCV is poorly understood. Like in Flavi- and Pestiviruses, it appears that HCV RNA replicated by a direct RNA-to-RNA mechanism. It has been impossible to detect DNA-intermediates in serum or liver of infected individuals. The NS5 region (see below) encodes a protein with demonstrated RNA-dependent RNA polymerase activity (Chung and Kaplan, 1992). Antigenomic (minus) RNA strands have been detected in liver (Takehare et al., 1992) and plasma (Fong et al., 1991, Shindo et al., 1992a) of patients. The detection of minus-strand RNA in plasma is surprising, because replication is presumably taking place in the liver. Minus-strand RNA could also be detected in peripheral blood mononuclear cells (Müller et al., 1993, Zignego et al., 1992, Wang et al., 1992c), which suggests an extrahepatic replication site for HCV. Due to technical difficulty, detection of minus-strand RNA should be interpreted with care (Willems et al., 1993). As antigenomic strand synthesis should start at the 3' terminus, some small repeated 6-8 bp sequences, present in both the 5' and 3' UTRs may be involved in secondary structure formation or cyclization of the RNA genome (Inchauspe et al., 1991). The precise mechanism of viral replication remains to be elucidated.

Since the NS5 encoded protein is the last to be translated from the viral genome, translation and replication are linked processes. Efficient translation may lead to high levels of the viral replicase. This, in turn may lead to higher production of infectious viral particles, infection of more hepatocytes and eventually a higher virus titre in the circulation.

Table 1. HCV encoded proteins

gene	function	aa pos.	N-glyc[1]	name	N-cleavage[2]
C	capsid	1-191	-	p22	-
E1	envelope	192-383	+	gp33	signalase
E2/NS1	envelope	384-809	+	gp70	signalase
NS2	metalloproteinase	810-1009	-	p23	signalase?
NS3	ser-protease+ helicase	1010-1619	-	p72	metalloproteinase
NS4(a+b)	?	1620-2016	-	p10 p27	NS3 protease NS3 protease
NS5(a+b)	replicase/ polymerase	2017-3033	-	p58 p70	NS3 protease NS3 protease

[1]N-glyc. indicates presence of putative Asn-linked glycosylation sites.
[2]N-cleav. indicates the putative enzyme responsible for proteolytic cleavage at the N-terminus of the indicated protein. (Hijikata et al, 1993b and Grakoui et al.,1993).

The core region

The HCV core protein (p22) is highly basic due to the presence of a large amount of Arg and Lys residues (±20%). It also contains many Pro (12%) residues (Houghton et al., 1991, Choo et al., 1991a). The core protein lacks N-glycosylation sites. The 20 C-terminal amino acids are highly hydrophobic and act as a signal sequence (von Heijne, 1986, Takeuchi et al., 1990a,b, Kumar et al., 1992, Hijikata et al., 1991a). P22 may be subject to further processing to generate mature C protein to be incorporated in the virion (Hijakata et al., 1991a). P22 has an RNA binding capacity.

Specific association between P22 and HCV genomic RNA accounts for the formation of nucleocapsid particles (Takahashi et al., 1992a,b). P22 also contains several highly conserved immunoreactive epitopes. Both recombinant core proteins and synthetic peptides, representing linear core epitopes, can be used for efficient detection of antibodies in most patient sera (Katayama et al., 1992, Nasoff et al., 1991, Chiba et al., 1991, Sällberg et al., 1992a,b, Harada et al., 1991, Hosein et al., 1991, Ishida et al., 1993, Okamoto et al., 1990b, 1992d).

The envelope regions E1 and E2/NS1

E1 and E2/NS1 encode the putative viral envelope proteins. Glycoprotein gp33-36 (table 1) has a protein backbone of 18-21 kDa (Bradley et al., 1992) and corresponds to the envelope glycoprotein gp33 of BVDV and gp25 of HoChV of the Pestiviruses and the envelope proteins (M/E) of the Flaviviruses (Weiner et al., 1992, Houghton et al., 1991), although there are no significant sequence homologies. The protein contains many potential N-glycosylation sites, and glycosylation of gp33 has been demonstrated in a cell-free translation system (Hijikata et al., 1991a). Transmembrane transport of gp33 is presumably facilitated by an N-terminal stretch of approximately 20 hydrophobic amino acids that functions as a signal sequence, recognized by cellular signalase, thereby also cleaving the core protein C22 from the precursor protein. Between residues 350-383 a stretch of hydrophobic amino acids is present that contains a putative membrane anchor for the core protein (Heinz, 1992), and a signal sequence for E2/NS1 (Hijikata et al., 1991a).

Glycoprotein gp72, encoded by E2/NS1 (table 1) has a protein backbone of 38 kDa (Bradley et al., 1992, Houghton et al., 1991, Kato et al., 1991, Hijikata et al., 1991a). A repetitive amino acid sequence has been observed between residue 471 and 511: Pro-$(X)_5$-Pro-$(X)_6$-Pro-$(X)_6$-Pro-$(X)_6$-Pro-$(X)_6$-Pro-$(X)_5$-Pro (Kato et al., 1991). A Cys residue is present in each of the last 4 intervening (X) sequences, and their positions seem conserved. These findings suggest the importance of the complex secondary and tertiary structures of this glycoprotein. The corresponding region in Flaviviruses encodes a non-structural homodimeric protein which is not present in extracellular virions (Spaete et al., 1992, Lee et al., 1989). In contrast, the analogous

Pestivirus region encodes the major envelope protein (gp53-55). The HCV gp72 is more heavily N-glycosylated than corresponding Flavivirus NS1 and Pestivirus gp53-55. Because the primary sequence homology between HCV is higher with Pestiviruses than with Flaviviruses, it has been postulated that gp72 may be a structural envelope protein rather than a non-structural protein (Houghton et al., 1991, Spaete et al., 1992, Rümenapf et al., 1993). The full-length form of E2/NS1 is not secreted from the cell, like the Flavivirus NS1 protein, but remains cell-associated, presumably by the presence of a single transmembrane anchor, since the C-terminally truncated E2/NS1 protein is rapidly secreted (Spaete et al., 1992). While the C-terminus is membrane-anchored, the N-terminus of E2/NS1 is located at the outside, and would be subject to surveillance of the immune system. E1 and E2/NS1 proteins appeared to form complexes (Ralston et al., 1993), but whether intermolecular disulfide bondings exist, remains unclear (Grakoui et al., 1993b).

Comparison of the available E2/NS1 sequences revealed the presence of 2 hypervariable regions (Hijikata et al., 1991b, Kato et al., 1992a,b, Weiner et al., 1991a). The first hypervariable region (HVR1) is located directly downstream of the putative cleavage site between E1 and E2/NS1 (between residue 383 and 384, Hijikata et al., 1991a) and covers the 30 N-terminal residues (384-414) of the E2/NS1 protein (Weiner et al., 1991a,b). HVR1 seems to lack a conserved secondary structure and resembles the V3 loop of human immunodeficiency virus 1 gp120 (Weiner et al., 1992). Specific antibody reactions were detected against peptides corresponding to linear epitopes in HVR1, indicating that the N-terminal part of the E2 region encodes antigenically distinct variants, subjected to immune selection (Weiner et al., 1992, Lesniewski et al., 1993). The observed hypervariability may result from sequential mutations leading to escape from immunereactivity (Kato et al., 1993a). Although HVR1 shows hypervariation during most chronic infections, conservation has also been observed (van Doorn et al., 1993c). This is presumably caused by absence of an adequate immune response against the HVR1 epitope due to absence of selection pressure favouring new mutants. HVR1 can be used to specifically distinguish HCV variants, which provides an important tool to study treatment and epidemiology of HCV (Weiner et al., 1993, Okada et al., 1992).

The second hypervariable region (HVR2) is much smaller and comprises amino acid positions 474 to 480 (Hijikata et al., 1991b, Kato et al., 1992a,b). The variability of gp72 may have significant implications for the development of a protective immune response and is important with regard to vaccine strategies.

Based on all these data, it is very likely that gp33 and gp72 are the actual envelope proteins, although their presence in the mature HCV virion envelope has never been shown.

The NS2 and NS3 proteins

The non-structural protein 3, designated p72, shows limited amino acid homology with corresponding Flavi- and Pestivirus proteins (Miller and Purcell, 1990). The N-terminal one-third of NS3 resembles a trypsin-like serine protease, similar to that found in Flaviviruses (Chambers et al., 1990) and Pestiviruses. If the Ser-1165 in the proposed catalytic site is replaced with another amino acid, the proteolytic activity of the p72 is abolished (Tomei et al., 1993, Eckart et al., 1993). This protease is responsible for some processing steps of the precursor polyprotein into the mature proteins. All four cleavages that occur C-terminally of the protease domain (NS3-NS4a, NS4a-NS4b, NS4b-NS5a, and NS5a-NS5b) where abolished by modification of His-1083 and Ser-1165. These cleavage sites show several common features, which probably determine the substrate specificity of the NS3 protease (Grakoui et al., 1993a).

Cellular proteases, such as signal peptidase, are involved in sequential cotranslational processing of the cleavage sites on the N-terminus of E1, E2, and possibly NS2. The NS2-NS3 cleavage is mediated *in cis* by a Zn^{2+} requiring metalloproteinase. The region between residue 898 to 1233, which comprises parts of both NS2 and NS3, was shown to be essential for the detection of this second viral protease (Hijakata et al., 1993a). NS3 acts *in cis* on its own C-terminus, but the remaining 3 C-terminal sites can be only processed by NS3 *in trans* (Tomei et al., 1993). There is also homology between NS3 and nucleoside triphosphate-binding helicase enzymes that are presumably involved in the unwinding of the dsRNA replicative intermediate necessary for genome replication (Houghton et al., 1991).

The NS4 protein(s)

The function of this protein, which is processed into NS4a and NS4b, remains unknown. The proteins contain highly immunogenic epitopes and several predicted transmembrane regions. The first cloned C100 antigen, which is used in most antibody detection assays, is an unnatural antigen spanning the C-terminus of NS3, NS4a and the N-terminus of NS4b.

The NS5 protein(s)

The non-structural protein 5 is processed into NS5a and NS5b. The latter shows significant amino acid sequence homology with the putative RNA-dependent RNA replicase from Carnation Mottle Virus (CARMV; Miller and Purcell, 1990). This is remarkable as CARMV is a member of the Carmovirus group of plant viruses. Furthermore, the NS5 region contains a Gly-Asp-Asp sequence (res. 2737-2739), which is a well-known characteristic sequence motif for all RNA-dependent RNA polymerases of positive-strand RNA viruses (Koonin, 1991). Therefore, NS5b encodes the viral replicase, and the activity of this enzyme has been demonstrated in vitro recently (Chung et al., 1992). The function of NS5a is unknown, but the protein contains important B-cell epitopes (DeLeys, 1993).

Processing of NS5 into NS5a and b is observed in Pestiviruses but not in Flaviviruses (Selby 1993). The C-terminal part (p70) contains the consensus polymerase sequences.

It has been suggested (Okamoto et al., 1992b) that in some isolates a secondary structure of the genomic RNA exists at the C-terminal part of the NS4 region that could possibly form an IRES. This IRES would be located just upstream of a common in frame ATG codon, allowing earlier production of NS5 encoded replicase. Consequently, this would accellerate the replication rate of the genomic RNA. However, no experimental data are yet available to confirm this hypothesis.

The 3' untranslated region

The stop codon of the large open reading frame is preceeded by a stable DNA stem-loop structure at the 3' end of the NS5 region (van Doorn, unpublished observations). This secundary structure might be equally important for proper termination of translation as the structure at the 5' UTR is for initiation of translation. The reported 3' untranslated regions show significant variations, both in length and in sequence. The most important difference is the observation of a poly(rA) tail in some isolates (Choo et al., 1991a, Han et al., 1991), whereas in other isolates (Kato et al., 1990a, Takamizawa et al., 1991, Chen et al., 1992, Okamoto et al., 1991, 1992a, Tanaka et al., 1992) a poly(U) tail is found. It is unknown whether the reported 3' terminal sequences are complete. A small putative hairpin structure has been described (Inchauspe et al., 1991) but the calculated thermodynamic stability is low and significance remains obscure.

Morphology of the HCV virion

Little is known about the precise morphology of the HCV viral particle, as the virus has never been seen (Overby, 1993). Since 1975, a variety of particles have been observed by electron microscopy and were attributed to NANBH (Spichtin, 1985), but these findings could not be substantiated.

Recent filtration studies, using the quantitative reverse transcription polymerase chain reaction (RT-PCR) technique to detect the viral genome, estimated a particle diameter between 30 and 38 nm (Yuasa et al., 1991).

Several authors described the determination of the buoyant density of the HCV virions by gradient ultracentrifugation, followed by testing the fractions for infectivity, presence of core antigen or HCV RNA. (Bradley et al., 1991c, Carrick et al., 1992, Takahashi et al., 1992, Miyamoto et al., 1992, Hijikata et al., 1993b). Usually, two virus containing fractions were obtained, with buoyant densities of $<$1.03-1.08 g/ml and 1.11-1.25 g/ml, respectively. Differences between several reports may be either due to differences in the fractionation protocols or may indeed reflect the density variation among HCV isolates. The average density of the

presumptive HCV particles (±1.08 g/ml) is similar to that of BVDV (1.09-1.15 g/ml), but slightly different from that of the Flaviviruses (±1.20 g/ml, Bradley et al., 1991c).

The HCV containing fractions with the lower density probably represent the native HCV virions (Takahashi et al., 1992a). Higher density fractions putatively contain nucleocapsid particles, consisting of core protein and RNA. Icosahedral-shaped particles with a diameter of approximately 33 nm, were observed after specific capture onto anti-core monoclonal antibody coated grids, indicating the presence of native core antigen in such particles. Furthermore, these nucleocapsid particles could be released from the larger virus-like particles by treatment with mild detergents (Takahashi et al., 1992a,b).

Interaction between HCV virions and anti-HCV immunoglobulins can affect the buoyant density and seems associated with infectivity of the sample (Hijikata et al., 1993b). HCV with low infectivity, associated with immunoglobulins was present in high density ultracentrifugation fractions, whereas HCV with high infectivity, without immunoglobulins, was predominant in low density fractions. Furhtermore, examination of serial samples from an HCV-infected chimpanzee revealed changes in buoyant density during the course of infection, parallel to the development of anti-HCV. These data suggest that HCV is bound to anti-HCV antibodies in antigen-antibody complexes.

In gradient fractions, spherical particles with a diameter of 36-62 nm were observed, which morphologically resemble toga- and Flaviviruses, with a unit membrane envelope with surface projections. Inside hepatocytes, 37-40 nm particles were found, often in lysosomes (Abe et al., 1991a). Toga-like viruses with a diameter of 50-60 nm had been observed previously in nuclei of hepatocytes from patients with fulminant non-A, non-B hepatitis (Fagan et al., 1989).

Although these findings are suggestive for the nucleocapsid particles, there is still no direct proof that the observed larger virus-like particles indeed represent native HCV virions.

Sequence variation and genotyping

During replication of the viral genome by the NS5 encoded RNA-dependent RNA polymerase, errors may occur, resulting in mutations. Some mutations are tolerated whereas others may abolish or hamper proper functioning of the resulting virion. By this method of 'error and trial', variant RNA genomes evolve. The coexistence of several closely related sequences in a single patient has been observed (Oshima et al., 1991, Okada et al., 1992, Murakawa et al., 1992, Tanaka et al., 1992).

The distribution of mutations along the genome is not random. First, this depends on the impact of each mutation on the efficacy of viral replication. Mutations that disturb the virus life cycle will obviously not be detected in the population. The immune system of the infected host offers the second level of selection pressure on mutant viral genomes.

The 5' UTR is remarkably conserved, whereas E2/NS1 contains a hypervariable region at the N-terminus. The overall mutation rate of the HCV RNA genome has been estimated between 0.9 and 1.92 x 10^{-3} base substitutions per genome site per year (Ogata et al., 1991, Okamoto et al., 1992b, Abe et al., 1992). However, it is difficult to assess the real mutation rate. First, the sequence analysis is performed on independent clones or directly on amplified DNA. Both methods have statistical limitations to detect rare sequences in a population of slightly different viral genomes. Secondly, most patients are not infected by a single virion. Especially infectious materials derived from plasma pools, such as factor VIII preparations, may contain a large variety of HCV sequences. Consequently, the observation of closely related HCV sequences (designated as 'quasispecies') in a single patient at different timepoints may be caused by both selection and mutation of sequences present in the inoculum. Sequentially emerging HCV sequences, able to escape neutralizing immune activities (Kato et al., 1993), possibly play a crucial role in the persistence of viremia during chronic HCV infection.

The first full-length HCV sequence was derived from a chimpanzee infected with a contaminated American Factor VIII concentrate (Houghton et al., 1990, Choo et al., 1991a,b). This sequence became known as the HCV prototype, HCV-US or HCV-1, to which each new

Table 2. Nomenclatures for HCV genotyping

representative sequence	Stuyver	Bukh/ Purcell	Chan/ Simmonds	Cha/ Urdea	Enomoto/ Date	Okamoto
HCV-1, H	1a	1a	1	I	K-PT	I
HCV-J, HCV-BK	1b	1b	1	II	K-1	II
HC-J6	2a	2a	2	III	K-2a	III
HC-J8	2b	2b	2	III	K-2b	IV
E-b1, Ta	3a	3a	3	IV	nc	V
Tb, Tr	3b	nc	nc	IV	nc	VI
EG-16, GB116, Z44a	4a	4	nc	nc	nc	
GB549, Z1	4b	4b	nc	nc	nc	nc
GB809, Z6	4c	4c	nc	nc	nc	nc
DK13	nc	4d	nc	nc	nc	nc
SA1, BE95	5a	5a	5	V	nc	nc
HK1	nc	6a	6	nc	nc	nc

Origin of reported isolates:
HCV-1: Choo et al., 1990; H: Ogata et al.,1991 and Inchauspe et al., 1991; HCV-J: Kato et al.,1990; HCV-BK: Takamizawa et al., 1991; HC-J6: Okamoto et al., 1991; HC-J8: Okamoto et al., 1992; E-b1 : Chan et al., 1992; Ta: Mori et al., 1992; Tb: Mori et al.,1992; Tr: Chayama et al., 1993; EG-16: Simmonds et al., 1993a; GB116, 549, 809: Stuyver et al., 1993c; Z1, Z4, Z6, DK13: Bukh et al., 1993b; SA-1, Bukh et al., 1992a; BE95: Stuyver et al., 1993c; HK1: Simmonds et al., 1993a. nc= not classified

sequence is compared. Later, full-length sequences from Japanese HCV isolates (HCV-J, Kato et al., 1990a and HCV-BK, Takamizawa et al., 1991) were shown to be significantly different from the prototype. The different strains were provisionally designated as 'American' and 'Japanese' strains. Since then, several full-length (Okamoto et al., 1991, 1992a, Chen et al., 1992, Tanaka et al., 1992) and numerous partial nucleotide sequences (e.g., Kubo et al., 1989, Kato et al., 1990b, Enomoto et al., 1990, Mori et al., 1992) from different geographic areas were reported.

Comparison of various HCV sequences (Kubo et al., 1989, Simmonds et al., 1990, Li et al., 1991) revealed the presence of similar viral variants in different areas of the world, and initiated attempts to classify the HCV isolates into distinct groups. Initial classifications were based on various genomic regions, and the associated nomenclatures using individual isolate names were confusing (table 2).

Analysis of NS5 sequences among Japanese isolates also revealed the presence of two types, designated as K1 and K2 (Enomoto et al., 1990). K1 is closely related to the American prototype HCV-1. K2 is more homologous to two other sequences, HC-J6 and HC-J8 (Okamoto et al., 1991, 1992a) and could be further divided into K2a and K2b with an homology of about 80%. Based on these findings, a phylogenetic tree containing four branches was proposed, comprising genotypes I-IV (Okamoto et al., 1992a). This tree was extended with new branches representing types V and VI by HCV isolates from Thailand (Mori et al., 1992) and similar European isolates (Chan S.W. et al., 1992). This provisional classification system was based on chronological order of discovery and did not properly reflect the relationship between sequences.

Recently, a new classification system was proposed (Chan S.W. et al., 1992, Stuyver et al., 1993a, Simmonds et al., 1993a) based on sequence alignments of a large number of HCV sequences derived from several parts of the genome. Construction of phylogenetic trees revealed that comparison of 5' UTR, core, NS3 or NS5 resulted in similar classifications of HCV isolates into types and subtypes (Chan S.W. et al., 1992). Isolates belonging to the same subtype show an average nucleotide homology of 88%, those belonging to different subtypes 78% and isolates of different types about 68% (Weiner et al., 1991b, Stuyver et al., 1993a). The proposed nomenclature is more functional as it is associated with the degree of homology between sequences, and new (sub)types can easily be added to the system. Table 2 shows a summary of the different classification systems.

Geographic distribution of HCV genotypes is subject of current investigations (Takada et al., 1993, Bréchot and Kremsdorf, 1993, Cha et al., 1992, Simmonds et al., 1993a, Chan S.W. et al., 1992, Kleter et al., 1994, Stuyver et al., 1993a, Chayama et al., 1993, Li et al., 1991). Genotype 1b seems to be the most abundant variant worldwide, with a high incidence in Japan and

Western Europe. Prototype 1a is relatively rare in these areas, but more prevalent in America. Genotype 2 is found less often in Europe than in Japan and in China. Genotypes 3 to 6 have been identified only recently and their distribution is not known at the moment. It is likely that most HCV genotypes coexist worldwide, but the incidence may vary considerably between locations. There are indications that different (sub)types of HCV may have different clinical relevances, such as the success of interferon treatment (Okamoto et al., 1992c, Pozzato et al., 1991, Yoshioka et al., 1992, Takada et al., 1992) and may be the risk to develop cirrhosis or hepatocellular carcinoma. Also different HCV genotypes may evoke different serological responses (Chan et al., 1991, van Doorn et al., 1993b). Sequence diversity may result in the production of type-specific antibodies in infected hosts. Genotype-specific antibodies have been found to react with the core protein (Machida et al., 1992), the NS4 protein (Stuyver et al., 1993b, Simmonds et al., 1993b, Tsukiyama-Kohara et al., 1993) and the E1 envelope protein (Maertens et al., 1993). However, specificity is still limited and prevents routine applications, RNA analysis remains the only available technique for HCV genotyping.

Currently, genotyping can be performed by type-specific PCR primers (Okamoto et al., 1992c, Chayama et al., 1993), direct sequencing (Kleter et al, 1994, Cha et al., 1992), restriction fragment length polymorphism (Nakao et al., 1991), Southern blot hybridization with type specific probes (Enomoto et al., 1990, Takada et al., 1993) or reverse hybridization to (sub)type specific probes (Stuyver et al., 1993a).

As each distinct genotype seems to be maintained throughout the HCV genome, the possibility to use the 5' UTR for genotyping has been investigated recently and offers several advantages. First, many 5' UTR sequences from isolates obtained in various parts of the world have been determined (Bukh et al., 1992a, Simmonds et al., 1993a, Kleter et al., 1994). Sequence alignments revealed extreme conservation in this region, except two more variable motifs that allow discrimination between (sub)types (Stuyver et al., 1993a). These motifs, as shown in figure 2, are partly complementary and can form a stable double-stranded RNA stem-loop structure (Brown E.A. et al., 1992, Tsukiyama-Kohara et al., 1992). Genotypic-specific variations in these motifs often occur as covariants, thereby leaving the secundary structure unchanged (Simmonds et

al., 1993a). This implicates structural and functional conservation of this sequence. Furthermore, the 5' UTR is used extensively for determination of viremia by the polymerase chain reaction (PCR), and subsequent use of the same PCR product for genotyping is complementary to routine HCV diagnostics.

Clinical aspects and Epidemiology of HCV infection

Most HCV infections are subclinical, often even asymptomatic, and up to 25% of the patients (Alter, 1990) develop jaundice. If symptoms are present during the acute phase they are typical of viral hepatitis and indistinguishable from infection with HAV or HBV. Fulminant hepatitis due to HCV infection is extremely rare (Theilmann et al., 1992, Liang et al., 1993a, Féray et al., 1993). Elevated levels of liver-specific transaminases, such as ALT, in the serum, provide an important tool to assess liver injury due to viral infection. Different patterns of ALT elevations have been observed in HCV infections. A serum ALT peak is often detected after about 8 weeks. It is assumed that complete clearance of the infection after acute resolving hepatitis is possible, although it has been speculated that eventually all HCV infections may become chronic (Prince et al., 1993). It has been established that a relatively high percentage (50-75%) of HCV infected individuals proceed towards chronicity. Chronic hepatitis is defined by ALT elevations persisting for more than a year after the onset of hepatitis (Alter, 1990). Development of cirrhosis has been documented but the factors affecting this course have been incompletely identified. HCV infection has been found to be a significant risk factor for the development of hepatocellular carcinoma in a number of studies (Saito et al., 1990, Kew et al., 1990, Tsukuma et al., 1993, Bukh et al., 1993) although causality has not been proven. Progression towards liver cancer is slow, and it usually follows the development of cirrhosis, although it may evolve more rapidly in elderly patients (Yano et al., 1993).

Most infected patients develop antibodies against HCV epitopes, after a variable period after infection. Individual antibody responses may be very different due to the variability of the

virus or the host immune system. HCV RNA is detectable (see below) very rapidly after infection, usually within one week. During chronic infection, anti-HCV and HCV RNA may become transiently undetectable, indicating that the viral titer may vary considerably. Figure 3 illustrates a typical clinical profile of a chronic HCV infection.

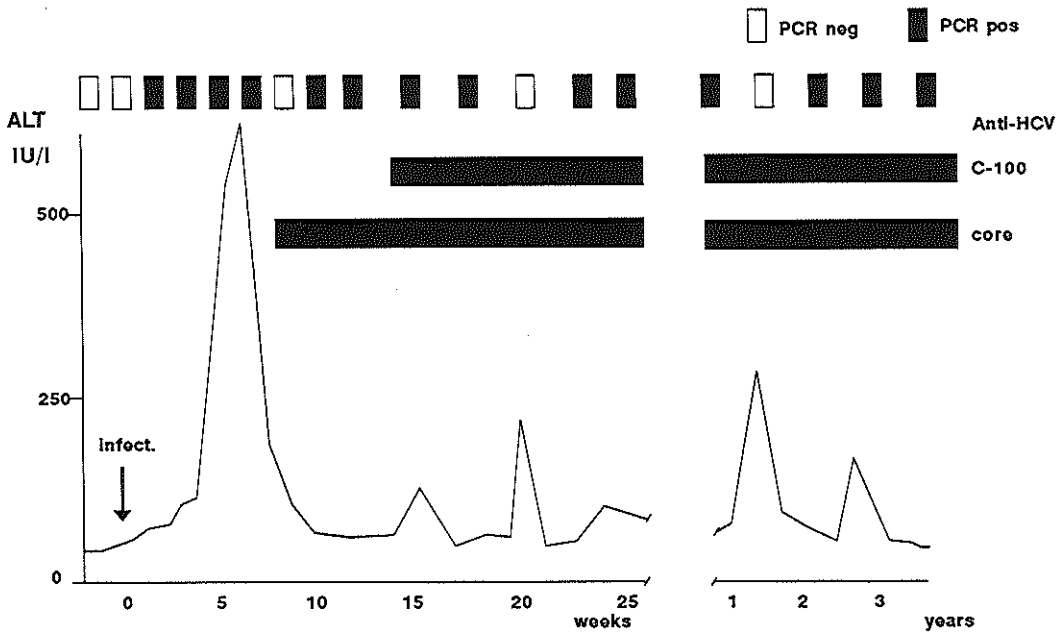

Figure 3. Schematic representation of a clinical pattern of a chronic HCV infection.

Percutaneous or parenteral transmission of HCV has been documented extensively (Aach et al., 1991, Mosley et al., 1991, Esteban et al., 1991, Alter, 1991, Donahue et al., 1992, van der Poel et al., 1990).

The positivity rates of antibodies against HCV in normal blood donors range from approximately 0.2% in Northern Europe to 1.5% in Japan. However, positivity rates as high as 20% in Egypt (Saeed et al., 1991) and 6% in Africa have been described (Alter, 1990). Anti-HCV screening programs for blood donations resulted in a significant reduction of posttransfusion cases (Alter H.J. et al., 1989, Alter, 1990, Japanese Red Cross NANB Research Group, 1991). However, antibody assays have limited sensitivities, and up to 40% of the infectious blood samples may remain undetected (Sugitani, 1992).

Although it has traditionally been considered a transfusion-associated disease, the majority of HCV infections occur outside the transfusion setting (Alter M.J., 1991). Intravenous drug users and hemophiliacs are at high risk for HCV infection. Prevalence of anti-HCV antibodies among these groups can be higher than 50% (Esteban et al., 1989, Stary et al., 1992, Weiland and Schvarcz, 1992). Transmission of HCV by sexual contact seems to be inefficient (Brettler et al., 1992, Kolho et al., 1991, Stary et al., 1992). However, although the sexual transmission is less efficient than for HIV, it may not be unimportant for the spread of the disease (Kao et al., 1992, Alter M.J. et al., 1989, 1991). Coinfection with HCV and human immunodeficiency virus (HIV) increases the risk for sexual HCV transmission considerably, which is important in high risk populations (Stary et al., 1992, Eyster et al., 1991, Botti et al., 1992). Mother-to-child (vertical) transmission seems a rare event (Fortuny et al., 1991, Nagata et al., 1992, Inoue et al., 1992). There may be discrepancies between results of anti-HCV determinations and HCV-RNA assays (Roudot-Thoraval et al., 1993, Lam et al., 1993, Kuroki et al., 1993). Coinfection of HCV and HIV in the mother may increase the risk of HCV transmission to the infant (Giovannini, 1990, Perez Alvarez et al., 1992, Novati et al., 1992). The positive influence of HIV infection on the efficiency of sexual and vertical HCV transmission may be due to increased HCV titers in the circulation, probably caused by reduced immune activity of the host.

Approximately 50% of HCV cases in the USA can be traced to parenteral modes of transmission. The remaining half the cases are due to community-acquired or sporadic transmission. The epidemiology of community-acquired HCV is complicated by the various definitions and differences between studies. The percentages range from 40-50% (Alter MJ, 1991, Bortolotti et al., 1991) to lower than 5% (Hollinger and Lin, 1992, Pohjanpelto, 1992). It is often difficult to elucidate the source of infection, as it is unknown whether, e.g., mucosal exposure is a possible route of transmission. Some reports (Abe et al., 1991b, Takamatsu et al., 1990, Wang et al., 1992b, Couzigou, 1993) claim the presence of HCV-RNA in saliva, although others challenge this (Hsu et al., 1991, Fried et al., 1992). Infectivity of saliva was shown in experimental infection in chimpanzees (Abe et al., 1987) but whether HCV transmission via saliva is an important epidemiologic factor remains unknown. Mucosal transfer of HCV might explain the (limited) increased risk for health care workers, such as oral surgeons and dentists (Jochen, 1993, Klein et al., 1991). The presence of HCV in body secretions, such as saliva, probably reflects a high level of viremia in the circulation. The higher the virus load, the higher the risk of transmission of HCV via sexual or household contacts. HCV RNA could not be detected in semen of 6 men with HCV-RNA positive serum (Terada et al., 1992).

Since the infectious titer of HCV is usually much lower than, e.g., HBV, the risk of transmission via needlestick accidents seems very low (Mitsui 1992), although repeated exposures to possibly low-level contaminated equipment, such as dialysis units, increase the risk of HCV transmission (Schlipköter et al., 1992, Chan T.M. et al., 1992)

Initial epidemiological surveys of HCV transmission used first generation anti-HCV tests. Since these tests proved insufficient, data should be interpreted with care. Now that second and third generation antibody assays, as well as sensitive HCV-RNA detection are available, epidemiological studies are more reliable. HCV seems to have a worldwide distribution, different from the distribution of HBV. Whether this reflects other or additional modes of transmission of HCV is not clear (Yano, 1992).

In the near future the importance of the viral (sub)type in epidemiology will be studied extensively. Preliminary results show that type 1 is the most common HCV genotype worldwide

and subtype 1b is less sensitive to treatment with interferon-α. Also the putative correlation between prolonged HCV infection and the development of hepatocellular carcinoma (Kew et al., 1990) must be investigated in more detail.

The recent discovery of HCV and HEV and the availability of sensitive assays for hepatitis viruses A through E allows identification of most hepatitis cases. However, some cases still cannot be attributed to any of these viruses and remain undiagnosed (Favorov et al., 1992, Arankalle et al., 1993, Thiers et al., 1993, Azar et al., 1993).

Diagnosis of Hepatitis C virus infection

Specific diagnosis of HCV instead of diagnosis by exclusion is an important achievement since the discovery of HCV in 1987. Identification of infected individuals prevents them from donating blood and limits the spread of the disease. Elimination of contaminated materials, derived from human blood, such as factor VIII, also prevents infection in recipients of these products. Furthermore, current diagnostic tools allow efficient monitoring of antiviral therapy.

Detection of specific antibodies against viral epitopes (Kuo et al., 1989) is an indirect indication of viremia. However, antibody detection is relatively cheap, easy to perform in large quantities and often correlates well with the presence of the virus itself (van der Poel et al., 1991, Reuter et al., 1992). However, there are also important limitations. Firstly, antibodies are only produced after a variable interval following infection. During this 'seronegative window' the patient is infectious and should be excluded from blood donations. Secondly, there are considerable differences between individual immune responses of patients, also affecting the length of this window.

Several antibody detection systems were developed during the past years, with increasing sensitivity and specificity. The first cloned antigen of HCV, designated C100-3, was produced in E. coli as a fusion protein with SuperOxide Dismutase (SOD; Kuo et al., 1989) and was used in the first generation anti-HCV assays, together with the smaller 5-1-1 protein. Both sensitivity and

specificity of this test were shown to be insufficient (van der Poel et al., 1991, Yatsuhashi et al., 1992, Wong et al., 1990, McFarlane et al., 1990). Anti-C100-3 negative blood may still be infectious and transmit HCV to recipients (Inaba et al., 1991). This is probably due to immunological incompetence in some individuals (Hosada et al., 1992) and to sequence variation in NS4.

Second generation assays were developed to overcome these problems and additional HCV antigens were included in the tests, such as recombinant NS3/NS4 (C33c, C200) and core (C22c) proteins. These assays detect more true positives among various risk groups than the first generation assays (McHutchinson et al., 1992). Routine diagnosis is performed by Enzyme Immunoassays (EIA) in microtiter plates. Initial positive samples can be further confirmed by a neutralization-based test or by immunoblot assays, such as RIBA (recombinant immunoblot assay; van der Poel et al., 1991, Tanzi et al., 1992) and LIA (Line Immuno Assay, Chan et al., 1991, Peeters et al., 1993). These confirmation assays are necessary to exclude false positive reactions (Chaudhary et al., 1991) and also allow the discrimination of the immune response against distinct viral epitopes. An increasing number of anti-HCV tests are based on the use of synthetic peptides (Okamoto et al., 1990b, Hosein et al., 1991, Kotwal et al., 1992, Ching et al., 1992, Brown D. et al., 1992) or chimeric recombinant antigens (Chien et al., 1992), resulting in more efficient diagnosis of HCV infection.

Anti-core assays are particularly useful for HCV diagnosis, as these increase the detection rate and limit the 'seronegative window' after inoculation (Vallari et al., 1992, Katayama et al., 1992, Okamoto et al., 1990b). The nucleotide sequence of the core gene is relatively well conserved, allowing serological detection of different HCV genotypes.

Detection of HBV surface and core antigens (HBsAg and HbcAg) in the bloodstream are important diagnostic tools. Direct detection of HCV proteins in blood seems possible (Takahashi et al., 1992b) but due to the limited sensitivity and usually low HCV titers this is not applicable for routine diagnostics.

The only method to determine viraemia is specific detection of HCV RNA. This can be achieved by direct detection of circulating HCV RNA by slot blot hybridization to radiolabelled

riboprobes (Hu et al., 1992) or inside hepatocytes by *in situ* hybridization (Blight et al., 1992). However, the sensitivities of these methods are limited. Currently, HCV RNA is assayed by a combined reverse transcription polymerase chain reaction (RT-PCR). This method is extremely sensitive and allows detection of minute quantities of HCV RNA even in plasma pools. During the early phase of primary HCV infection, HCV-RNA is the only diagnostic marker of infection (Kato et al., 1993b). Although detection of HCV RNA correlates very well with viraemia, is superior to anti-HCV detection (Sugitani et al., 1992) and can predict infectivity, it is important to note that lack of PCR positivity does not absolutely rule out infectivity (Garson et al., 1990, Barbara et al., 1993).

A variety of RT-PCR assays for HCV RNA detection have been described and allow HCV RNA detection in plasma and liver samples (Weiner et al., 1990, Wolff et al., 1992). Most systems employ primers from the highly conserved 5' UTR (Cha et al., 1991), since these allow detection of all known genotypes with a single set of primers. To enhance sensitivity the size of the PCR product can be reduced (Garson et al., 1991) and nested PCR can be performed (Okamoto et al., 1990c, Lin et al., 1992, Gretch et al., 1993). The use of degenerated primers allows detection of all currently known genotypes (Stuyver et al., 1993b). Using sense cDNA primers, RT-PCR allows specific detection of antigenomic HCV RNA, indicative of viral replication (Takehara et al., 1992).

Despite the availability of a wide range of RNA isolation protocols (van Doorn et al., 1992, Ulrich et al., 1993) and PCR primer combinations, the reliability of RT-PCR for HCV RNA detection remains a problem (Zaaijer et al., 1993). Both sensitivity and specificity need to be improved considerably to compare experimental results from different laboratories. Furthermore, the handling and storage of blood samples, suitable for HCV RNA detection must be standardized to prevent degradation of HCV RNA (Busch et al., 1992, Wang et al., 1992a, Cuypers et al., 1992).

Quantitative analysis of HCV RNA can be achieved by two methods at present. Competitive PCR employs a modified RNA transcript or cDNA which is processed along with the sample of interest in serial dilutions (Kaneko et al., 1992, Hagiwara et al., 1993). This method is

tedious and very contamination prone. More importantly, competitive PCR should use known quantities of control molecules not only during PCR but throughout the entire detection method, to assess influences from isolation and cDNA procedures.

An alternative procedure is not based on RT-PCR, but uses specific capture and branched DNA to detect HCV RNA specifically (Urdea, 1993). Although the latter method is easy to perform, relatively contamination proof and omits exponential enzymatic DNA amplification, sensitivity is limited to approximately 350.000 equivalents/ml.

HCV RNA quantitation will become more important, since it can have significant clinical implications (Lau, 1993), such as the efficacy of interferon-α treatment in chronic HCV infections (Shindo et al., 1991, Weiland et al., 1993) and the relation between viral titer and infectivity of body secretions.

Therapy of HCV infection

In 1986, Hoofnagle et al. treated chronic non-A, non-B hepatitis patients with interferon alpha-2b (Hoofnagle et al., 1986) and succeeded in inducing remission in eight of the ten patients. Several other trials with interferon treatment followed (Thomson et al., 1987, Davis et al., 1989, Di Bisceglie et al., 1989). Based on improvement of serum ALT, and liver histological abnormalities, these studies suggested a therapeutic effect of interferon. Interferon has pleiotropic effects that are antiviral and immunomodulatory. Long-term follow-up of interferon-treated patients showed that normalization of ALT levels is often accompanied by disappearance of detectable HCV RNA from the serum (Shindo et al., 1992b). Failure to normalize ALT levels during interferon treatment is consistent with resistant viral infection. Treated patients can be classified into different categories. Complete responders show complete normalization of ALT and disappearance of circulating HCV RNA. Partial responders exhibit a significant reduction, but not a complete normalization of ALT, and HCV RNA often remains detectable at a lower level. Nonresponders do not reveal any improvement after interferon treatment. During posttherapy follow-up, a high percentage of the

initial responders show a relapse of disease, with returned elevated ALT and detectable serum HCV RNA (Shindo et al., 1992b, Douglas et al., 1993).

The reported success rates of interferon treatment differ significantly. The relevances of the dose and administration schedule of interferon are not completely clear (DiBiceglie et al., 1989, Benelux Multicentre Trial Study Group, 1993, Liang et al., 1993b). The severity of chronic HCV infection, assessed by liver histology, is negatively correlated with the outcome of interferon treatment (Uchida et al., 1993). Differences between HCV genotypes may also have important implications. Sensitivity of genotype 1 to interferon treatment seems lower than that of genotype 2 (Takada et al., 1992, Yoshioka et al., 1992, Pozzato et al., 1991). This phenomenon is possibly correlated with the infectious titre of HCV before onset on treatment. Patients with a high quantity of HCV RNA in the serum are less likely to respond than patients with low HCV RNA concentrations (Lau, 1993, Hagiwara et al., 1993, Yamada et al., 1992). Levels of viremia of the different HCV genotypes seems to be significantly different (Kleter et al., unpublished observations). Furthermore, the mode of acquisition is found to be an important determinant of the level of HCV viremia (Lau 1993).

In conclusion, interferon therapy can lead to complete and sustained response, but the success depends on multiple virus- and host-related factors.

Developments

In the past 5 years substantial progress has been made after the discovery of HCV. The virus itself has been characterized intensively and our insight in its biology increased tremendously. However, the picture is far from complete. Little is known about the mechanism of virus persistence during chronic infections, evolution of the viral genome, and response to antiviral treatments.

The clinical diagnosis of HCV-RNA must be improved and standardized, and quantitative analysis should be included, given the recent indications of clinical importance. Antibody screening must be improved and should possibly lead to serologic typing assays to differentiate

between the various HCV genotypes. A special problem are seronegative samples that remain undiagnosed by the current screening assays. Direct detection by molecular biological methods could solve this problem but due to technical limitations, this approach seems not feasible yet.

The question remains whether protective immunity against HCV can be achieved, and how vaccine strategies should be developed. Initial vaccination experiments permit little optimism and progress towards an effective vaccine might take many years.

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Outline of the thesis

This thesis describes several aspects of hepatitis C virus (HCV) infections. The first part focuses on the diagnosis of HCV viraemia, by detection of the viral RNA genome. Chapter 2 describes the development of a novel technique for isolation and purification of HCV RNA from blood samples. This RNA-capture method is applied to investigate the presence of HCV RNA in chimpanzee plasma samples that were obtained at least 2 years after experimental Non-A, non-B hepatitis (NANB) inoculations. HCV RNA results are compared with results from a Line Immuno Assay (LIA), which detects anti-HCV antibodies. Chapter 3 focuses further on the HCV RNA-capture technique described in chapter 2. The method is optimized, and the performance is evaluated by comparing specificity and sensitivity with a highly sensitive standard HCV RNA detection technique in a coded panel of patient samples. HCV RNA results are correlated with anti-HCV determination by a Line Immuno Assay (LIA).

The second part of this thesis (chapter 4) describes experimental HCV infections in a cohort of chimpanzees. The presence of HCV RNA is monitored by the optimized RNA-capture assay and anti-HCV is measured by the LIA in serial serum samples obtained before and during HCV infection. In order to determine the genomic stability of this particular HCV isolate during chronic infection or after transmission to other chimpanzees, sequence variation of the putative hypervariable region in the E2/NS1 region is studied.

The third part of the thesis focuses on the recognition of HCV genotypes. HCV isolates can be classified into different genotypes based on sequence variation of the RNA genome. Whether the highly conserved 5' UTR allows sufficient discrimination between HCV genotypes is studied in chapter 5. In chapter 6, 5' UTR based genotyping with a reverse hybridization Line Probe Assay (LiPA) is evaluated by comparison with sequence analysis. Furthermore, the correlation between the viral genotypes and detection of antibodies to specific HCV epitopes is investigated to assess the possibility of serological discrimination. To substantiate genotyping by 5' UTR analysis, a large cohort of HCV patients was genotyped by LiPA, as described in chapter 7. For each genotype several isolates were further characterized by sequence analysis of both the 5' UTR and the N-terminus of the core region. Classifications of isolates based on the 5' UTR and on the core region are compared.

Finally, chapter 8 summarizes and discusses the results and conclusions from this thesis.

CHAPTER 2

Hepatitis C virus antibody detection by a line immunoassay and (near) full length genomic RNA detection by a new RNA-capture polymerase chain reaction

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Abstract

A rapid and simple RNA-capture polymerase chain reaction assay (RCPA) for detection of hepatitis C virus (HCV) is described. The assay detects specifically the presence of (near) full length genomic RNA of HCV by capturing HCV-RNA at the 3' terminus on magnetic beads, followed by cDNA synthesis and PCR with 5' end specific primers.

Sera were obtained from 30 chimpanzees inoculated with non-A, non-B hepatitis from various sources, 28-122 months after infection. The sera were tested for the presence of HCV-RNA by RCPA and for HCV antibodies by the Line Immuno Assay (Inno-LIA). Both tests were compared and show a high degree of agreement. Screening of 30 chimpanzee sera revealed either clearing of the virus below detection level (22/30) or development of an HCV carrier state (8/30). Only 1 of 11 LIA-indeterminate samples was positive by RCPA. As the RCPA is more sensitive, it can be used to test for the presence of HCV in sera that are classified as indeterminate by the LIA.

The outcome of the infection seems to be independent of the nature of the inocula, suggesting that the individual immune response could determine either clearing of the virus or the development of chronic infection.

Introduction

Hepatitis C virus (HCV) is the major cause of post-transfusion non-A, non-B Hepatitis (NANB) (Choo et al., 1989; Kuo et al., 1989). The virus possesses a positive-sense, single stranded RNA genome of about 9,500 nucleotides, and the genome organization closely resembles that of Flavi- and Pestiviruses (Choo et al., 1991; Miller and Purcell, 1990). As the virus is transmitted by blood and blood products, highly sensitive methods for detection of the virus are important. The estimated average titre of HCV in blood is relatively low (Bradley et al., 1983; Simmonds et al., 1990; Bradley et al., 1985), and so far it has been impossible to assay viral proteins directly in serum. Specific antibodies against several viral protein components can be detected by ELISA.

These, however, provide indirect information about the presence of virus in the sample. On the other hand, viral RNA can be detected directly by cDNA synthesis and by PCR.

Most protocols for isolation of viral RNA from serum involve phenol extractions and ethanol precipitation of total RNA, with considerable risk of contamination in the PCR reactions. Furthermore, considerable amounts of material are lost during the process. These factors hamper routine testing of larger numbers of samples in clinical laboratories. A very simple method for specific purification of full length genomic HCV-RNA was developed by selective RNA-capture, with low risk of contamination. The purification process eliminates phenol-extraction and precipitation steps completely and requires minimal sample handling. Furthermore, the RCPA allows the specific detection of (near) full length HCV-RNA only.

A number of chimpanzees have been used for NANB studies during the past decade. These chimpanzees, with well-documented NANB infections were tested for the presence of HCV. The aims of this study were to determine the current HCV status of these animals, and to evaluate the RCPA against the LIA, a confirmatory assay for detection of HCV antibodies. The results of both tests were compared and discussed.

Material and methods

Chimpanzee sera

Chimpanzee serum samples were obtained 28-122 months after inoculation with NANB infectious material from various sources. Several animals have been infected by the material described by Tsiquaye et al. (1980). Other chimpanzees (Hellings et al., 1987) received inocula derived from either a Factor VIII pool, or human serum (personal communication J.A. Hellings, Organon Technika, Boxtel, the Netherlands). Since the majority of the inocula were derived from plasma pools, the presence of several types of viruses in one inoculum cannot be excluded.

All animals developed clear symptoms of acute infection, including elevated serum alanine transaminase and gamma glutamyltranspeptidase and appearance of characteristic alterations in hepatocytes visualized by electron microscopy.

RNA capture

Using the RNA-capture PCR assay, as outlined in figure1, (near) full length HCV RNA was detected. Oligo LD 40 captures genomic RNA, released from viral particles, at the very 3' end. The captured RNA was reverse transcribed at the very 5' end and the cDNA was amplified by PCR using 5' end specific primers NCR1 and NCR2.

Capture oligo LD 40 (5'biotin- GAAAAAAAAAAAAGGGAATGGCCTATTGGC-3'; synthesized by Pharmacia, Woerden, the Netherlands) is complementary to the extreme 3' end sequence (position 9055-9086; numbering starting at first codon of polyprotein) of the HCV genome, as published by Kato et al. (1990). Reverse primer NCR2 (5'-ATACTCGAGGTGCACGGTCTACGAGACCT-3', pos. -2 to -21) and primer NCR1 (5'-GTATCTCGAGGCGACACTCCACCATAGAT-3', pos. -324 to -304) were described by Garson et al. (1990). Paramagnetic streptavidin-coated particles were from Promega (Streptavidin MagneSphere™ particles no. Z524).

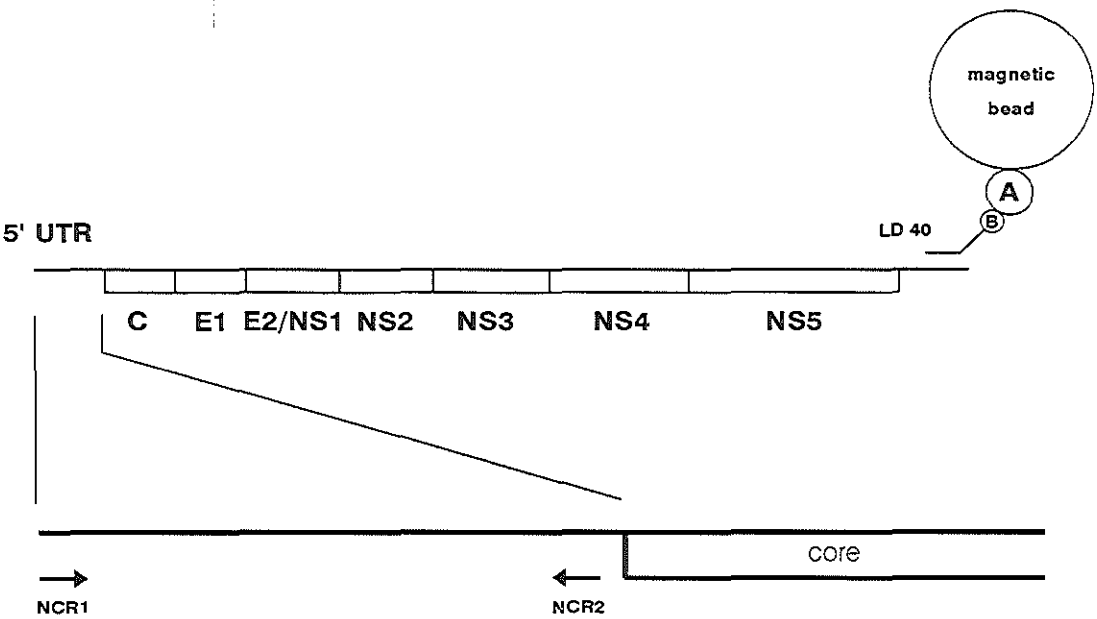

Figure 1. Oligonucleotides LD40 are linked to magnetic beads by a avidin-biotin interaction. LD40 sequence is complementary to the extreme 3' end of the HCV-RNA genome and therefore able to capture the RNA by specific hybridization. cDNA synthesis on purified HCV-RNA is primed by NCR2.

Biotinylated oligonucleotide LD 40 was coupled to the magnetic beads by mixing 100 ng of LD 40 with 0.1 mg beads (prewashed twice with one volume of 0.5xSSC) in 100 µl 0.5xSSC at room temperature and left 30 minutes at room temperature. Uncoupled LD 40 molecules were removed by washing the beads three times with one volume of 0.5xSSC. The beads were washed by attachment to the tube wall in a magnetic stand (Promega). Finally, the oligobeads were resuspended in 0.5xSSC at a concentration of 0.1 mg/500 µl and stored at 4°C.

The actual RNA capture protocol was modified after Jakobsen et al. (1990). Fifty µl of serum was diluted in 800 µl lysisbuffer (0.5 M LiCl, 0.1 M Tris-HCl pH 8.0, 0.01 M EDTA, 1% SDS, 5 mM DTT) and incubated at 50°C for 20 min. 500µl of the oligobeads suspension was added and incubated further on ice for at least 1 hour, mixing slowly every 15 minutes without vortexing. After RNA capture, beads were washed five times with 500 µl volumes of ice-cold buffer; twice with LiCl washing buffer (0.15 M LiCl, 0.01 M Tris-HCl pH 8.0, 1 mM EDTA, 0.1% SDS.), twice with 0.5xSSC and once with 0.1xSSC. After the last washing step, the tubes were centrifuged very briefly and all fluid was removed.

cDNA synthesis.

Immediately after RNA capture and washing, the beads were resuspended in 16 µl of water containing dATP, dCTP, dGTP and dTTP and reverse primer NCR2, heated to 75°C for three minutes and snap-cooled on ice. 9 µl of a mixture, containing buffer, RNasin and reverse transcriptase were added, giving a total volume of 25 µl with final concentrations of 50 mM Tris-HCl pH 8.0, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 800µM each dNTP, 10 pmoles of reverse primer, 0.25 units/µl RNasin (Promega) and 8u/µl M-MLV reverse transcriptase (Gibco-BRL). After mixing, the suspension was incubated at 40°C for at least 1 hour, and mixed every 15 minutes.

cDNA amplification by PCR

From 5-10 µl of the cDNA reaction mix, including magnetic beads, were included in a 100 µl PCR reaction, containing 10 mM Tris pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.1% Triton X-100, 10 pmoles of primers NCR1 and NCR2 and 1.5 units of Taq DNA polymerase (Promega). The sample was covered with mineral oil. The PCR consisted of 40 cycles of 1 min

95°C, 2 min 42°C, 3 min 72°C. Fifteen μl samples were analyzed on 1% agarose gels, blotted to Hybond N^+ membranes (Amersham), hybridized to a labelled [NCR1-NCR2] 320 bp amplified DNA fragment and autoradiographed, according to standard procedures (Sambrook et al., 1989).

Line Immuno Assays

HCV antibodies were detected by the Inno-LIA (Line ImmunoAssay, Innogenetics N.V., Belgium; Pollet et al., 1991). Assays were carried out according to the manufacturer's instructions. Briefly, 10 μl of serum was diluted 1/100 and incubated with a LIA antigen strip, which exposes several highly specific HCV antigens from the core, NS4 and NS5 regions of the polyprotein (see also figure 2). After staining, the antibody reaction against every line was read semi-quantitatively. Three positive control lines consist of various amounts of human IgG whereas one line is anti-human IgG as sample addition control.

Results

Thirty chimpanzee sera were tested for the presence of HCV. Using the LIA system, antibodies against highly specific antigens derived from the NS4, NS5 and core region of HCV were detected. With the new RNA-capture PCR assay, the presence of near full length HCV genomic RNA was assayed. Specificity of the RCPA was determined by using magnetic beads with or without oligo LD40 to capture RNA from an HCV containing serum sample. A positive signal was obtained in the presence of LD40, whereas without LD40 a signal was not visible after autoradiography (results not shown). The results of both HCV antibodies and HCV-RNA tests are summarized in table I.

Figure 2 illustrates results of both LIA and RCPA for selected samples. Hybridization with a ^{32}P-labelled specific oligonucleotide probe yielded identical results.

Table 1. Results of LIA and RCPA on chimpanzee sera.

chimpanzee	NS4	NS5	core1	core2	core3	core4	LIA	RCPA	concl.
Inoculum provided by Tsiquaye et al. (1980)									
Peggy(89)	2+	2+	-	±	-	-	+	+	chron.
Phil(82)	2+	2+	-	±	-	±	+	+	chron.
Hans(95)	2+	-	-	±	-	-	+	+	chron.
Riet(95)	1+	-	-	-	-	-	ind	-	res.
Sophie(44)	±	-	-	-	±	-	ind	-	res.
Natasha(67)	±	-	-	-	-	-	ind.	-	res.
Beatrix(46)	±	-	-	-	-	-	ind	-	res.
Walter(67)	±	-	-	-	-	-	ind	-	res.
human serum 1.									
Billy(84)	±	2+	-	-	-	-	+	+	chron.
Factor VIII									
Randy(47)	2+	±	3+	±	1+	2+	+	+	chron.
Thijs(59)	1+	±	-	±	±	-	ind	-	res.
Ton(52)	1+	-	-	-	-	-	ind.	+	chron.
human serum 2.									
Ruud(65)	±	2+	-	-	-	-	+	+	chron.
Cor(80)	±	2+	-	-	-	-	+	+	chron.
Bam-Bam(89)	-	±	-	-	-	-	ind	-	res.
Bie(72)	±	-	-	-	-	-	ind	-	res.
Peer(93)	±	-	-	-	-	-	ind	-	res.
Dirk(80)	±	-	-	-	-	-	ind	-	res.

Chimpanzees Socrates, Jaqueline, Agnetta, Anna-Clara, Zeef, Laurens, Marlies, Barney, Centa, Robin, Bart, and Oscar were negative by LIA and RCPA. ind. = indeterminate, res.= presumably resolved, chron.= chronic carrier, concl. = conclusion. Numbers between brackets after chimpanzee names indicate how many months after infection the tested serum sample was obtained. Chimpanzees are grouped according to the inoculum they received. By comparing color intensity with the internal standards on the LIA strips, individual lines are scored -, ±, 1+,2+,3+ or 4+ according to the indications of the manufacturer.
Samples are classified non-reactive if all lines have a negative scoring. Samples are classified reactive if either one line has a reactivity of 2+ or higher or if at least two lines have a reactivity of at least 1+. A sample is considered indeterminate if it doesn't meet the criteria for non-reactive or reactive. A sample is classified RCPA positive if a NCR1-NCR2 amplified DNA fragment of the right size (320 bp) is detected by autoradiography in duplicate.

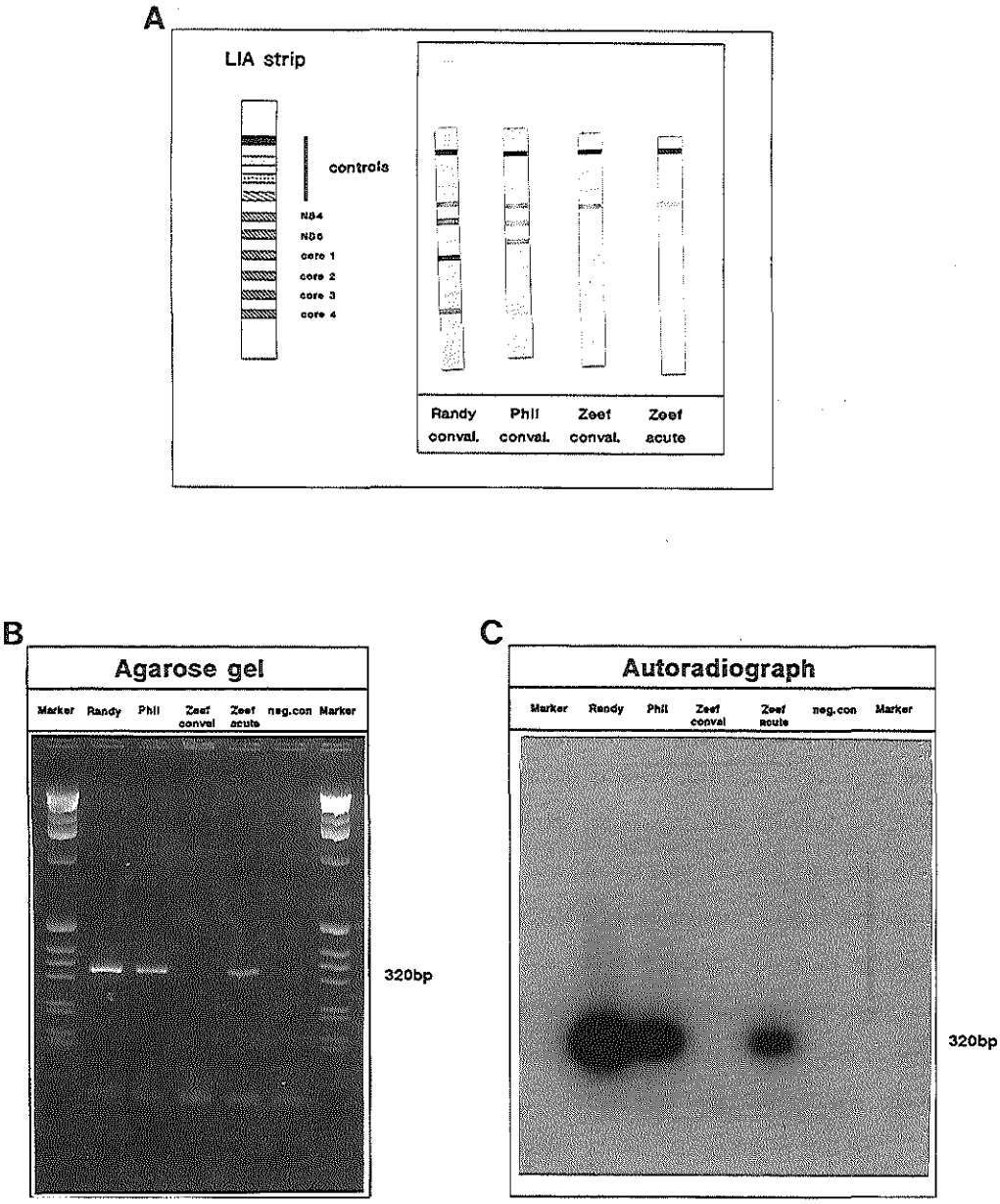

Figure 2. Example of results of Line Immuno Assays (LIA) and RNA-capture PCR assays (RCPA). (a) LIA strips contain 4 control and 6 HCV specific antigen lines. Incubation with serum and consecutive staining detects the presence of captured antibodies against these specific antigens. (b) 15 μl PCR samples are electrophoresed on a 1% TAE-agarose gel, showing specific NCR1-NCR2 amplimers of 320 bp. (c) The same agarose gel was blotted to Hybond N^+, which was hybridized to a random primed ^{32}-P labelled NCR2-NCR1 fragment, followed by autoradiography for 30 minutes.

Discussion

RNA-capture PCR Assay

A new method for detection of (near) full length HCV-RNA in serum is described by HCV-RNA capture specifically at the 3' end and amplifying the 5' end. This method, which resembles the sandwich ELISA in using two separate epitopes on one molecule, has several advantages.

First, the RNA capture method is rapid and simple and eliminates the need for hazardous chemicals such as phenol or guanidiniumisothiocyanate. Secondly, although there is uncertainty about the exact termini of the viral RNA genome, the RCPA detects only HCV-RNA which is at least near full length. When using conventional RNA isolation methods (Chomczynski and Sacchi, 1987), even separate detection of both 3' and 5' ends of the HCV-RNA is not a proof that they are derived from the same viral genome. So far, there is no evidence for the existence of defective HCV particles, carrying only a fragment of the genome. The capture assay described may also be a good method to investigate this issue. A third important advantage of the method is the relatively low risk of contamination during RNA purification with material amplified earlier. The specific isolation of HCV molecules is based on sequences not present in the amplified fragments, as the 3' end sequence is captured, whereas the amplification primers are detecting the 5'end of the HCV-RNA. A fourth advantage is the specific purification of HCV-RNA, whereas most other methods use total RNA or total nucleic acid from plasma, which may increase the number of nonspecific annealing reactions during cDNA synthesis and PCR considerably. By using a specific capture oligonucleotide together with two specific PCR primers, the overall specificity of the test is very high. Positive samples indeed show very clean bands on gel with hardly any background and results may be improved further by increasing the stringency of washing of the HCV-RNA on the beads, e.g., by prolonged washing at elevated temperature.

Another advantage is that the capture method can be used to purify selectively single stranded RNA or DNA from various sources, e.g., purification of specific messenger, genomic or subgenomic RNA from viruses in serum. The lithium and SDS present in the lysis buffer obviously inactivate the high levels of ribonucleases present in serum very efficiently. This method may therefore also be effective for whole blood or for biopsy material.

HCV diagnosis by antibody detection

Availability of a reliable and easy HCV detection method is of major importance for routine HCV screening. The first generation anti-HCV tests, detecting antibodies against c-100 antigen only, has been shown to be inadequate (Inaba et al., 1991) and have now been replaced by more sensitive assays. LIA is a confirmatory assay similar to RIBA (recombinant immunoblot assay, Ortho Diagnostics), but using a larger number of other epitopes, with high sensitivity and specificity.

It was shown previously that the LIA (Pollet et al., in press), as well as other systems using synthetic peptides (Hosein et al., 1991), resulted in low numbers of false positive or false negative reactions. The predictive value of LIA for infectivity is therefore better than that of the anti-C-100 test. Significant association between infectivity of anti-C100 positive blood donors and persistence of antibody reactivity has already been established (van der Poel et al., 1990). Furthermore, LIA provides information on the presence of antibodies against specific epitopes. This might be useful for determination of relations between the LIA antibody profiles and clinical progression of the disease.

LIA and RCPA results using chimpanzee sera.

The data presented describe the outcome of HCV infection in a population of chimpanzees tested long after inoculation. Based on biochemical and electron microscopical data obtained before and after inoculation, it was concluded that all these chimpanzees have been infected with NANB hepatitis virus.

The LIA can yield three possible results.

1. Some of the animals reacted positive on LIA, and apparently developed a chronic infection (Billy, Hans, Peggy, Randy, Ruud, Cor and Phil). Phil has been a carrier for almost 10 years now, indicating that a prolonged carrier state is possible without obvious clinical complications, in agreement with earlier reports (Tabor et al., 1980; Ogata et al., 1991).

The agreement between positive LIA and positive RCPA is 100% in this population of chimpanzees. However, there are several remarkable differences between carriers with respect to the LIA antibody profile. Chimpanzee Billy has a low titre of anti-NS4, but a high level of anti-NS5, whereas chimpanzee Hans shows the opposite. Chimpanzees Peggy and Phil have high

antibody levels against both NS4 and NS5. Chimpanzee Randy shows antibodies against all epitopes tested.

Since identical or closely related inocula were used in several chimpanzees, the progression towards chronic infection seems to depend on variation among the chimpanzees. Individual differences in immune response between animals might be reflected in the different LIA antibody patterns.

2. Some chimpanzee serum samples show indeterminate reaction by LIA (e.g., Riet, Ton, and Thijs). These indeterminate results hamper definite classification by LIA only. Most (10/11) indeterminate sera were negative by RCPA; only chimpanzee Ton is positive. The animals are likely to be clearing the virus. Examination of Ton's serum during the next few years may provide information on whether clearance beyond RCPA detection level occurs. So far, Ton is still classified as a carrier. By testing earlier serum samples from this group of LIA-indeterminate animals by RCPA we should be able to estimate the average time required to clear the virus below detection level. To determine beyond doubt whether these sera are still infective, they would have to be inoculated into another chimpanzee.

Another possibility is that the samples were taken at a time between two peaks of viraemia during chronic infection (Bradley et al., 1981), although this is less likely, because considerably higher titres of persisting antibodies are expected.

HCV-RNA assays are therefore necessary, in addition to the antibody test, not only for sera showing borderline level antibodies, but also for sera from the acute phase of the disease, when antibodies are not yet detectable. Several samples, including acute phase sera, obtained throughout the course of infection of chimpanzees Socrates, Zeef and Sophie were all negative for anti-C100. However, RCPA shows that the virus was present during the acute phase of the disease. In Socrates, infectivity of this acute phase serum was proven by transmission to chimpanzee Sophie, resulting in infection. Acute phase serum from Zeef was also negative by LIA, but positive by RCPA (figure 2).

3. The last group of chimpanzees was found negative by LIA. These sera were also negative by RCPA, indicating the clearance of the virus from blood beyond detection level. The agreement between negative LIA result and negative RCPA result is also 100%, indicating the high specificity of both tests.

By comparing results from both HCV antibody assays and HCV-RNA assays, it has been claimed that all 4 possible combinations of antibody and RNA findings are possible (Ohkoshi et al., 1990). In this study, however, samples negative by LIA and positive by RCPA were not found. This might reflect the specificity of both test systems.

HCV subtypes

There is heterogeneity among the reported 3' terminal sequences of HCV-RNA (Choo et al., 1991; Kato et al., 1990; Han et al., 1991; Takamizawa et al., 1991) as summarized in figure 3. Based on this diversity two subtypes of HCV can be distinguished. The Kato (Kato et al., 1990) and Takamizawa (Takamizawa et al., 1991) sequences have a high degree of homology, but differ considerably from the sequences of Choo (Choo et al., 1991) and Han (Han et al., 1991), which are almost identical. Capture oligo LD 40 is based on the sequence published by Kato et al. (1990).

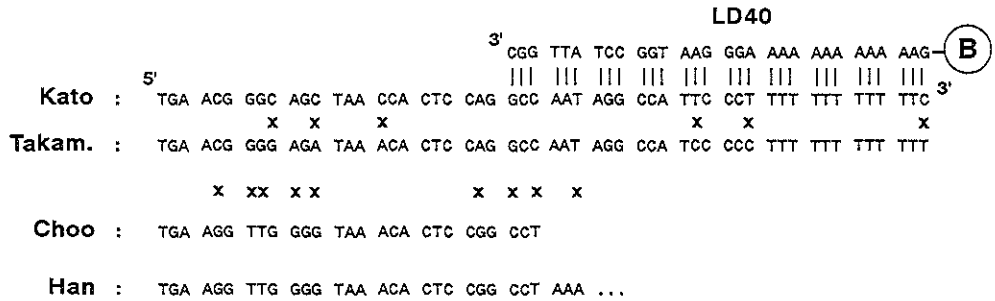

Figure 3. Comparison of 3' terminal sequence of HCV-RNA as published by the indicated authors. Shown are sequences downstream of the last codon of the polyprotein. Mismatches are indicated by a cross. Position and sequence of capture oligonucleotide LD40 is shown.

Sequences of several amplified HCV-cDNA fragments obtained from chimpanzee Phil convalescent serum, have a much higher degree of homology with the Japanese sequences than to the Choo subtype (data not shown). Therefore, the HCV-RNA derived from Phil was very likely to possess 3' terminal sequences complementary to LD 40, as confirmed by the results. However, some of the chimpanzees, e.g., Randy, Ton and Cor, were infected with other inocula, derived from the same factor VIII preparation which was used by Choo et al. (1991), and Han et al. (1991). HCV-RNA from these animals can also be captured by oligo LD 40, suggesting the presence of this common sequence at the 3' end of both subtypes of HCV. As LD 40 is a 31-mer and during RNA isolation the RNA-LD40 hybrid is washed with 0,1xSSC, specificity of purification is high. Using other capture oligos might be useful for rapid discrimination between subtypes of HCV, simply by using a different oligo for capturing HCV-RNA of different subtypes. The cDNA and PCR reactions are based on highly conserved sequences at the 5'end, so these are effective for most subtypes. The RCPA system will be compared with other PCR assays and subjected to further modifications. Sensitivity might be increased, e.g., by capturing HCV-RNA at the 5' untranslated region, by using random primers for reverse transcription and optimal primers for PCR amplification. The efficacy of the LIA will also be subject of further study.

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CHAPTER 3

Rapid detection of Hepatitis C virus RNA by direct capture from blood

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Abstract

A new diagnostic assay for hepatitis C virus RNA detection is described. HCV genomic RNA is captured onto streptavidin-coated magnetic beads by solution hybridization with biotinylated complementary oligonucleotides. The specificity of the capture assay is confirmed using different capture oligonucleotides as well as sera representing different types of HCV. Sensitivity was determined by testing serial dilutions of an HCV infected plasma. A panel of 50 sera was tested for anti-HCV by a Line Immuno Assay (LIA) and for HCV-RNA by both a conventional guanidinium extraction method and the new capture assay. The specificity of the capture assay was 95.8% and the sensitivity was 92.3%, compared to the standard protocol. This method provides a fast and simple alternative for HCV-RNA detection in blood samples.

Introduction

Since the discovery of the Hepatitis C virus (HCV) as the main cause of non-A, non-B hepatitis (Choo et al., 1989) numerous studies have rapidly increased insight in the biology of this virus. The viral genes have been mapped, leading to the classification of HCV as a new member of the Flaviviridae (Choo et al., 1991). From several isolates the entire sequence of the cDNA, obtained from the RNA genome of about 9400 nucleotides has been determined. (Kato et al., 1990; Choo et al., 1991; Takamizawa et al., 1991; Okamoto et al., 1991, 1992a; Chen et al., 1992a). Based on sequence comparisons, several types of HCV (Houghton et al., 1991; Chan et al., 1992; Cha et al., 1992; Okamoto et al., 1992b) can be identified, although no consensus about nomenclature has been reached yet.

Antibodies against HCV epitopes can be assayed using expression products from partial cDNA clones (Chiba et al., 1991; Chen et al., 1992b; Mori et al., 1992; Wang et al., 1992) or synthetic peptides (Kotwal et al., 1992; Ching et al., 1992; Hosein et al., 1991). It also seems possible to detect HCV antigen directly in serum (Takahashi et al., 1992), although sensitivity of this method is low and relatively large quantities of plasma are needed.

The detection of HCV-RNA in serum has been described by others (Kaneko et al., 1992; Widell et al., 1991; Bukh et al., 1992a; Cha et al., 1991; Schlauder et al., 1992; Novati et al., 1992; Lazizi et al., 1992). Basically, total nucleic acids are isolated from serum by phenol and chloroform extraction and precipitation with ethanol. The RNA is reverse transcribed into cDNA, which is subsequently amplified by the Polymerase Chain Reaction (PCR), enabling detection by

Southern blotting and hybridization. Methods for preparation of viral nucleic acids for PCR are tedious, involve multiple manipulations and are a potential source of contamination. To reduce these problems, simplified procedures have to be developed. One of the possibilities is to isolate and purify the organism of interest by antigen capture, and subsequent detection of viral RNA by PCR, as described for Hepatitis A and Hepatitis E viruses (Jansen et al., 1990; McCaustland et al., 1991). Specific antibodies, preferably monoclonal, are necessary for effective and specific capture of the organism. At this moment sufficient quantities of antibodies, directed against HCV surface epitopes are not available. Direct capture of RNA from crude cell lysates has been described earlier, but sensitivity was limited because PCR was not used (Albretsen et al., 1990; Thompson et al., 1989).

Therefore, a combined HCV-RNA capture PCR assay was developed (van Doorn et al., 1992). This assay is based on hybridization of a complementary biotinylated oligonucleotide to HCV-RNA, followed by capture of the resulting hybrid onto streptavidin-coated paramagnetic particles. Further evaluation of the capture method for sensitivity and specificity is described in this paper. The applicability of the assay is determined by testing a coded panel of patient sera by both the conventional extraction method (Kleter et al., 1993) and the new capture assay.

Materials and methods

Plasma samples

Fifty plasma samples were obtained by venapuncture. Aliquots were quickly frozen in liquid nitrogen and stored at -70°C.

Diagnosis of non-A, non-B hepatitis (NANB) was based on classical parameters as elevated ALT, detection of histological indication of hepatitis in liver biopsies, absence of serological markers for hepatitis A or B, and exclusion of other possible causes.

Based on HCV-RNA assays using the standard protocol, one of us (B.K.) prepared a coded panel for the HCV-RNA capture method. The panel was complemented with samples from 7 HBV patients, 2 autoimmune patients and a healthy control.

The efficiency of the capture assay for different types was examined using human plasma samples containing either HCV type 1, 2 or 3 sequences (classification according to Chan et al., 1992). Sensitivity of both HCV-RNA assays was determined by testing diluted plasma samples from the Eurohep HCV-RNA panel, which was prepared by dr. P.N. Lelie (CLB, Amsterdam).

Oligonucleotides

Capture oligonucleotides

LD40E (9055.. 9086)(-)	5'-B-GGC CGG GGC GGC CGC GAA AAA AAA AAA AGG GAA TGG CCT ATT GGC -3'
LD57 (-29.. 3)(-)	5'-B-GGC CGG GGC GGC CGC CAT GTT GCA CGG TCT ACG AGA CCT CCC GGG GC -3'
LD58 (-66.. -35)(-)	5'-B-GGC CGG GGC GGC CGC CAA GCA CCC TAT CAG GCA GTA CCA CAA GGC -3'
LD53 (-278..-248)(-)	5'-B-GGC CTT TGC GGC CGC TAA CGC CAT GGC TAG ACG CTT TCT GCG TGA -3'
LD52 (9261..9200)(-)	5'-B-GGC CGG TGG AGT GAG TTT A/GAG C/TTT G/TGT C/TC/TT C/TAC C/TGC CCA GTT GAA GAG -3'
BG1 (16S rRNA)	5'-B-CAG ACT GAG TAA GGA GGT GAT CCA ACC GCA GGT TCC CCT ACG GTT ACC TTG TTA CGA CTT -3'

cDNA and PCR primers

NCR3 (-314..-288)(+)	5'- GGG GCG GCC GCC ACC ATA/G A/GAT CAC TCC CCT GTG AGG -3'
LD66 (-55..-79)(-)	5'- ACC ACA AGG CCT/G TTC GCG/A ACC CAA C -3'
HCV18 (-323..-304)(+)	5'- GGC GAC ACT CCA CCA TAG AT -3'
HCV19 (-1..-20)(-)	5'- GTG CAC GGT CTA CGA GAC CT -3'

hybridization probe

HCV17 (-88..-69)(+)	5'- GAG TAG TGT TGG GTC GCG AA -3'

Underlined sequences are additional and not complementary to the target. B indicates biotinylation. Polarity of the oligonucleotides are indicated in parentheses.

Standard HCV-RNA assay

RNA was isolated by a modified version of the guanidinium method (Chomczynski et al., 1987) as described elsewhere (Kleter et al., 1993). Briefly, 60 μl of plasma was diluted in denaturing guanidinium thiocyanate buffer, extracted with phenol and chloroform, and precipitated with isopropanol. One third of the RNA was used for reverse transcription with primer HCV19 in a volume of 25μl. After heat inactivation of the reverse transcriptase, PCR ingredients including primer HCV18 were added to a final volume of 100 μl. PCR consists of 40 cycles of 1 min. 95°C, 2 min. 48°C, and 3 min. 72° C.

RNA capture assay

Chimpanzee serum from the acute phase of an HCV infection served as a positive control for the capture assay. This chimpanzee had been infected with type 1 HCV (Takamizawa et al., 1991; Kato et al., 1990) as was confirmed by sequence analysis (data not shown).

Fifty μl plasma was diluted in 800 μl lysisbuffer (0.5 M LiCl, 1% SDS, 0,2M EDTA, 1 mM DTT, 100 mM Tris-HCL pH 8,0) and incubated at 37°C for 10 minutes; 100 μl of 20x SSC and 50μl of 100x Denhardt's solution were added together with 100 ng of capture oligo. After careful mixing without vortexing, the sample was heated at 80°C for 5 minutes to denature the RNA, followed by 1 hour shaking at room temperature to hybridize the capture oligo to the RNA.

Paramagnetic streptavidin-coated particles (Dynabeads M-280 Dynal, Oslo, Norway) were

prewashed once with 0,5x SSC. Fifteen μl of beads (0.15 mg) was added to each sample and incubated for 30 minutes at RT. Beads were concentrated at the tube wall using a magnetic separation stand. Fluid was removed by sterile disposable pastettes and 500 μl of fresh buffer was added. The beads were washed twice with 0.15M LiCl, 0.1% SDS, 0.01M Tris-HCl pH 8.0, and twice with 0,5xSSC. After the last wash, the suspension was collected at the bottom of the tube by very brief centrifugation and fluid was removed completely. Beads were resuspended in 18 μl of DEPC treated water, 0.75 μl 20mM dNTP's (Pharmacia) and 1 μl (0.2 μg) of random hexamers (Pharmacia). After heating at 80°C for 5 minutes tubes were quickly chilled on ice and a mix of 6 μl 5x buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 3 μl 0.1 M DTT, 1 μl M-MLV reverse transcriptase (200 u) and 0.5 μl RNAsin (20 u) was added. Suspensions were incubated at 37°C for 90 minutes, resuspending the beads every 30 minutes. The reverse transcriptase was inactivated at 95°C for 5 minutes. (Sellner et al., 1992). Ten μl of the cDNA solution, including beads, were used in a 100μl PCR assay. Reaction conditions were: 10mM Tris-HCl pH 9.0, 0.1% Triton X-100, 0.01% gelatin, 200μM dNTP's, 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 units SuperTaq (Sphaero Q, Leiden, the Netherlands) and 10 pmoles of primers LD66 and NCR3. (Note that the cDNA solution already contains dNTP's, KCl and $MgCl_2$).

The PCR program consisted of 1 minute pre-incubation at 94°C, followed by 40 cycles of 1 min 94°C, 1 min 45°C and 2 min 72°C. After the last cycle an extra incubation for 5 min at 72°C was carried out.

Analysis of the PCR products

PCR products (15 μl) were analyzed on a 1,5% TBE agarose gel, followed by blotting onto Hybond N^+ membranes (Amersham) and hybridization with ^{32}P labeled HCV17. Results were based on examination of autoradiographs. Outcome of an experiment was only accepted if duplicates showed comparable signals. If discrepant results were obtained, samples were retested in duplicate using the same sense primer (either NCR3 or HCV18) in both assays.

HCV antibody assay

Each plasma sample from the coded panel was tested for the presence of antibodies against core, NS4 and NS5 epitopes by a Line ImmunoAssay Inno-LIA HCV Ab II (Innogenetics N.V., Gent, Belgium) according to the manufacturer's instructions.

Results

Several aspects of the new HCV-RNA capture assay like sensitivity, specificity and practical applicability were analyzed. First, the efficiency of different oligonucleotides to capture type 1 HCV-RNA was analyzed, as shown in figure 1. Capture oligo's LD53, LD57 and LD58 are specifically capturing highly conserved sequences from the 5' untranslated region (5'UTR). LD52, containing 7 degeneracies, is aimed at the C-terminal part of NS5. LD40 is annealing at the extreme 3' end of some of the type 1 RNA (type 1b sequences; Kato et al., 1990; Takamizawa et al., 1991), which allows the specific detection of (near) full length HCV-RNA (van Doorn et al., 1992). Negative control was oligo BG1, specific for 16S rRNA sequences. There were clear differences in capture efficacy of the various oligonucleotides (figure 1). LD40, LD58 and LD53 all efficiently captured HCV-RNA. Oligo LD53, which hybridizes between the annealing sites of the two PCR primers, did not seem to obstruct cDNA synthesis and subsequent amplification. LD57 yielded consistently high signals. Because the target sequence for LD57 is highly conserved among all known HCV isolates (Bukh et al., 1992b), this capture oligo was used for further capture experiments. LD52 was less efficient, whereas negative control oligo BG1 was not able to capture HCV-RNA at all. A combination of LD57 and LD58, to improve binding of HCV-RNA to the magnetic beads, resulted in a considerable reduction of signal. Results of capture assays on positive serum without any capture oligo were negative, illustrating the specificity of the capture (data not shown).

Second, the capture system was evaluated on isolates from three different types of HCV. Three plasma samples containing either type 1 (Kato et al., 1990; Takamizawa et al., 1992; Choo et al., 1991), type 2 (HCJ-6, Okamoto et al., 1991), or type 3 (Lee et al., 1992; Chan et al., 1992) HCV sequences were tested. HCV-RNA was captured with LD57, and amplified using primers LD66-NCR3. Each sample yielded PCR fragments of the right size. Direct sequencing confirmed the expected type specific differences in these 5' UTR fragments. (data not shown). These results illustrated that the capture method is able to detect RNA of distinct HCV types.

The sensitivity of the capture assay was determined by testing 10-fold serial dilutions of a HCV RNA-positive human serum, provided as part of the Eurohep panel. The standard assay was shown to be approximately 10-fold more sensitive than the capture assay. The sensitivity of the standard protocol was approximately 1 Chimpanzee Infectious Dose (CID) per ml.

A coded panel of 50 patient plasma samples was tested for the presence of HCV-RNA by both the standard protocol and the capture assay. Each sample was tested in duplicate. Plasma samples giving discrepant results in the two HCV-RNA assays were retested using identical sense

primers NCR3 or HCV18 in both assays to exclude this as a cause of discrepancy. It was not possible to use HCV19 as antisense primer in the capture assay, because it overlaps with capture oligo LD57. Only one sample, found positive with NCR3 in the capture assay, was initially scored RNA negative by the standard protocol using HCV18, but became positive when using NCR3.

All sera were also tested for the presence of HCV specific antibodies by the Inno-LIA HCV Ab II assay. Table 1 shows a summary of the results. Twenty-four samples were RNA positive in both assays; 21 of these are anti-HCV positive, 2 were indeterminate and 1 was anti-HCV negative. Twenty-three samples were RNA negative in both assays, of which 19 were anti-HCV negative, 2 were indeterminate and 2 were positive. Among the RNA negative and anti-HCV negative samples were the 7 HBV and 2 auto-immune patients and the healthy control sample.

Two samples were HCV RNA negative by both assays but did contain antibodies against viral epitopes. One sample was only RNA positive by the capture assay and was also anti-HCV positive. Two samples were exclusively positive by the standard protocol, 1 being anti-HCV positive and 1 negative. The capture assay has a specificity of 95.8% and a sensitivity of 92.3% compared to the standard protocol. Compared to both RNA assays, the LIA had a sensitivity between 85-88%.

Table 1. Comparison between the standard protocol and the HCV RNA capture assay in 50 patient plasma samples.

		HCV RNA capture assay		
		+ anti-HCV	-	anti-HCV
Standard Protocol	+	24	21 +	1 +
			2 ind.	2
			1 -	1 -
	-	1	1 +	2 +
				23
				2 ind.
				19 -

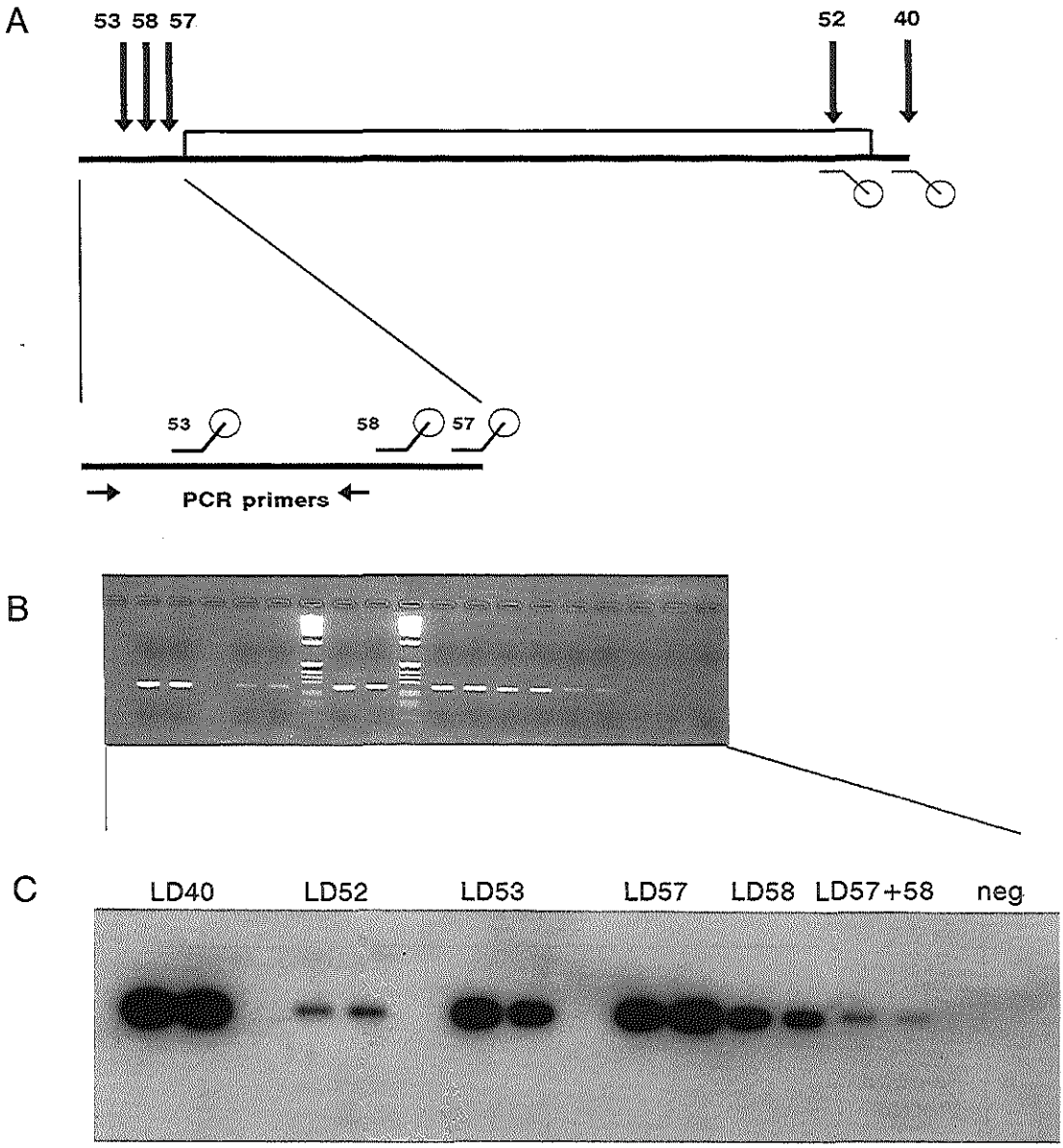

Figure 1. Different oligonucleotides were used for HCV RNA capture. The position of these capture oligos is shown in A. After capture, cDNA synthesis and PCR, samples were analyzed on agarose gel (B) and by Southernblot hybridization (C).

Discussion

Detection of HCV-RNA in serum by the capture assay described here is an alternative for the more complex and laborious extraction and precipitation methods. Several advantages favour the capture method. It is less contamination prone, because the specificity of HCV-RNA isolation prevents co-purification of amplimers and it is essentially a 1-tube assay. The method also omits the use of hazardous chemicals such as phenol, chloroform and guanidiniumthiocyanate. Furthermore, ethanol precipitation and multiple centrifugations are omitted. The method is fast; 20-30 samples can be processed in duplicate by one person. Because this method specifically purifies the HCV RNA, it can be used to assay samples that contain substances that are inhibitory for PCR, like heparin.

The efficiency of the capture assay was tested by using several oligonucleotides aimed at different regions of the HCV genome. Oligo's LD53, LD57 and LD58 are annealing at various 5'UTR sequences. The target sequence for LD52 is located at the C-terminal region of NS5, whereas LD40 hybridizes to the extreme 3' UTR. There were considerable differences in sensitivity among the tested HCV specific oligo's (figure 1). This may be caused by the secondary structure of the RNA genome, which makes some sequences more accessible than others (Brown et al., 1992). If a non HCV-specific capture oligo like BG1 was used, or no capture oligo at all, no positive signal could be obtained from an HCV-RNA positive plasma, which indicates the specificity of the capture system. The reduced efficiency of using both LD57 and LD58 together in one assay was probably due to steric hindrance of the oligo's while capturing target sequences that are only a few nucleotides apart. Consequently, either the capture efficiency was low or the conformation of the captured RNA prevented proper cDNA synthesis. Oligo LD52 was less efficient for RNA-capturing, and this target region also shows variation among isolates. Despite several degeneracies in the oligo, LD52 apparently does not anneal well to this type 1 HCV-RNA. Oligo LD40 is interesting, because it hybridizes at the extreme 3' end of type 1b RNA. By subsequent amplification of 5' end sequences it was possible to detect specifically full-length RNA efficiently. However, the 3' UTR shows a high degree of sequence variation (Han et al., 1991). It was therefore impossible to develop a consensus capture sequence for this region.

Results from the limiting dilution experiments performed on serum show that the capture assay is about 10 times less sensitive than the standard protocol. There are several factors that affect the overall efficiency of the assay, like degree of biotinylation of the capture oligo, and optimal hybridization conditions. Recent experiments have shown that use of proteinase K to release HCV-RNA from the viral particles considerably improves the sensitivity (data not shown).

Further studies are aimed at increasing the sensitivity of the system.

Although the sensitivity of the capture assay at the moment is slightly lower than the standard protocol, the method can be very useful as a screening test. Most infected individuals have high enough HCV titers to be detected by the capture assay, as shown by results of the comparison of the two methods using the patient panel.

To determine the applicability of the capture assay for routine diagnostics, the results of the capture assay were compared with those of the standard protocol. The sensitivity and specificity of our standard assay have shown to be very high (Kleter et al., 1993).

There were only three discrepancies in the entire patient panel. One serum sample was positive by capture assay only and did contain HCV antibodies. Two serum samples were exclusively positive by the standard protocol and only one of those contained detectable HCV antibodies. The discrepant results are probably due to sequence variation among HCV types. As stated, there is a difference in anti-sense PCR primer between the two tests. Sequence variations in the target sequences for these antisense primers might explain different test results, although this region is extremely conserved among reported HCV isolates (Bukh et al., 1992b). Based on the strong signals obtained from the 3 samples, it seems not likely that the discrepancies are due to low HCV titers. The real cause for the 3 discrepancies remains obscure.

The sample, exclusively positive by the capture assay was obtained from a hemophiliac, with clinical symptoms of hepatitis. No histological data of liver biopsies are available. This sample also contained HCV antibodies, which indicates at least previous contact with HCV. Of the 2 samples, exclusively positive by the standard protocol, 1 was LIA negative, and was derived from a patient which was immunosuppressed. The other sample was anti-HCV positive.

As shown previously (van Doorn et al., 1992) for chimpanzee serum samples there is a very good correlation between the presence of HCV antibodies detected by the LIA and the detection of HCV-RNA. One patient was RNA positive by both methods, but did not contain detectable HCV antibodies, which can be explained by the severe immunosuppression in this patient after receiving a heart transplant. Generally, a small fraction of all HCV antibody positive samples are PCR negative, but up to 30% of HCV-RNA positive serum samples are not detected with current antibody assays (Sugitani et al., 1992). The sensitivity of the LIA, compared with both HCV-RNA assays in our patient panel, was approximately 85%. Two sera are RNA positive and LIA indeterminate, and 2 are RNA negative and LIA indeterminate, which emphasizes the necessity of HCV-RNA assays to complement serological tests.

Among 23 RNA-negative samples, two are LIA positive. This may reflect stages of infection where the virus concentration has dropped below detection level, and antibodies are still

detectable. The only sample exclusively positive by the capture assay does contain HCV antibodies, an indication that this patient had been infected with HCV.

In the future, subtyping of HCV will become an important issue, because of possible clinical implications (Yoshioka et al., 1992; Pozzato et al., 1991). Therefore, the HCV capture assay, combined with methods for rapid analysis of the amplified cDNA like reverse hybridization assays (Stuyver et al., 1993), will provide powerful tools in HCV diagnosis and analysis.

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CHAPTER 4

Longitudinal analysis of Hepatitis C virus infection and genetic drift of the hypervariable region

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Abstract

Hepatitis C virus (HCV) infections in a cohort of chimpanzees were studied retrospectively. All animals had been inoculated intravenously with materials derived from a single source chimpanzee plasma implicated in non-A, non-B hepatitis, prepared by extensive ultracentrifugation. Anti-HCV and HCV RNA were monitored by the confirmatory Line Immuno Assay and by a RNA-capture PCR method, respectively. In a chronically infected chimpanzee, HCV RNA was first detectable after 32 days, throughout the acute phase, dropped transiently below detection level, and became positive again. In three other chimpanzees with acute resolving infections, HCV RNA was detected 7-11 days after inoculation, and became permanently undetectable after ALT normalization. Various anti-HCV profiles were detected among the chimpanzees. Analysis of the hypervariable region in E2/NS1 in seven chimpanzees suggested genome stability upon transmission, revealed different mutation frequencies during chronic infection and suggested the importance of immune selection during chronic HCV infection.

Introduction

Hepatitis C virus (HCV) has been identified as the major etiologic agent of posttransfusion non-A, non-B hepatitis (NANB; Choo et al., 1989, Kuo et al., 1989). The virus, classified as a distinct relative of the Flavi- and Pestiviruses, contains a positive-sense, single-stranded RNA genome of approximately 9400 nucleotides and encodes a single large open reading frame of about 3010 amino acids. The viral polyprotein is processed into several structural and non-structural proteins. Antibodies against several viral proteins can be detected in the serum of infected individuals and are used in routine diagnostic screening assays. Serum HCV-RNA can be detected by reverse transcriptase polymerase chain reaction (RT-PCR) and provides a direct measure of viremia (Weiner et al., 1990). Several HCV types have been distinguished among HCV isolates worldwide, and at least 5 types have been identified so far (Simmonds et al., 1993, Kleter et al., 1994, van Doorn et al., 1994a, Cha et al., 1992, Chan et al., 1992, Okamoto et al., 1991, 1992).

During the past two decades, NANB hepatitis was studied in vivo in the only susceptible animal, the chimpanzee. (Bradley et al., 1979, 1981). Experimental infections of these animals provided data and material that were crucial for the molecular characterization of HCV (Bradley et al., 1985). Several retrospective studies in chimpanzees have elaborated our understanding of this virus and its interaction with the infected host (Beach et al., 1992, Shindo et al., 1992, Farci et al., 1991, 1992a,b, Abe et al., 1992). In this study, experimental HCV infections were examined retrospectively in chimpanzees via biochemical, serologic and virologic parameters. The animals had been inoculated with processed infectious chimpanzee plasma material from a single source, containing genotype 1b HCV (typing according to Simmonds et al., 1993). Several sequential passages are included in this study, and the hypervariable region of HCV (Weiner et al., 1991, Kato et al., 1992a,b) was analyzed by direct sequencing in a total of 7 chimpanzees, in order to assess genetic drift of this particular HCV isolate.

Materials and methods

Preparation of inocula

The original NANB inoculum was obtained from an acute phase plasma from chimpanzee George (Tsiquaye et al., 1980). This chimpanzee had been infected with hepatitis A and hepatitis B viruses and had recovered from acute NANB hepatitis induced by an implicated factor IX preparation. Subsequently, this animal was challenged with an implicated factor VIII preparation, which was kindly supplied by Dr. Daniel Bradley (Bradley et al, 1979). The chimpanzee developed a severe acute NANB hepatitis after an incubation period of only 12 days, when a volume of acute phase plasma was obtained. Samples (5.0 ml) from this plasma were centrifuged at 52,000 rpm. (200,000 x g) for 18 h at 4°C. Aliquots of the supernatant (4.0 ml), collected from the top of the tube were pooled, distributed to new tubes and centrifuged again under the same conditions. This procedure was repeated twice more in new tubes. At the end of the 4th cycle of centrifugation, an aliquot (100 μL) was carefully collected from the surface layer of the supernatant, diluted in 9.9 ml of PBS pH 7.4 and labeled "Top Fraction". About 3 ml of the first supernatant was collected from below the meniscus (top to the middle part of each tube) and ultracentrifuged again. After 18

h centrifugation at 200,000 x g, a second aliquot of 100 μL was collected at the meniscus of the supernatant and added to 9.9 ml of PBS. This diluted sample was labeled "Middle Fraction".

Inoculation of chimpanzees

All chimpanzees were inoculated intravenously, and the infection schedule is represented in figure 1 and table 1. Chimpanzee **Phil** received 2.5 ml of the "Top Fraction" in December 1982. Three ml of the diluted "Middle Fraction" were inoculated into chimpanzee **Peggy**. Furthermore, 0.3 ml of the "Middle Fraction" were mixed with 2.7 ml plasma obtained from chimpanzee **Phil** at 165 days after infection and inoculated into chimpanzee **Hans**. At 165 days after infection, chimpanzee **Phil** had biochemical and histological signs of recovery and it was presumed that this plasma might contain neutralizing antibodies. Chimpanzee **Coen**, who had been inoculated previously with HIV-1, was inoculated in 1983 with 1 ml plasma from **Phil** obtained at 35 days after infection. Chimpanzee **Socrates** was inoculated in 1985 with 0.1 ml plasma from **Phil** obtained at 62 days after infection, which was mixed with 0.9 ml plasma from **Coen** obtained at 192 days after infection. Antibodies would possibly neutralize the infectious virions and anti-human IgG was added to generate large immune complexes. Briefly, plasma samples from **Phil** and **Coen** were mixed, incubated at 37°C for 1 h, and then with anti-IgG, overnight at 4°C. After clarifying the mixture at 2,500 rpm for 10 min, 1 ml was inoculated into **Socrates**. **Sophie** was infused in 1987 with 40 ml acute phase plasma from **Socrates** obtained 74 days after infection. Serial blood samples were obtained from the chimpanzees by venipuncture and serum was stored at -20°C. Infections were monitored by testing serum samples for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyltranspeptidase (g-GT) activity.

HCV-RNA capture assay

HCV-RNA was purified from serum samples by a modified version of the HCV-RNA capture assay described earlier (van Doorn et al., 1992, 1994b). Serum (50-100 μL) was diluted in 200 μL lysis buffer (50mM Tris-HCl pH 8.0, 5 mM EDTA, 0.5% SDS, 1mM Dithiothreitol, 1% Triton X100) containing 0.5 mg/ml proteinase K, 10 μg carrier tRNA and incubated at 55°C for 30 minutes. Afterwards, proteinase K was heat-inactivated by incubation of the mixture at 80°C for 5 min. Biotinylated capture oligo LD57 (table 2) was coupled to streptavidin-coated paramagnetic

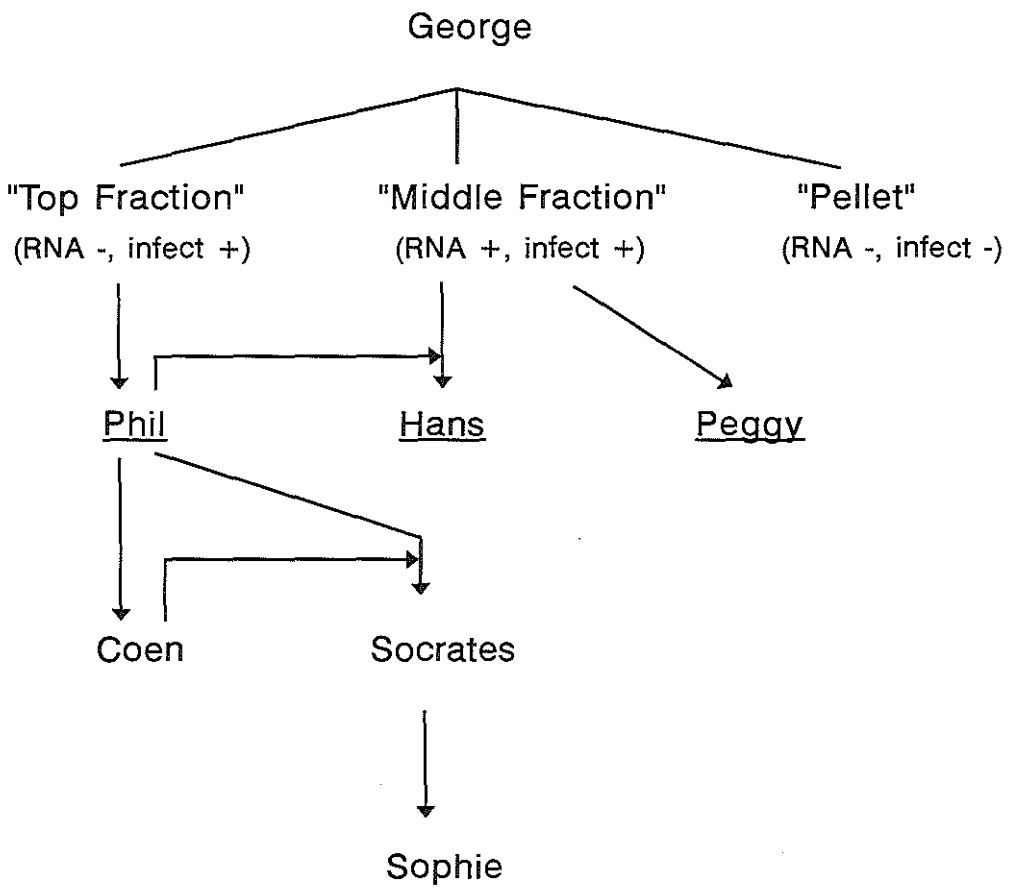


Figure 1. Inoculation scheme of chimpanzees. Underlined chimpanzee name indicates chronic HCV infection.

particles (Dynabeads M280, Dynal, Oslo, Norway). Beads were prewashed twice with 0.5 x SSC (20 x SSC is 3 M NaCl, 0.3 M Na-citrate pH 7.0) and LD57 was coupled (250 ng LD57/0.15mg beads) by mixing with the beads in 5 x SSC. After 30 min, the oligobeads were washed twice with 5xSSC to remove uncoupled LD57. Fifteen μL oligobeads and 100 μL 10 x SSC were added to the lysate. To denature the RNA and facilitate hybridization to the capture oligo, samples were heated to 80°C for 5 min and shaken for 60 min at ambient temperature. Beads were washed twice with 5 times diluted lysis buffer and twice with 0.5 x SSC using a magnetic stand. cDNA synthesis was performed as described (van Doorn et al., 1992, 1994b) using random primers. Ten μL cDNA was amplified by PCR using primers derived from the 5' untranslated region (5' UTR; all primers are represented in table 2). NCR4 and NCR3 were used as outer primers. Two μL from the first PCR mixture was transferred into nested PCR reactions with LD55 and LD56 as inner primers. Fifteen μL samples were analyzed by agarose gel electrophoresis and Southern blot hybridization, using HCV17 as a probe. The sensitivity of the capture assay is slightly lower than the conventional assays, using extractions with organic solvents and precipitation of the HCV RNA. The detection limit has been estimated at between approximately 150 and 1500 HCV RNA genomes (unpublished data).

Anti-HCV assay

Antibodies to core, NS4 and NS5 epitopes were determined by a confirmatory Line Immuno Assay (Inno-LIA HCV AbII, Innogenetics NV, Gent, Belgium) according to the manufacturer's instructions.

Sequence analysis of the hypervariable region

The hypervariable region was analysed by direct sequencing of nested, biotinylated PCR products (van Doorn et al., 1994a). HCV36 and HCV31 served as outer primers in the initial PCR. The product was reamplified with primers HCV38B and HCV37, resulting in a biotinylated DNA fragment of which 356 bp were sequenced. Sequences of both strands were determined by the T7-DNA sequencing system (Pharmacia, Uppsala, Sweden) using PCR primers and internal sequencing primers LD79 and LD80.

Table 1. HCV inocula and transmissions

	inoculum	passage	year of infection
George	factor VIII concentrate	0	1982
Phil	George 12 dpi, top fraction	1	1982
Coen	Phil 35 dpi	2a	1983
Socrates	Phil 62 dpi + Coen 192 dpi + anti Human IgG	2b + 3	1985
Sophie	Socrates 74 dpi	3	1987
Peggy	George 12 dpi, middle fraction	1	1983
Hans	George 12 dpi, middle fraction + Phil 165 dpi	1 + 2c	1983

Dpi indicates number of days after infection.

Table 2. Oligonucleotides

oligo	pol.	pos.	sequence (5'-3')
LD57	-	-29..3	Bio-GGCCGGGGCGGCCGCCATGiT GCACGGTCTACGAGACCTCCCGGGGC
NCR3	+	-314..-288	GGGGCGGCCGCCACCATARRATCACTCCCCTGTGAGG
NCR4	-	-66.. -47	CACTCTCGAGCACCCTATCAGGCAGTACC
LD55	-	-99.. -81	CRCTACTCGGCTAGCAGTCT
LD56	+	-263..-241	CTAGCCATGGCGTTAGTATGA
HCV17	+	-88.. -69	GAGTAGTGTTGGGTCGCGAA
HCV37	-	1874..1852	AAGCAATACACYGGRCCACAYAC
HCV36	+	1281..1290	GGTCAYCGCATGGCWTGGGA
HCV38b	+	1287..1298	Bio-CGiATGGCiTGGGAiATGATG
HCV31	-	1894..1864	CTGCAGGCATGCGGGCTBGGRGTGAAGCARTA
LD79	+	1414..1435	TCCATGGTRGGGAACTGGGC
LD80	-	1746..1727	TGATGGGACCCCACCCCTG

+ indicates sense, - indicates antisense orientation. Positions are according to Choo et al., 1991 [24]. Underlined sequences are non-HCV specific R= G or A, Y = C or T, W = A or T, i= inosine, Bio = 5'-terminal biotin.

Results

HCV infections in chimpanzees

Colony-bred chimpanzees were experimentally infected with NANB hepatitis (see figure 1 and table 1). The inocula for chimpanzees Phil, Peggy and Hans were prepared by extensive ultracentrifugation of infectious chimpanzee George plasma. All chimpanzees developed hepatitis with elevated levels of liver-specific enzymes in the serum. In chimpanzee Phil, infection was also proven by light- and electronmicroscopical investigation of liver biopsies, which revealed characteristic histological and ultrastructural changes in hepatocytes (data not shown). Serial blood samples from 4 chimpanzees, Phil, Coen, Socrates, and Sophie, were tested for the presence of HCV-RNA and anti-HCV. Results are summarized in figure 2A-D and table 3.

Chimpanzee Phil (figure 2a) received a diluted low-density fraction of the centrifuged plasma and clearly developed a chronic HCV infection. A biphasic ALT profile was observed during the acute phase. g-GT showed only one peak, which coincided with the second ALT peak. Enzyme elevations were relatively low compared to levels observed in the other chimpanzees, and the infection seemed mild. HCV-RNA was first detected at 32 days after infection, remained detectable till 84 days after infection, followed by a transient period during which HCV-RNA was undetectable. Later, RNA became and remained detectable for more than 10 years. Anti-NS4 was first detected at day 53 and was the only serologic marker for at least several months. Anti-NS5 was not detected during the first 5 months and anti-core positivity was observed for the first time at day 2527. Infectivity of Phil's acute-phase plasma was proven by inoculation of other animals.

Chimpanzee Coen (figure 2b) was inoculated with acute-phase plasma from Phil. HCV RNA was detected in the first available sample after inoculation at day 11. RNA became permanently undetectable immediately after the ALT peak at day 67 suggesting complete resolution of the infection. Anti-core antibodies were first detected at day 40 and titers increased till day 74. At day 54 anti-NS4 was also detected, but anti-NS-5 remained consistently undetectable throughout the entire infection.

Chimpanzee Socrates (figure 2c) experienced an acute resolving HCV infection, after inoculation of mixed sera from Phil (34 days after infection) and Coen (192 days after infection), which had been complexed with anti-human IgG. At 192 days after infection, Coen serum

contained antibodies against NS4 and various core epitopes while HCV RNA was undetectable. In Socrates, HCV-RNA was below detection level at day 4 but was positive from day 8 till the ALT peak at 88 days after infection. Between day 89 and 92 the HCV-RNA concentration rapidly dropped till below the detection limit of our HCV-RNA assay. This change coincided with the sharp decline of the ALT levels in the serum. At day 54 antibodies against one core epitope were first observed. Levels of antibodies to core epitopes remained low, whereas anti-NS4 was undetectable in all tested samples. Anti-core antibodies gradually diminished until they became completely undetectable.

Chimpanzee Sophie (figure 2d) was infused with a large volume of acute phase plasma from Socrates. The infectious dose of this inoculum was not determined but PCR results suggested the presence of a high virus titer. The serum g-GT level reached a very high peak of 740 u/L at day 57, whereas ALT elevations were relatively mild. This chimpanzee also experienced only transient viremia. HCV-RNA was detected in the first available sample after inoculation at day 7. RNA was not detected after day 56. As observed in Socrates, the sharp decrease of the RNA concentration coincided with normalization of ALT levels. Anti-core antibodies were first detected at day 49, and titers diminished till almost complete seronegativity at 140 days after infection. (The sample at 140 days after infection would be scored as indeterminate on LIA). Later, only antibodies against the second core epitope were detectable from 407 to 1127 days after infection.

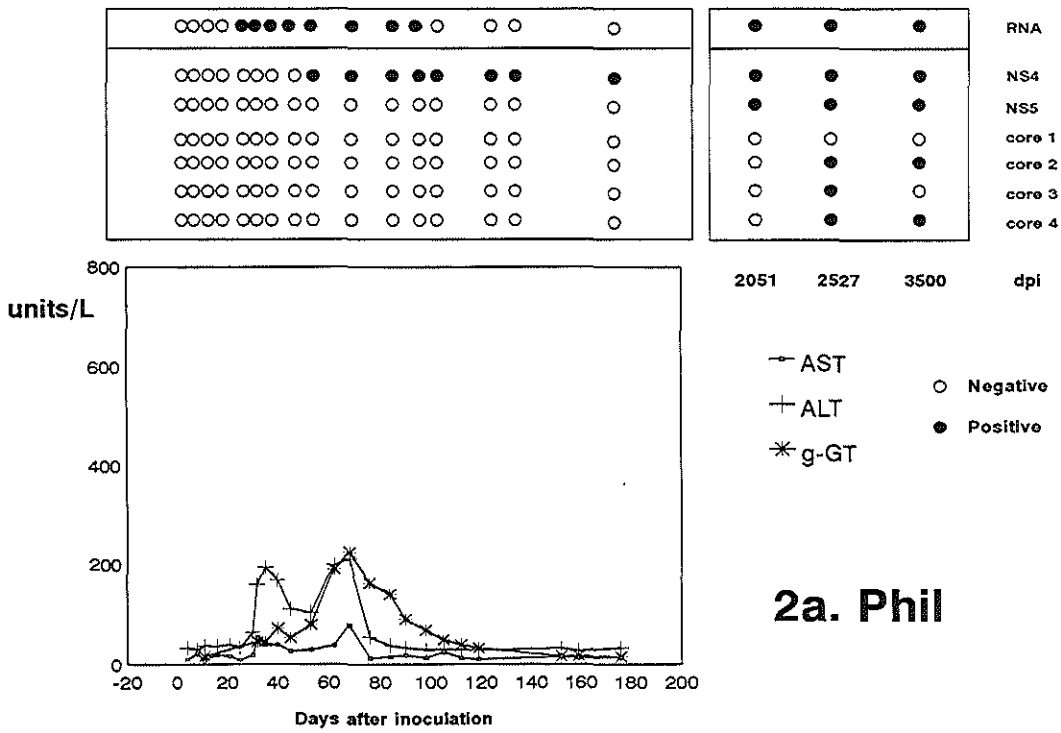
Chimpanzee Hans was inoculated with fractioned HCV positive plasma from George that had been mixed with plasma from Phil (165 days after infection), in an attempt to neutralize the virus by antibodies against essential epitopes. Hans developed a chronic HCV infection. The acute phase plasma from George was positive for both HCV-RNA and anti-HCV antibodies detected by second generation ELISA (Tsiquaye KN, unpublished data). Phil plasma at 165 days after infection does contain antibodies against non-structural NS4 epitopes (figure 2a) but not against NS5 or core epitopes. The antibody status against E1 and E2 proteins is not known. The overall anti-HCV response in Phil was poor, and therefore it is not likely that Phil antiserum at 165 days after infection could neutralize virus. HCV-RNA was undetectable in Phil at 165 days after infection, but during chronic HCV infection, infectivity of this plasma sample cannot be completely excluded (Beach et al., 1992). After inoculation of the mixture, Hans developed a chronic HCV infection.

Chimpanzees Peggy and Hans were not included in the longitudinal HCV-RNA and anti-HCV analysis. Both animals developed acute hepatitis with elevated ALT levels. Samples obtained in 1990 were tested positive by RT-PCR previously (van Doorn et al., 1992), indicating chronic HCV infections. Genetic drift was also studied in these animals.

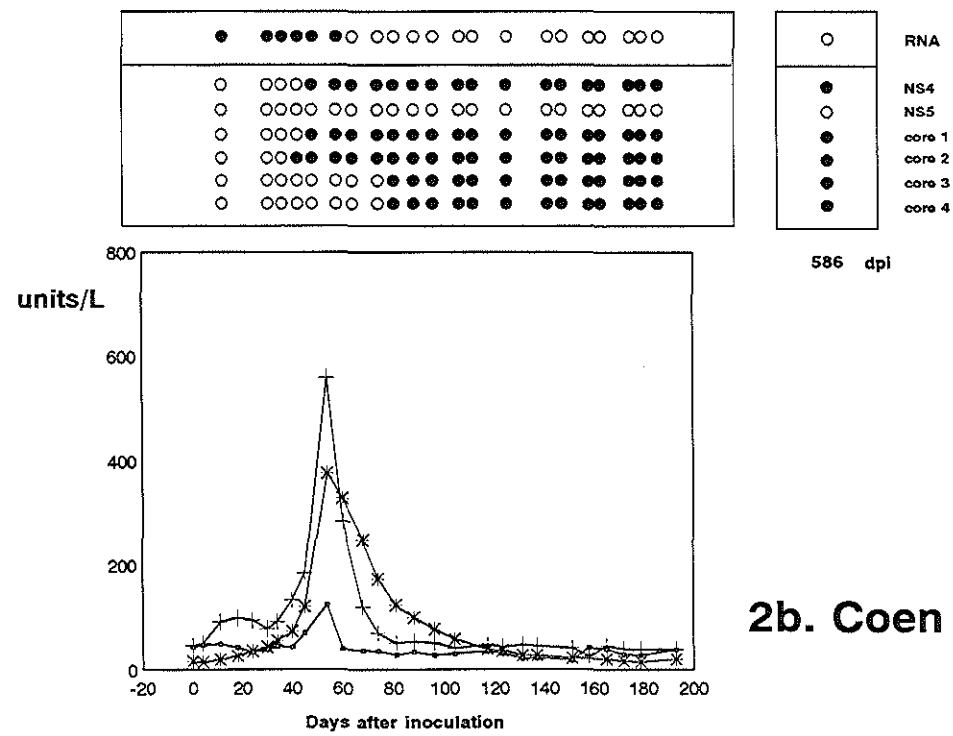
Sequence analysis

HCV genomes from Phil and Socrates were characterized by sequencing of amplified cDNA fragments derived from 5' UTR, E2/NS1 and NS5, and revealed the presence of genotype 1b (data not shown). Isolates from Phil (1983, 1987, 1989, 1993), Coen (1983), Socrates (1985), Sophie (1987), Peggy (1983, 1986, 1987, 1990), and Hans (1986, 1989) were used for sequence analysis of the hypervariable region. Furthermore, the original plasma sample from George as well as the derived "Middle Fraction", used to inoculate the chimpanzees Peggy and Hans, were analyzed. The sequences determined from the E1-E2/NS1 boundary (nucleotides 1016-1372, encoding amino acids 339-457), including the hypervariable region, are represented in figure 3. Sequences of cDNA fragments obtained during the early acute phase of hepatitis in Phil, Coen, Socrates and Sophie are completely conserved. Genetic drift of HCV during the chronic HCV infection in Phil between 1982 and 1993 is remarkably low. Three mutations were observed in the 356 bp fragment and only one located in the postulated hypervariable region. The deduced amino acid sequences of the 1982 and 1993 sequences differ in only 2 positions. Viral genetic drift in Peggy and Hans is much higher in this region. (The 1983 sample from Peggy was obtained after the acute phase, 4 months after inoculation). In both animals 17 nucleotide mutations, mainly non-silent mutations were observed, resulting in significant amino acid replacements in the putative hypervariable region. Sequence from the "Middle Fraction" showed some sequence ambiguities but was almost identical to the sequence obtained from the acute phase plasma from George (figure 3a). It was consistently impossible to detect HCV RNA in the original "Top Fraction", and therefore could not be subjected to sequence analysis.

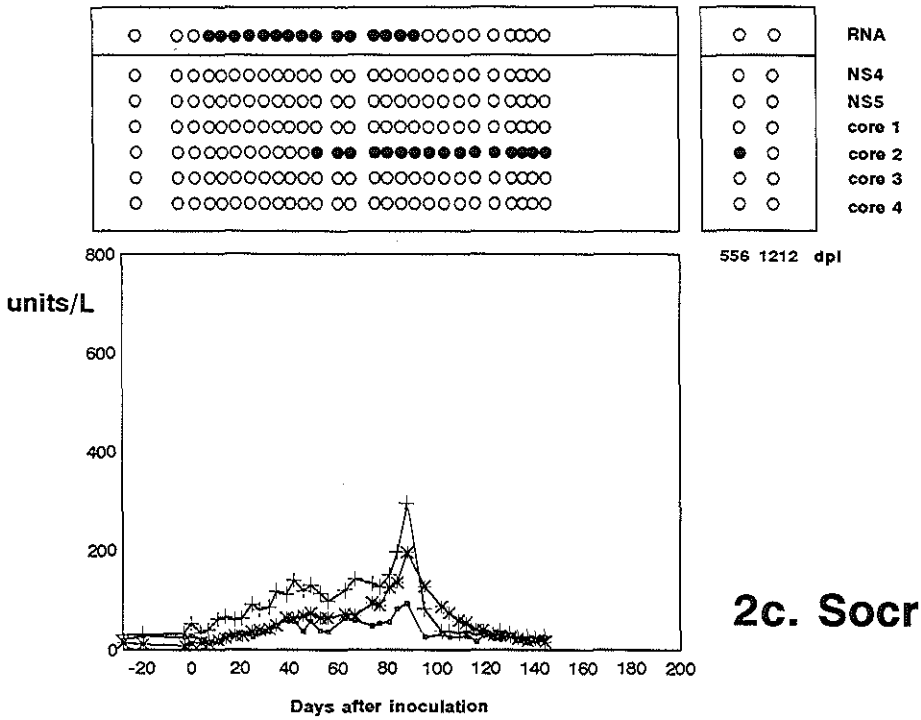
Figure 2. Schematic representation of longitudinal analysis of HCV infections in Phil (a), Coen (b), Socrates (c) and Sophie (d). Dpi = days after infection. AST= aspartate amino transferase, ALT= alanine aminotransferase, g-GT= gamma-glutamyltranspeptidase.



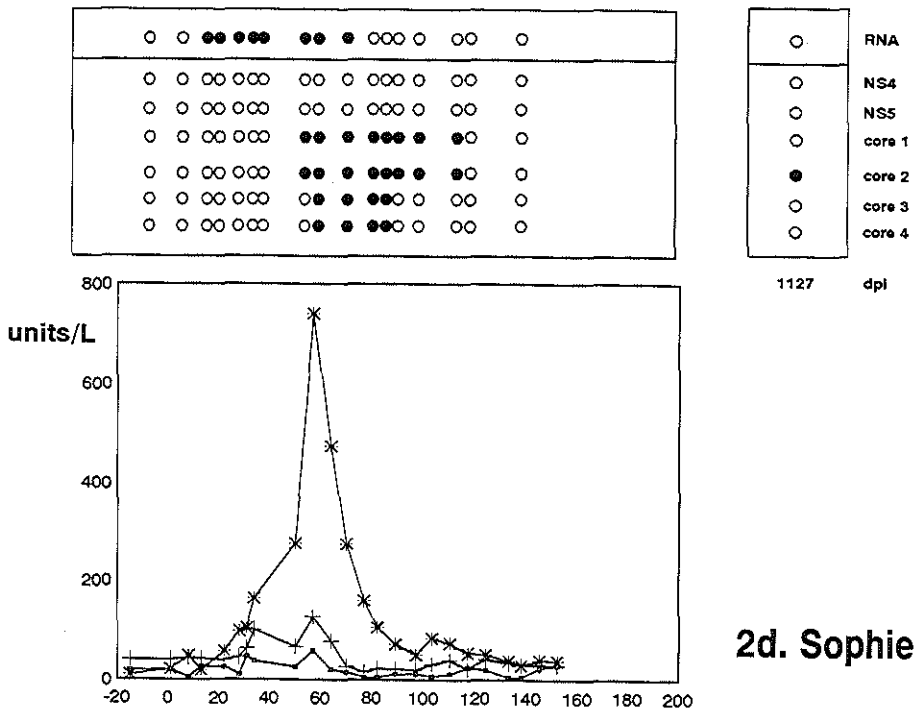
2a. Phil



2b. Coen



2c. Socrates



2d. Sophie

Table 3. Clinical and serological data of HCV infection in experimentally infected chimpanzees

Chimpanzee		Acute phase		Outcome	LIA anti-HCV			HCV RNA PCR detection	
	start[1] (dpi)	peak ALT (dpi)	peak ALT (units/L)		NS4 (dpi)	NS5 (dpi)	core (dpi)	first (dpi)	duration
Phil	30	35[2] 68	194 211	chronic	53	2051	2527	32	> 10 years
Coen	11	54	560	resolving	54	-[3]	40	11[4]	57 days
Socrates	25	88	294	resolving	-	-	54	8	80 days
Sophie	22	57	126	resolving	-	-	49	7	49 days

[1] Start of acute phase illness is defined as the day post-infection where either ALT (alanine aminotransferase), AST (aspartate aminotransferase), or g-GT (gamma glutamyltranspeptidase) is above normal values.

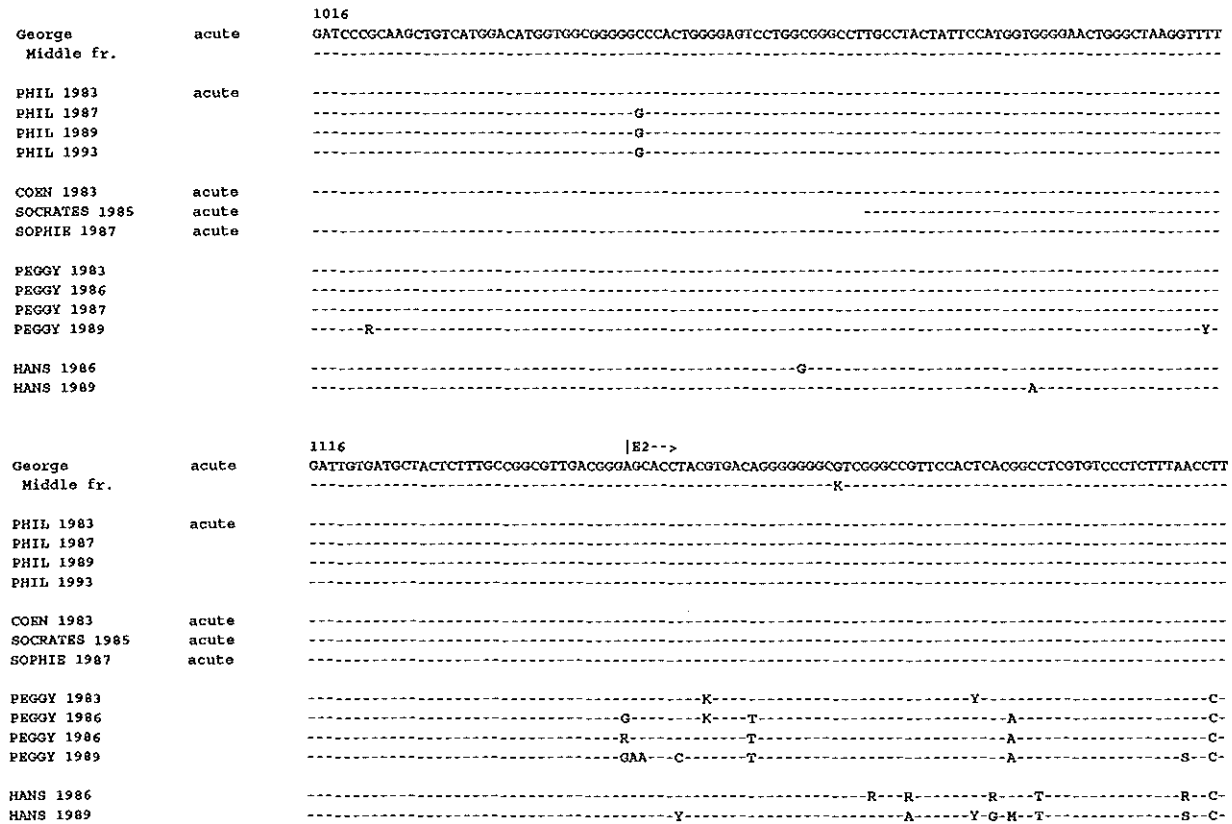
[2] A biphasic ALT profile was observed during the acute phase.

[3] - indicates absence of antibodies.

[4] first available sample after inoculation.

LIA = Line Immuno Assay. Dpi indicates number of days after infection.

Figure 3a. Nucleotide sequence alignments of E1-E2/NS1 region



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                                1216
George              acute       GGGGCGCAGCAAAAAGTCCAGCTCATAAACACCAACGGCAGCTGGCACATCAACAGAACTGCCCTGAACTGCAATGACTCCCTCCAGACTGGGTTCCTCG
 Middle fr.                     ----------------------------------------------------------------------------------------------------Y----

PHIL 1983           acute       ----------------------------------------------------------------------------------------------------
PHIL 1987                       ----------------------------------------------------------------------------------------------------
PHIL 1989                       ----------------------YR--------------------------------------------------------------------------------
PHIL 1993                       ------A---------------------------------------------------------------------------------------------

COEN 1983           acute       ----------------------------------------------------------------------------------------------------
SOCRATES 1985       acute       ----------------------------------------------------------------------------------------------------
SOPHIE 1987         acute       ----------------------------------------------------------------------------------------------------

PEGGY 1983                      ---------------A----------------------------------------------------------------------------------------
PEGGY 1986                      ---------------A-------------------------T----------------------------------------------------------
PEGGY 1987                      ---------------A-------------------------T----------------------------------------------------------
PEGGY 1989                      ---------------A-------------------------T----------------------------------------------------M---

HANS 1986                       ----------------------------------------------------------------------------------------------------
HANS 1989                       ---------------W----------------------------------------------------------------------------------A---

                                1316
George              acute       CTGCGCTGTTCTACACGCACAAGTTCAACGCGTCCGGATGCTCGGAACGCATGGCCC
 Middle fr.                     -----------------------------------------------------------

PHIL 1983           acute       -----------------------------------------------------------
PHIL 1987                       ---------------R-------------------------------------------
PHIL 1989                       ---------------A-------------------------------------------
PHIL 1993                       ---------------A-------------------------------------------

COEN 1983           acute       -----------------------------------------------------------
SOCRATES 1985       acute       -----------------------------------------------------------
SOPHIE 1987         acute       -----------------------------------------------------------

PEGGY 1983                      -----------------------------------------------------------
PEGGY 1986                      ------------------------------AT---------A---------------
PEGGY 1987                      ------------------------------AT---------A--------R------
PEGGY 1989                      ------------------------------AT---------A--------R------

HANS 1986                       -----------------------------------------------------------
HANS 1989                       --------------------------ASS-KY-------Y---------------
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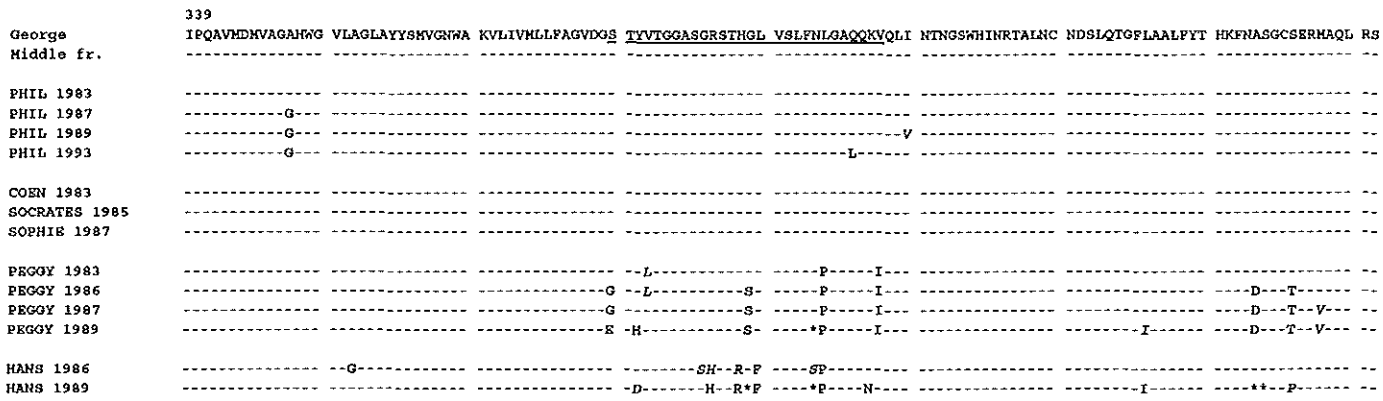
Alignments of E1-E2/NS1 sequences (nt 1016-1372), including the hypervariable region from 7 chimpanzees.
Middle fr. represents sequences from the "Middle Fraction". Sequences obtained during the acute phase of the disease are indicated.

Figure 3b. Deduced amino acid sequence alignment E1-E2/NS1 region



Deduced amino acid sequences (residues 339-457). Underlined amino acid sequence indicates the hypervariable region. Italics indicates the possibility of the presence of both the original and the indicated amino acid, due to sequence ambiguity. * indicates the possible presence of multiple amino acids.

Discussion

The courses of NANB hepatitis infections in chimpanzees were studied retrospectively by detection of HCV RNA and anti-HCV. All chimpanzees described in this study were infected with inocula from, or derived by passage of, a single acute phase plasma from chimpanzee George, containing genotype 1b HCV. It is important to note that George previously went through 2 distinct episodes of NANB hepatitis, caused by contaminated Factor IX and Factor VIII preparations respectively (Tsiquaye et al., 1980). Therefore, it is difficult to trace the real source of the predominant HCV variant as detected in George.

Chimpanzee Phil showed a biphasic ALT profile, considered characteristic for a specific Factor VIII preparation (Bradley et al., 1979, Beach et al., 1992. HCV-RNA was first detected 32 days after infection in Phil, in contrast with the much earlier detection in the other 3 chimpanzees involved in this and other studies (Shimizu et al., 1990). This phenomenon has been reported earlier (Hilfenhaus et al., 1992) but the amount of inoculated HCV and the first detection of viremia did not seem to be correlated.

Chimpanzee Coen had been infected by HIV-1 before inoculation with HCV containing acute phase plasma from Phil. It is unknown how the replication of these viruses may interfere, although an increased risk of vertical transmission has been reported (Giovannini et al., 1990).

Chimpanzee Sophie received a large inoculum of possibly high-titer material, obtained during the acute phase of infection in Socrates. The incubation period in chimpanzee Sophie did not differ significantly from the other animals, although a shorter incubation period after inoculation of a high titer HCV preparation in a chimpanzee has been suggested (Beach et al., 1992). The peak level of g-GT observed in Sophie was much higher than in the other chimpanzees and there may be a correlation between the infectious dose and the g-GT level, indicating the severity of primary infection.

In all animals, RNA levels dropped below detection level immediately after the sharp decline of the serum ALT level, a phenomenon that has been observed earlier (Beach et al., 1992, Shindo et al., 1992, Farci et al., 1991, 1992b). The level of serum ALT is considered to be a direct measure of hepatic injury (Ohosone et al., 1992). During acute illness, HCV-infected hepatocytes are lysed, releasing large quantities of liver specific enzymes in the blood. After a

massive destruction of infected hepatocytes, the number of infected hepatocytes producing HCV virions will be reduced significantly, causing very low levels of HCV RNA in the serum immediately after the ALT peak. It is unknown whether development of chronicity depends on an extrahepatic reservoir of HCV such as lymphocytes (Hellings et al., 1988, Müller et al., 1993) during this primary immune response.

Antibody profiles

Antibody patterns in the 4 chimpanzees showed significant differences, and were unpredictive for the development of chronicity. Remarkably, anti-NS5 has been observed only in 3 samples from Phil, obtained more than 5 years after infection. This result was unexpected since NS-5 epitopes in the LIA are derived from homologous genotype 1 sequences and anti-NS5 has been observed in chimpanzees and humans infected with various genotypes (van Doorn et al., 1992, 1994a). Anti-core antibodies were the first evidence of immune response in chimpanzees Coen, Socrates and Sophie, but were not detected in the chronically infected chimpanzee Phil up till 176 days after infection. The remarkably delayed anti-core response in Phil may have facilitated the development of chronicity in this animal.

Neutralization experiments

Neutralization experiments were clearly unsuccessful in Socrates and Hans. Although virus and antibody titers in the various sera may have been incompatible, this result and other reports (Farci et al., 1992b) suggest, that protective antibodies against HCV are not produced. Farci et al. (1992b) also reported that antibodies against core, NS3 and NS5 epitopes could not prevent reinfection in chimpanzees. This phenomenon may also partly explain the high percentage of chronic HCV infections. On the contrary, recent experiments (Hijikate 1993) suggested that association of antibodies with HCV virions is correlated with a reduction of infectivity, but these results remain to be validated in vivo.

Genetic drift of the hypervariable region

The N-terminal part of E2/NS1 has been designated as a hypervariable region (E2 HV; Weiner et al., 1991, Kato et al., 1992a,b, Hijikata et al., 1991, Oshima et al., 1991, Tanaka et al., 1992).

The sequence of E2 HV differed considerably (data not shown) from earlier reported genotype 1b isolates (Kato et al., 1990, Takamizawa et al., 1991). Putative consensus amino acids (Lesniewski et al., 1993) at positions 385 (threonine), 406 (glycine), 409 (glutamine) and 413 (leucine) are also conserved in these isolates. No variations in HCV sequence were observed between George and early acute phase isolates from Phil, Coen, Socrates and Sophie. This indicates the relative stability of the HCV hypervariable region during passage of the virus in another host, and is in accordance with previous reports (Farci et al., 1992b).

The observed mutation rate of the E2 HV region during chronic infection in chimpanzee Phil was remarkably low. Mutation of the viral RNA, caused by errors of the RNA polymerase, may result in appearance of new sequences in the virus population. These mutants may experience different selective pressure than the wildtype. Also, if an inoculum already contained multiple coexisting sequence variants, both mutation and selection can account for the detection of multiple sequences at different timepoints during infection. Sequence analysis by either cloning or direct sequencing of amplified cDNA has limited sensitivity to detect variants with a low abundancy. The low mutation rate of the E2 HV region observed in Phil may be due to the inoculation of a very limited number of different HCV genomes. In several reports, the buoyant density of HCV virions (Carrick et al., 1992, Miyamoto et al., 1992, Takahashi et al., 1992, Bradley et al., 1991) was estimated and results varied considerably. Association of HCV with ß-lipoprotein (Thomssen et al., 1992) will cause a much lower density, and may explain the observed 'floating' of the virus, whereas formation of immune complexes can cause an increase in density (Hijikata et al., 1993). Preselection of a specific population of HCV virions by fractionation of the George plasma by ultracentrifugation cannot be excluded. On the other hand, it is more likely that hypervariability is caused by selective immune pressure (Weiner et al., 1992, Kato et al., 1993). If a predominant E2 HV epitope is neutralized by specific antibodies, mutations may lead to escape mutants, which evoke a new immune response that again selects for novel escape mutants. If such an antibody response is absent, there will be no driving force for selection of escape mutants. Thus, the low mutation frequency observed in Phil may be due to the absence of selective immune pressure against E2 HV. We have not yet studied the antibody response against epitopes from the E2 HV region. Interestingly, far more replacement changes than silent mutations are observed in Peggy and Hans, suggesting the importance of immune selection of E2 HV epitopes.

Since the hypervariable region is located in a putative envelope protein, sequence variation may have important clinical implications, such as the efficacy of interferon therapy (Okada et al., 1992). Therefore, analysis of sequence variation should be accompanied by a thorough investigation of the immunological implications of host factors such as the HLA genotype. Understanding the interaction between virus and host will be crucial for the future development of vaccines or antiviral therapies.

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CHAPTER 5

Sequence analysis of the 5' untranslated region in isolates of at least four genotypes of Hepatitis C virus in the Netherlands

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Abstract

The RNAs of Hepatitis C virus (HCV) isolates from 62 patients with chronic HCV infection were analyzed by direct sequencing of the 5' untranslated region. Two important sequence motifs were recognized; one between position -170 and -155 and the other between -132 and -117. These motifs are partly complementary. All three previously published genotypes were observed: 34 (55%) isolates were classified as type 1 (including prototype [from the United States] and HCV-BK [from Japan] sequences), 11 (18%) as type 2 (including HC-J6 and HC-J8), 12 (19%) as type 3 (including EB1), and one patient was coinfected with genotype 1 and 2. Four (6%) isolates showed aberrant sequences and were therefore provisionally classified as genotype 4. These results indicate the significance of 5' UTR sequence variation among different HCV genotypes, and the possibility to use this region for consistent genotyping of HCV isolates.

Introduction

Since the discovery of hepatitis C virus (HCV), a flavi-like virus with a positive-sense, single stranded RNA genome of approximately 9.400 nucleotides (Choo et al., 1989), several full length sequences have been obtained from various isolates (Choo et al., 1991, Kato et al., 1990, Okamoto et al., 1991, Okamoto et al., 1992a, Takamizawa et al., 1991) and proposals have been made to classify HCV isolates into different (sub)types (Cha et al., 1992, Chan et al., 1992, Enomoto et al., 1990, Houghton et al., 1991, Mori et al., 1992, Okamoto et al., 1992c). Based on all available sequence information, Chan et al., (1992) distinguished 3 HCV genotypes. Type 1 isolates include the prototype HCV-1 (Choo et al., 1991), the HCV-H strain (Ogata et al., 1991), HCV-K1 (Enomoto et al., 1990), HCV-J (Kato et al., 1990) and HCV-BK (Takamizawa et al., 1991); type 2 includes HCV-K2 (Enomoto et al., 1990), HC-J6 (Okamoto et al., 1991) and HC-J8 (Okamoto et al., 1992a); type 3 includes HCV E-b1 (Chan et al., 1992), HCV-T (Mori et al., 1992) and HCV1196 (Lee et al., 1992).

HCV genotyping is of interest in viral transmission studies and HCV epidemiology. Furthermore, the success of interferon treatment (Pozatto et al., 1991, Yoshioka et al., 1992) may be (sub)type related.

There are several reasons to choose the 5' untranslated region (5' UTR) for genotyping: (a) analysis of a large number of HCV isolates resulted in similar phylogenetic trees for the 5' UTR, the core, NS-3 and NS-5 regions (Chan et al., 1992); (b) the observed mutation rate of the 5' UTR is extremely low (Ogata et al., 1991, Okamoto et al., 1992c); (c) sequence variation within the conserved 5' UTR is mainly limited to specific regions; and (d) the putative secondary structure of the 5' UTR, as established on biochemical and phylogenetic data (Brown et al., 1992), suggests (functional) conservation of this region.

In the study described here, 62 HCV isolates from patients in the Netherlands with a chronic HCV infection were investigated to determine whether HCV genotyping by sequence analysis of PCR products derived from the 5'UTR could be performed.

Materials and methods

Patients

Sixty-two patients from the Netherlands, between 26 and 74 years of age with elevated alanine aminotransferase (ALT) levels, a biopsy-proven chronic non-A, non-B hepatitis, antibodies to hepatitis C virus, and no recent history of infection with hepatitis B virus, hepatitis A virus, Cytomegalo virus or Epstein-Barr virus, were analyzed.

Anti-HCV

Antibodies to HCV were tested by a second generation enzyme immuno assay (EIA) (Abbott, North Chicago, IL, USA) and confirmed by the recombinant immunoblot assay (RIBA-4, Ortho Diagnostics, Raritan, NJ, USA) according to the instructions of the manufacturer.

Blood plasma

For HCV RNA detection, EDTA-blood was collected by venipuncture and plasma was prepared within 2 hours after sampling. One ml aliquots were quickly frozen in liquid nitrogen and stored at -70°C until use.

HCV RNA PCR

HCV RNA was isolated from 100 µl plasma by a modified version of the guanidinium method as described previously (Chomczynski et al., 1987). cDNA synthesis was performed on one-third of the isolated RNA in a 25 µl reaction volume (Kleter et al., 1993) using 20 pmol antisense primer HCV19 (GTGCACGGTCTACGAGACCT, position -1 to -20), 200 units Moloney murine leukemia virus reverse transcriptase (M-MLV, Gibco-Bethesda Research Laboratories, Gaithersburg, Md, USA), 30 units RNAsin (Promega, Madison, WI, USA), 0.5 mM of each deoxyribonucleotide (Boehringer, Mannheim, Germany) at 42°C for 30 min after brief denaturation at 80°C. PCR was performed (40 cycles, 1 min 94°C, 2 min 48°C, 3 min 74°C) with antisense primer HCV19 and sense primer HCV18 (GGCGACACTCCACCATAGAT, position -304 to -324) or sense primer HCV35 (TTGGCGGCCGCACTCCACCATGAATCACTCCCC, position -296 to -318; underlined sequences are not complementary to HCV). For diagnosis, the first round PCR products were analyzed by Southern blot hybridization with probe HCV17 (GAGTAGTGTTGGGTCGCGAA, position -86 to -67), followed by washing at low stringency.

Direct sequencing of PCR products

For direct sequencing (Hultman et al., 1991), a second round of PCR (40 cycles, 1 min 94°C, 2 min 48°C, 3 min 74°C) was performed with sense primer NCR3 (GGGGCGGCCGCCACCATRRATCACTCCCCTGTGAGG, position -288 to -314) and antisense primer LD58 (5' bio-GGCCGGGGCGGCCGCCAAGCACCCTATCAGGCAGTACCACAAGGC, position -37 to -64). LD58 is biotinylated at the 5' end. Biotinylated PCR products (estimated by agarose gelelectrophoresis at approximately 100 ng) were captured onto streptavidin-coated paramagnetic particles (Dynabeads M-280, Dynal, Oslo, Norway). Single stranded DNA was prepared by denaturation of

the captured amplification product by alkaline treatment according to the instructions of Dynal. Separate strands were sequenced using the T7 DNA sequencing kit (Pharmacia, Uppsala, Sweden) and [α-^{32}P]-dATP (Amersham, Buckinghamshire, UK). NCR3 served as a sense primer on the minus strand captured on the beads, and NCR4 (CACTCTCGAGCACCCTATCAGGCAGTACC, position -57 to -29) was used as antisense primer on the plus strand in the supernatant. DNA sequences were read manually from autoradiographs and analyzed with the PC/Gene computer-program (Intelligenetics Inc., Mountain View, CA, USA).

Results

Sixty-two patients with chronic HCV infection were anti-HCV positive by EIA, confirmed by RIBA-4, and were HCV RNA positive by RT-PCR aimed at the 5' UTR. Sense primer HCV18, based on the first published HCV sequences (Garson et al., 1990, Okamoto et al., 1990) was initially used for diagnosis of HCV viraemia by PCR. In the case of low yields of PCR products, sense primer HCV35 improved PCR results considerably (data not shown), and in these cases, PCR products obtained by HCV35 were used for sequence analysis.

Nested PCR products were directly sequenced, and the results are represented in figure 1. If identical sequences were obtained from a number of isolates, only one representative is shown. Several sequences have been reported previously, and are identified by their original names. Limited sequence variations were observed essentially in 2 motifs: motif 1 is located between nt -170 and nt -155, and motif 2 between nt -132 and nt -117 (figure 1). Based on these motifs, 58/62 isolates could be classified into the 3 genotypes as proposed previously. Thirty-four (55%) isolates were classified as type 1, which includes the prototype HCV sequence HCV-1. Isolate HC1-N8 showed a single nucleotide insertion at position -138. In one patient (isolate HC1/2) a double infection with type 1 and 2 was observed, as deduced from bands with identical mobility in two different lanes on the gel (figure 2). Type 2 sequences, like HC-J6, were observed in 11 (18%) patients. These isolates are also recognized by the presence of a T at position -72 and a C at

position -80. HC2-N2 contains a mutation at nt -127 in motif 2. Within genotype 2, sequence heterogeneity was observed at position -119, showing either a T or C. Type 3 sequences were detected in 12 (19%) patients. In addition to the type-specific sequence motifs, type 3 isolates can also be identified by a TCA sequence at positions -93 to -95. HC3-N2 contains a point mutation at position -118 in motif 2. Four (6%) isolates were provisionally classified as type 4. These isolates showed additional sequence heterogeneity between position -238 and -235, compared to types 1, 2, and 3.

Comparison between obtained (nt -262 to -66) and all previously reported 5' UTR sequences (Bukh et al., 1992), Cha et al., 1992, Chan et al., 1992, Han et al., 1991), revealed a number of new 5' UTR mutations within all genotypes (figure 1).

Table 1. Mean nucleotide variation among four HCV genotypes in (A) the entire 197 bp 5' UTR fragment and (B) motifs 1 and 2.

(A)

Type	Number of isolates*	nucleotide variation (%) 1	2	3	4
1	34	0.025			
2	11	0.071	0.020		
3	12	0.071	0.112	0.010	
4	4	0.041	0.061	0.061	0.020

(B)

1	0.000			
2	0.280	0.025		
3	0.280	0.355	0.003	
4	0.125	0.235	0.158	0.015

Figure 1. Alignment of 5' UTR sequences from different genotypes.

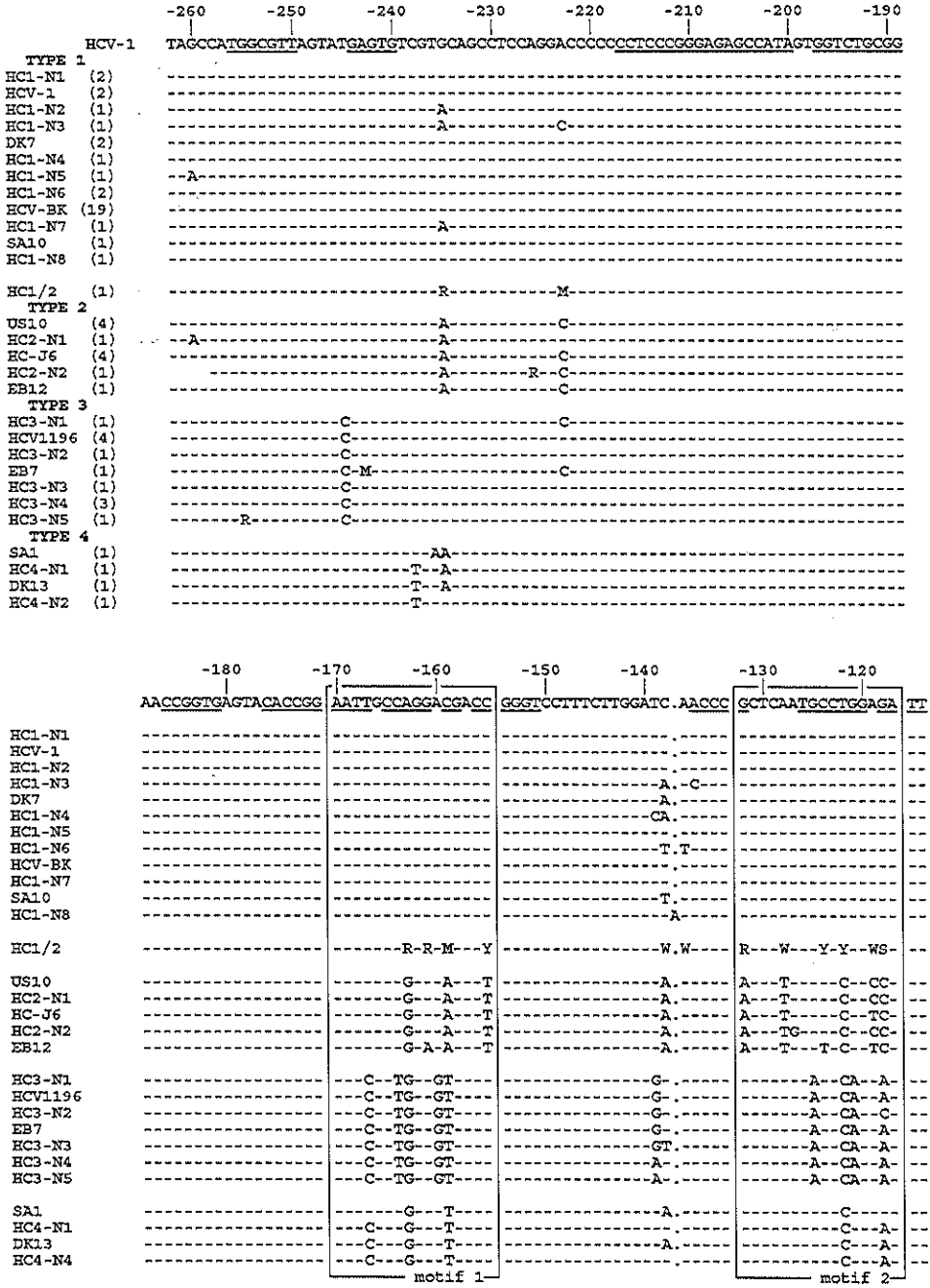
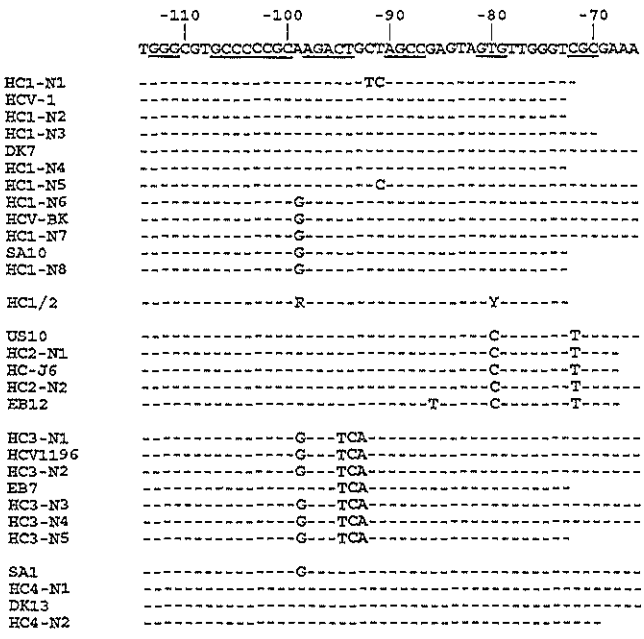


Figure 1. (continued)



Alignment of 5' UTR sequences from 62 patients. Sequences between position -262 and -66 were classified into genotypes 1, 2, 3 (as proposed by Chan et al., 1992), and 4. Previously published sequences are identified by their original name: HCV-1 (7); DK7, SA10, US10, SA1, and DK13 (2); HCV-BK (26); HC-J6 (21); EB-12 and EB-7 (4) and HCV1196 (17). Numbers in parentheses indicate the number of isolates with that sequence. Hyphens indicate presence of identical nucleotides as in prototype. Sequence motifs 1 and 2 are boxed. HCV-1 underlined nucleotides are involved in the putative dsRNA stem structure (1). Abbreviations: M (A or C); R (A or G); Y (T or C); W (A or T) and S (G or C).

The overall sequence heterogeneity among the 4 genotypes in this study ranged from 4% between types 1 and 4 to 11% between types 2 and 3, whereas heterogeneity within each type was less than 2.5% (Table 1a). Mutations are not randomly distributed along the 5' UTR, but are clustered in motifs 1 and 2. Comparison of these motifs among the four genotypes revealed strong conservation within each genotype and showed significant differences between the genotypes (Table 1b). Further analysis of the sequence variation in motifs 1 and 2 revealed the presence of

covariants, i.e., complementary mutations in each motif, maintaining the postulated secondary structure of the 5' UTR genomic RNA (Brown et al., 1992). The P-values for the occurrence of one, two, or three covariant mutations in motifs 1 and 2 to have arisen by chance were very low (*P= 0.06, P=0.004, P=0.0002,* respectively). Covariance occurred at positions -164, -163, -161 and -155 in motif 1, and positions -132, -124, -122, and -121 in motif 2. This covariance was consistently observed in all 62 sequences, indicating the importance of this phenomenon.

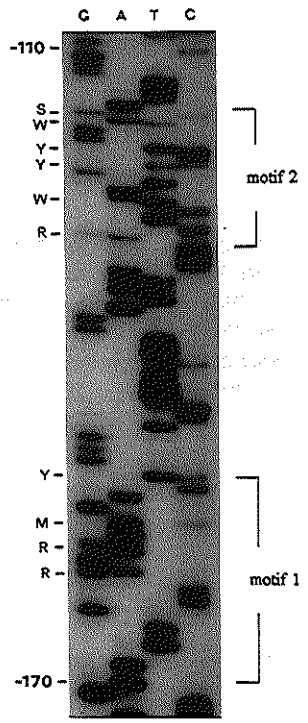


Figure 2. Direct sequence analysis of the 5' UTR from isolate HC1/2 with sense primer NCR3. Abbreviations: S (G or C); W (A or T); Y (T or C); R (A or G) and M (A or C).

Discussion

Sequence analysis of the 5' UTR of HCV isolates from 62 patients with a chronic HCV infection allowed consistent and efficient genotyping. Fifty-eight (94%) isolates could be classified into the 3 different genotypes as proposed by Chan et al., (1992). Identification of the genotypes is essentially based on the sequence variation in the defined motifs 1 and 2.

All three genotypes reported so far were observed in this patient population in the Netherlands. Coexistence of HCV genotypes in several geographic regions has been indicated earlier (Cha et al., 1992, Chan et al., 1992. However, little is known about the distribution of HCV genotypes in Europe. The majority of published isolates belong to type 1 (Cuypers et al., 1991, Kremsdorf et al., 1991). Types 2 and 3 were detected in European isolates only recently (Bukh et al., 1992, Chan et al., 1992).

Although at a low frequency, isolates belonging to a new genotype, tentatively designated as genotype 4, were observed in the Netherlands. Similar sequences were first found in isolates from South Africa (Cha et al., 1992) and Denmark (Bukh et al., 1992). To justify classification of these isolates as a new genotype 4, sequence analysis of coding regions is necessary. Preliminary results (data not shown) indicate significant nucleotide sequence differences between the core region from type 4 isolates and corresponding sequences from types 1, 2, and 3. One sequence (SA1), classified here as type 4, showed minor differences with the other type 4 sequences, and might be classified as a separate genotype 5 (P. Simmonds et al, unpublished observations). Probably, all genotypes have a worldwide distribution but relative abundances per geographic region may differ considerably.

In one patient a double infection was found involving genotype 1 and 2. This is in accordance with the reported frequency of double infections which has been observed by others (Okamoto et al., 1992b, Yoshioka et al., 1992) using type-specific primers or probes.

New 5'UTR sequences were observed in 21 of the 62 analyzed isolates. The distribution of the sequence variation is not random. Recently, the putative secondary structure and possible functional elements of the 5' UTR of the HCV genome were postulated (Brown et al., 1992, Tsukiyama-Kohara et al., 1992, Yoo et al., 1992). The defined sequence motifs 1 and 2 show partial complementarity and are able to form a stable stem-loop structure, e.g., nt A at pos. -170

is complementary to nt T at pos. -115 and the nt C (or T for type 2) at pos. -155 is complementary to nt G (or A for type 2) at pos. -132. Covariance in motifs 1 and 2 consistently preserve the secondary structure. Therefore, mutations are more likely to be tolerated in single stranded regions. This is confirmed by the relatively high mutation rate in the ssRNA loop between position - 136 and -151. Most of the point mutations are located outside the defined sequence motifs and do not affect classification. Only 2 isolates showed single point mutations within the 2 motifs: HC2-N2 contains a G at position -127, and HC3-N2 contains a C at position -118. Despite these variations, classification of HC2-N2 and HC3-N2 as type 2 and 3 respectively, was obvious.

The overall sequence variation between the 197 bp 5' UTR fragments is not statistically significant, as only 6-21 mutations were observed among the four genotypes. Since the mutations are not randomly distributed along the 5' UTR, it is impossible to apply regular statistical methods to this problem, as these require random distribution of events. The secondary structure, i.e. the stem-loop structure formed by partial complementarity of motifs 1 and 2, must be maintained (with a sufficiently low free energy) to function properly as an internal ribosomal entry site (Tsukiyama-Kohara et al., 1992). Therefore, mutations in these motifs have functional restrictions. Comparison of the 5' UTR sequences as described in this paper, reveals the existence of covariants. These complementary mutations in motifs 1 and 2 indeed completely conserve the secondary structure, indicating the significance of covariance. Based on these findings, classification of HCV isolates should preferably be based on covariance in motifs 1 and 2.

In conclusion, consistent genotyping based on 5' UTR sequence analysis is possible and may complement studies on antiviral treatment and transmission of HCV.

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The sequences reported in this paper have been deposited in the EMBL data library (accession numbers X58937-X58953).

CHAPTER 6

Analysis of Hepatitis C virus genotypes by a Line Probe Assay (LiPA) and correlation with antibody profiles

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Abstract

In order to determine the (sub)type of Hepatitis C virus (HCV) the 5' untranslated regions (5' UTR) derived from 54 patients with a chronic HCV infection were analyzed. Biotin-labelled PCR products from the 5' UTR were used for reverse hybridization in a Line Probe Assay (Inno-LiPA) and results were validated by comparison with direct sequencing data. Five different genotypes could be distinguished based on 5' UTR sequence diversity. Results of typing by LiPA and direct sequencing were similar. Antibody responses against core, NS-3, NS-4 and NS-5 epitopes were detected by RIBA-4 and Inno-LIA HCV Ab II confirmatory assays. There was no clear correlation between genotype and anti-HCV response, although types 2 and 3 HCV isolates show poor reactivity with NS-4 epitopes.

Introduction

Hepatitis C virus, the main etiological agent of post-transfusion hepatitis, is a small enveloped virus and contains a positive sense, single-stranded RNA genome of approximately 9400 nucleotides (Choo et al., 1989). Based on genomic (Miller et al., 1990) and physico-chemical characteristics (Bradley et al., 1985) of the virus, HCV is classified as a distinct member of the Flaviviridae. From HCV isolates, obtained worldwide, several full length (Kato et al., 1990a, Takamizawa et al., 1991, Choo et al., 1991, Okamoto et al., 1991, 1992a, Chen et al., 1992) and numerous partial sequences (e.g. Chan et al., 1992, Han et al., 1991, Ogata et al., 1991, Takeuchi et al., 1990, Kato et al., 1990b, Mori et al., 1992, Enomoto et al., 1990, Weiner et al., 1991, Lee et al., 1992) have been obtained. Based on sequence diversity, several proposals were made to classify the different HCV isolates (Chan et al., 1992, Enomoto et al., 1990, Okamoto et al., 1992b, Houghton et al., 1991, Cha et al., 1992), but there is no consensus in HCV nomenclature so far. A useful HCV classification was proposed recently (Stuyver et al., 1993), based on phylogenetic trees determined by Chan and collegues (Chan et al., 1992), differentiating between types (approx. 68% average sequence homology) as well as between subtypes (approx. 79% sequence homology). Homologies between isolates belonging to the same subtype are about 88%. This system was further extended with new types, provisionally designated as types 4 and 5 (Simmonds et al., 1993).

There are indications that infections caused by different HCV (sub)types may have different clinical implications (Okamoto et al., 1992b). The effectiveness of antiviral treatment (Kanai et al., 1992, Pozatto et al., 1991, Yoshioka et al., 1992), efficiency of viral transmission, distribution among various patient populations and the development of hepatocellular carcinoma may also be subtype related. Preliminary results urge HCV subtyping which complements the routine diagnostic antibody and RT-PCR assays.

All available sequence data were used to develop type and subtype-specific probes for the reverse hybridization Line Probe Assay (LiPA), which has been described in detail recently (Stuyver et al., 1993). The assay is based on the observation that variation within the 5' UTR is mainly restricted to 2 short (sub)type specific sequence motifs.

In this study HCV isolates were analyzed from 54 patients by the novel LiPA which allows classification of isolates into genotypes 1, 2 and 3 (Chan et al., 1992, Stuyver et al., 1993), and the new types 4 and 5 (Simmonds et al., 1993). Results of reverse hybridization were compared with data from direct sequencing of the 5' UTR. Furthermore HCV antibody profiles, determined by RIBA-4 and Inno-LIA HCV Ab II, were compared with the 5 genotypes.

Materials and methods

Patient sera

Blood samples from 54 patients were obtained by venipuncture. EDTA-plasma was prepared within 2 hours after collection, aliquotted, quickly frozen in liquid nitrogen and stored at -70°C. All patients had a chronic HCV infection with elevated ALT levels, biopsy-proven liver abnormalities and were anti-HCV and HCV-RNA positive (Kleter et al, 1993).

RNA isolation, and reverse transcriptase PCR (RT-PCR)

HCV-RNA was isolated from freshly frozen plasma samples by a modified version of the acid guanidinium-phenol-chloroform method as described (Kleter et al., 1993). Briefly, cDNA was synthesized using antisense primer HCV19 (pos. -1 to -20; 5'-GTGCACGGTCTACGAGAC CT-3') and amplified by PCR using HCV19 and sense-primer HCV18 (pos. -323 to -304; 5'-GGCGACACTCCACCATAGAT-3') or HCV35 (pos. -318 to -296; TTGGCGGCCGCACTCCA-CCATGAATCACTCCCC). Fourty PCR cycles were performed, consisting of 1 min. 94°C., 1

min. 55°C, and 1 min. 72°C. Amplification products were analyzed by agarose gelelectrophoresis and Southern blot hybridization using probe HCV17 (pos -88 to -69; 5'- GAG-TAGTGTTGGGTCGCGAA-3').

Line Probe Assay (LiPA)

Based on all available 5' UTR sequences, several type-specific sequence motifs were recognized (see also Stuyver et al., 1993). Motif 1 is located between positions -170 and -155, and motif 2 between -132 and -117. These motifs already allow discrimination between the different genotypes, are partially complementary and can form a stable dsRNA stem structure (Brown et al., 1992). A number of positions displaying more subtle, but consistent variations allow consolidation of typing by means of motif 1 and 2, or enable more detailed subtyping. Type 1 can be divided into subtypes 1a and 1b, by the presence of an A or G at position -99, respectively. At pos -99 a G has been observed in all type 1b isolates studied so far, but is also present in most type 3 and some type 4 isolates. Type 2 isolates are also recognized by a T at -72 and a C at -80. Further subtyping into subtypes 2a and 2b is based on nucleotides at positions -161 (G or A respectively) and -124 (C or T respectively). Therefore, motifs 1 and 2 can be used for both typing and subtyping of type 2 isolates. Type 3 is also identified by the presence of a TCA motif at positions -95 to -93.

The LiPA is based on the hybridization of labelled PCR amplification products to specific oligonucleotides directed against the variable regions of the 5' UTR. These probes were immobilized as parallel lines on membrane strips (reverse hybridization principle). During nested PCR, the product is biotinylated, which allows detection of hybrids by alkaline phosphatase labelled streptavidin. The HCV-LiPA (figure 1) contains 15 probe lines, exposing 18 different 16-mer probes. Sixteen probes specifically recognize HCV genotypes, and 2 (no. 21 and 22) are general HCV probes (location of the probes in the 5' UTR sequence is shown in figure 2.). The development of the LiPA was described in detail recently (Stuyver et al., 1993).

From the first round PCR, 0.5 μl product was transferred into a new, 50 μl nested PCR reaction, containing primers HC3 (sense: -264 to -238: 5'-TCTAGCCAT GGCGTTAGTRYGAGTGT-3'), HC4 (antisense: -29 to -54: 5'- CACTCGCAAG-CACCCTATCAGGCAGT-3') and biotinylated ^{11}dUTP. These biotinylated nested PCR products were used as probe on the LiPA strips. Briefly, biotinylated DNA was denatured by mixing 10-20 μl of the nested PCR reaction with an equal volume of 400mM NaOH, 10mM EDTA, and incubating for 5 min. at room temperature. The denatured fragment was hybridized to the probes

on the LiPA in the presence of tetramethylammoniumchloride (TMACl, Merck) at 42°C for 2 hours. Strips were washed at 52°C and alkaline phosphatase conjugated streptavidin was added and coloured by NBT/BCIP substrate (4-Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate) for 15-30 min. at room temperature in the dark. Strips are finally rinsed in distilled water and air-dried. The results of the LiPA were determined by scoring the presence or absence of hybridization with each probe line.

Direct sequencing

PCR products were reamplified using sense primer NCR3 (pos. -314 to -288; 5' - GGGGCGGCCGCCACCATARRATCACTCCCCTGTGAGG-3'; underlined sequence is non-HCV specific) and LD58 (pos -66 to -35; 5'-Bio-GGCCGGGGCGGCCGCCAAGCACCCTA-TCAGGCAGTACCACAAGGC-3') carrying a 5' biotin moiety. Biotinylated nested PCR products were used as template for direct sequencing, using the protocol suggested by the manufacturer of the Dynabeads (Dynal, Norway). Briefly, nested PCR products were mixed with streptavidin-coated paramagnetic particles (Dynabeads M280, Dynal, Norway) in binding and washing (B&W) buffer (1x B&W buffer is 10 mM Tris-HCl, pH 7.5, 1 M NaCl, 1 mM EDTA), to allow the binding of the biotinylated DNA. Complementary strands were separated by addition of NaOH, and sequenced using the T7 DNA sequencing kit (Pharmacia, Uppsala, Sweden) and [α-^{32}P]-dATP (Amersham, Buckinghamshire, UK). DNA attached to the beads or in the supernatant was sequenced with either NCR3 as a sense primer on the captured minus-DNA strand or NCR4 (pos.-66 to -47; 5'-CACTCTCGAGCACCCTATCAGGCAGTACC-3') as an antisense primer on the plus strand in the supernatant. Sequencing products were separated on an 8% polyacrylamide:bisacrylamide gel (19:1 w/w). DNA sequences were read manually from autoradiographs and analyzed with the PC/Gene computer program (Intelligenetics Inc., Mountain View, CA, USA).

Anti-HCV assays

Antibodies to HCV were assayed by an Enzyme Immunosorbent assay (EIA; Abbott Chicago, Il, USA) and confirmed by RIBA-4 (Ortho diagnostics, Raritan, NJ, USA) and Inno-LIA HCV Ab II (Innogenetics, Gent, Belgium) according to the manufacturers instructions.

Results

HCV-RNA was isolated from 54 patients with a chronic HCV infection, and amplified by RT-PCR using 5' UTR specific primers. These PCR products were analyzed by direct sequencing as described elsewhere (Kleter et al., 1994). Biotinylated amplification products from nested reactions were used as probe in the reverse hybridization LiPA system. The location of the probes on the LiPA strip and typical LiPA results are shown in figure 1.

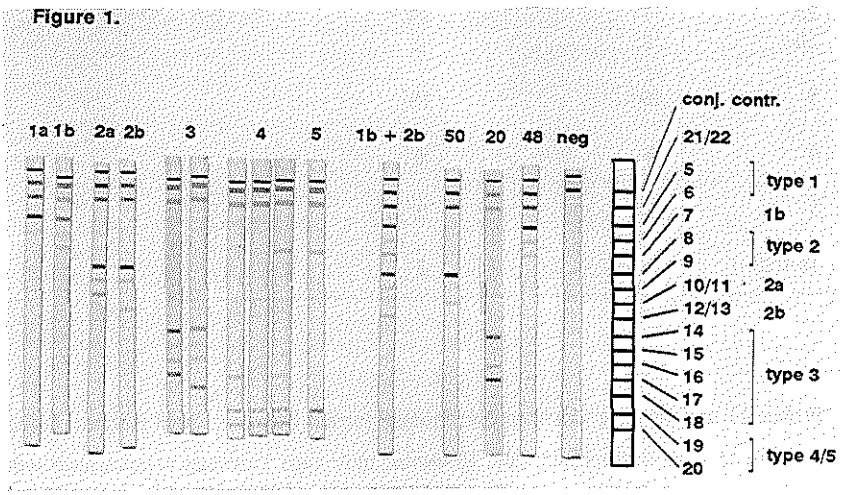


Figure 1. Positions of the probe lines on the LiPA strip and typical results. Probes 21 and 22 are general probes to identify general 5' UTR sequences. Probes 5-20 distinguish different types and subtypes of HCV as indicated. Representative results of isolates containing types 1a, 1b, 2a, 2b, and 3 are shown, together with all type 4 and 5 isolates. Furthermore, isolates 50 and 20, displaying sequence-specific variation and the single discrepant isolate 48 are shown.

All labelled nested PCR products hybridized to general probes 21/22 and with a subset of genotype-specific probes. Results of LiPA analysis of 54 patient isolates are summarized in table 1. Five different genotypes were detected and there were no isolates that could not be typed by LiPA. Observed hybridization patterns were consistent and classification into types and subtypes was obvious in all isolates, including one double infection with subtypes 1b and 2b.

Table 1. Genotypes of 54 patient HCV isolates determined by the LiPA

Type	# of isolates	%
1a	7	13
1b	24	44
2a	7	13
2b	1	2
3	10	18
4	3	6
5	1	2
1b+2b	1	2

Probes 17 and 18 were aimed to discriminate further between type 3 isolates, based on the presence of a G or A at -139 respectively. However, most type 1 isolates cross-reacted with probe 17, and to a lesser extent with probe 18. Overall background staining on the strips was very low or completely absent.

Reverse hybridization results from LiPA were compared with data obtained from direct sequencing. Sequences were determined on nested products derived from the same first round PCR products as used for LiPA. 5' UTR sequences and locations of LiPA probes are shown in figure 2. Comparison of LiPA and direct sequencing results revealed a small discrepancy in only one sample. Amplified HCV-cDNA from this isolate (no. 48, containing the DK7 sequence figure 2.)

hybridized with probe 7 (figure 1), indicating the presence of a G at pos. -99, but direct sequencing showed only an A. This isolate is therefore classified as 1b by LiPA but as 1a by direct sequencing. On the other hand, some aberrant hybridization patterns observed in LiPA were validated by direct sequencing results. Isolate 50 (HC2-N2) failed to hybridize with probe 9. This is in perfect agreement with the presence of a G at -127. Isolate 20 (HC3-N2) hybridized only very weakly to type 3-specific probe 15, which can be explained by the presence of a C at -118. The presence of such mutations in regions used for typing never hampered the classification because 2 or 3 type-specific probes are present in the LiPA. Probe 9 contains a C/T degeneracy at -167, which allows hybridization to both type 4 and type 5 sequences. Probe 20 does not hybridize to the type 5 sequence. In a number of isolates additional sequence variation outside the defined motifs 1 and 2 was detected by direct sequencing, but this did not affect the classification by the LiPA.

Antibodies against various HCV epitopes were assayed by RIBA-4 and LIA. Results are shown in table 2.

Table 2. Anti-HCV profiles of 54 patient isolates determined by RIBA-4 and Inno-HCV LIA HCVAb II

		RIBA-4					**Inno-LIA**			
type	# pat.	511 (NS4)	C-100 (NS4)	C33c (NS3)	C22-3 (core)	total	NS-4	NS-5	core	total
1a	8	8	8	8	8	8	8	5	8	8
1b	23	22	19	22	22	22	22	13	22	22
2a	7	1	2	7	7	7	2	5	6	6
2b	1	0	0	1	1	1	0	1	1	1
3	10	0	4	10	10	10	2	7	10	10
4	3	0	1	3	3	3	2	2	3	3
5	1	1	1	1	1	1	1	1	1	1
1+2	1	0	0	1	1	1	0	1	1	1

Anti-HCV detection by RIBA and LIA. 5-1-1 and C-100 are derived from the NS-4 region. C33c contains NS-3 and C22-3 covers core epitopes. Numbers in columns represent samples with a positive reaction against the specified epitope.

One patient was negative by RIBA and LIA. One was indeterminate by LIA, as only weak anti-core reaction was detected. From the single anti-HCV negative sample, weak HCV-RNA signals were obtained. However, a sample from this patient, obtained one month earlier, was anti-HCV positive in RIBA. Only one patient specifically lacked core antibodies in both confirmatory assays, all other anti-HCV positive isolates were anti-core positive in both assays. All patients contained anti-NS-3 antibodies as determined by RIBA.

Type 2 and type 3 infected patients show a low response rate against NS-4 epitopes, both in RIBA and LIA. NS-5 antibodies are detected by LIA in the majority of patients. The single isolate with a coinfection did not contain antibodies against NS-4 epitopes.

Figure 2. Alignment of 5' UTR sequences.

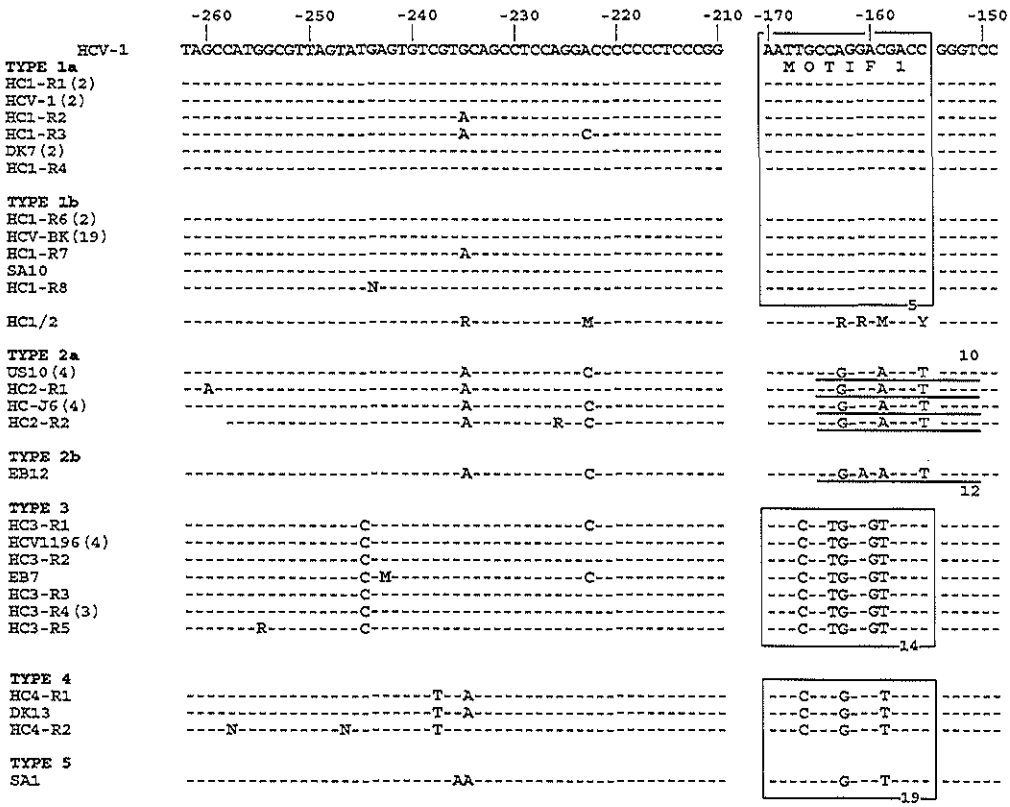
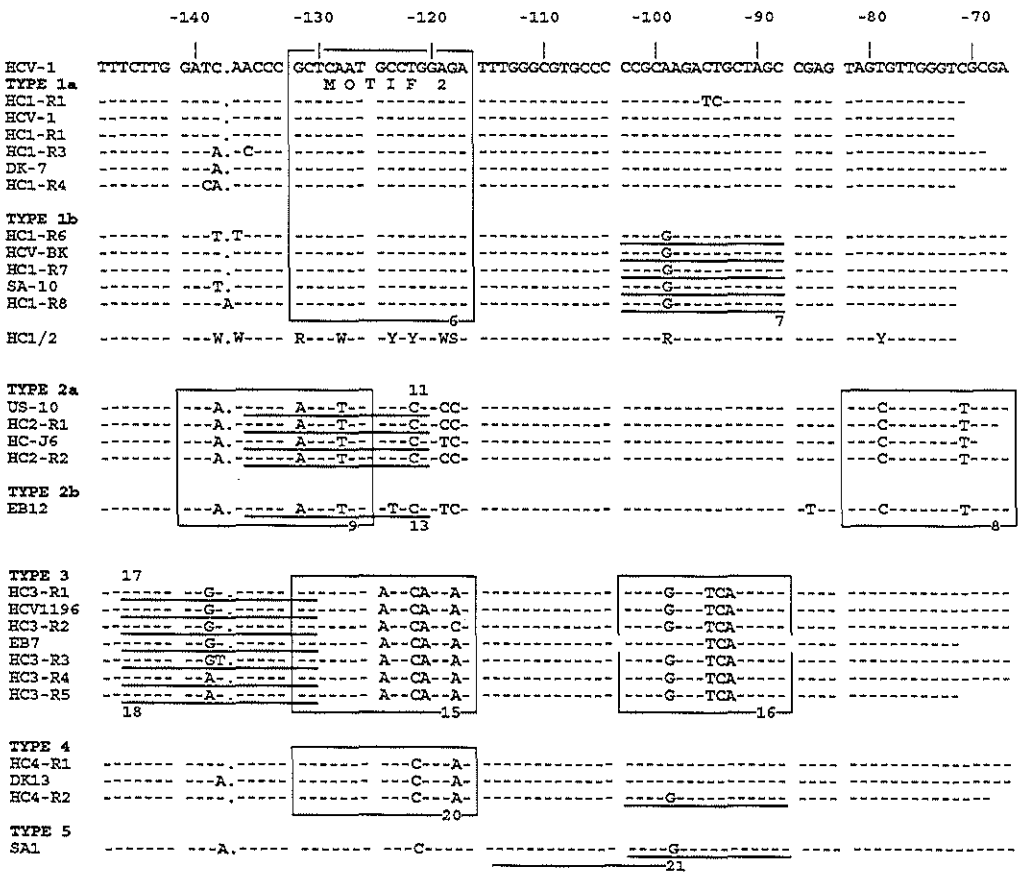


Figure 2. (continued)



Alignment of 5' UTR sequences (pos. -265 to -68) and positions of LiPA probes. Sequences between pos. -210 and -170 are completely conserved and omitted from the figure. Sequences are grouped into 5 different genotypes. HCV-1 is the prototype sequence (Choo et al. 1990). DK-7, SA10, US10, and DK13 (Bukh et al. 1992), HC-J6 (Okamoto et al 1992), EB-7, EB-12 (Chan et al 1992) were previously reported and identified by their original name.

Discussion

This study describes the use of a novel reverse hybridization Line Probe Assay to determine the (sub)type of HCV in 54 well characterized patient plasma isolates. The system was evaluated by direct sequening and results are highly similar.

There are several reasons to use the 5' UTR of the HCV RNA genome for genotyping. Firstly, the 5' UTR is generally used in diagnostic PCR assays to detect viremia by universal primers. Therefore it is convenient to perform subsequent typing analysis on the resulting DNA product. Secondly, the sequence variations within the 5' UTR are limited to specific regions, such as motifs 1 and 2, located between highly conserved flanking sequences, allowing the application of general PCR primers as well as (sub)type-specific probes. This relatively high degree of conservation in the 5' UTR omits the necessity of type-specific primers to classify HCV using more variable coding regions of the genome (Okamoto et al., 1992b). A large number of 5' UTR sequences has been published so far (Chan et al., 1992, Stuyver et al., 1993, Bukh et al., 1992). Discrimination between types has been described using amplified cDNA derived from the 5' UTR for restriction fragment length polymorphism (RFLP, Simmonds et al., 1993, Nakao et al., 1991, McOmish et al., 1993). Furthermore, the overall mutation rate of HCV has been estimated at approximately 1.5×10^{-3} base substitutions per site per year (Okamoto et al., 1992c, Ogata et al., 1991). However, mutations occur unevenly along the genome. A hypervariable region located at the N-terminus of E2/NS1 has a high mutation rate, whereas the 5' UTR has a very low rate. Studies on genetic drift of HCV for more than 8 years in a chronically infected chimpanzee (Okamoto et al., 1992c) and over 13 years in a chronic patient (Ogata et al., 1991) showed complete conservation of the 5' UTR. Finally, the putative secondary structure of the 5' UTR (Simmonds et al., 1993, Brown et al., 1992, Tsukiyama-Kohara et al., 1992) implies functional conservation with respect to initiation of translation of the single open reading frame and genome replication (Yoo et al., 1992). Therefore, it is expected that there is a high selective pressure on the function of this region. This is indicated by the existence of paired mutations (co-variants) in the complementary strands in the stem of the putative RNA-hairpin. This covariance, observed among different genotypes, conserves secondary RNA structure of the region (Simmonds et al., 1993). Finally, extensive sequence comparisons have shown (Chan et al., 1992) that sequence heterogeneity in the 5' UTR displays similar phylogenetic relationships between HCV genotypes as other regions such as core, NS3 and NS5. Therefore, sequence analysis of the 5' UTR should

allow consistent discrimination of HCV types.

HCV-RNA isolates from 54 chronic HCV patients were genotyped by 5' UTR analysis. Amplified cDNA from the 5'UTR was biotin-labelled during nested PCR and used as a probe for reverse hybridization in the LiPA. Reverse hybridization offers a fast method to screen for the presence of specific sequences in a PCR product. The probes used in the LiPA described here cover a considerable part of the entire 5' UTR sequence. The LiPA contains both general and (sub)type specific probes and therefore allows detection of known as well as unknown HCV genotypes. Each of the 54 isolates described here hybridized to the general probes and could be further (sub)typed by LiPA. New HCV types will fail to hybridize with the current (sub)typing probes on the strip, but will hybridize to the general probes. Also aberrant hybridization patterns can be observed on the LiPA, as shown in isolates 20 and 50 (figure 2.). Therefore the LiPA provides an instrument for rapid identification of new HCV types or subtypes. The entire LiPA post-PCR procedure takes only 5 hours and can be performed easily in a routine setting. If new types are identified, the sequence can be determined and corresponding probes can be added to the strip, making it a very versatile system. In contrast, the efficacy of RFLP analysis entirely depends on the presence of useful restriction sites within the variable regions.

To evaluate the efficiency of the LiPA system, the presence or absence of hybridization to each probe was compared with the corresponding results from direct sequencing. Single nucleotide differences were efficiently detected, e.g. to discriminate between type 2a and 2b by probes 10, 11, 12, and 13. In addition to published sequences, new variations were also detected by LiPA and confirmed by direct sequencing, as observed in isolates HC3-N2 and HC2-N2. There was only 1 discrepancy between LiPA and direct sequencing. PCR fragments from isolate 48 (DK7 sequence) weakly hybridized with probe 7, suggesting the presence of a G at -99. However direct sequencing showed the presence of an A exclusively. The question remains whether this isolate contains 1a or 1b sequences, although hybridization with probe 7 is much weaker than in all other 1b isolates. A possible explanation is a co-infection with types 1a and 1b. A large excess of 1a sequences over 1b sequences could explain the failure of direct sequencing to detect both A and G at -99 and the weak hybridization of the PCR product with probe 7. The directly obtained sequence represents the major sequence present in an isolate. Furthermore the LiPA does identify subtype 1a based by absence of hybridization with probe 7. Positive identification of each subtype could further improve the reliability of the LiPA. Probes 17 and 18 cross-reacted with most of the type 1 isolates. The reason for this is unclear. The value of probes 17 and 18 in discriminating between the

presence of a G or A at position -139, is doubtful. The target sequence for these probes is located in the region forming a single stranded RNA loop in the putative secundary structure of the genomic RNA (Brown et al., 1992). This might also explain the relatively high frequency of mutations detected in this region, including the insert in HC1-N8.

A single double infection with subtypes 1b and 2b was detected (figures 1 and 2; isolate HC1/2) both by LiPA and direct sequencing. It is much easier, both to detect and (sub)type the double infection by LiPA than by direct sequencing. Interpretation of double signals at one nucleotide position by direct sequencing can be difficult and may require advanced experience in reading sequence autoradiographs.

Although the version of the LiPA described here did not yet contain type 5 specific probes, it was possible to discriminate between type 4 and 5 sequences because probe 19 contains a degeneracy. Addition of type 5 specific probes onto the LiPA strip will further facilitate recognition of this genotype.

Profiles of antibodies against specific HCV epitopes were determined. Two confirmatory immunoblot assays, capable to detect antibodies against epitopes from core, NS-3, NS-4 and NS-5, could not discriminate between different genotypes. RIBA-4 uses expression products of recombinant cDNA clones, derived from various parts of the HCV genome, whereas the Inno-LIA exposes a number of synthetic peptides. All epitopes are based on sequences derived from type 1 HCV isolates, because no data from other genotypes were available at the time these tests were developed. At present much more data are available from genotypes 1, 2 and 3, and reveal significant sequence heterogeneity in various parts of the genome (Chan et al., 1992, Cha et al., 1992). Consequently, infection with other HCV genotypes may evoke antibodies against type specific epitopes, which are not optimally recognized by type 1 epitopes in the RIBA and LIA. This is illustrated by the absence of antibodies against NS4 epitopes in most type 2 and 3 isolates (Chan et al., 1991, table 2). Antibodies against 5-1-1 are even completely absent in type 3 isolates. The single indeterminate LIA result on an isolate classified as type 2a may be partly explained by genotype-specific immune reactions.

Antibodies against core and NS-5 epitopes show a higher degree of cross-reactivity. It was possible to distinguish a number of type 1 and 2 isolates by assaying anti-core antibodies, directed against 2 different type-specific core peptides (Machida et al., 1992), although specificity was limited. Type-specific serological assays would facilitate discrimination between different HCV genotypes considerably. Sequence information of antigenic regions is still limited. Therefore, much

more sequence data from all different genotypes must be obtained, in order to develop (sub)type-specific antigens for antibody assays. However, considerable differences in immune reactivity among individual patients exist. In some patients, antibody responses are poor; e.g. in isolate 23, antibodies against core were undetectable. Also in immunocompromised patients serological testing is difficult, due to lack of sufficient antibody titers. These conditions would prevent serological HCV typing.

In summary, sequence analysis of the 5' UTR of HCV allows consistent genotyping of all HCV isolates presently known. Furthermore, reverse hybridization systems in a strip test format like the described LiPA, provide fast and reliable typing of HCV isolates and identification of new HCV types. This assay allows the use of the amplification products from 5' UTR and therefore conveniently complements the routine diagnostic RT-PCR assays. Screening of large patient populations is feasible, and could lead to rapid identification of new HCV genotypes.

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CHAPTER 7

Analysis of Hepatitis C virus genotypes 1 to 5 by the Line Probe Assay (LiPA) and correlation with sequences from the 5' UTR and core region

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Abstract

Hepatitis C virus (HCV) isolates were genotyped by means of the Inno-LiPA HCV genotyping assay. From a large cohort of 278 patients with a chronic HCV infection, 31 isolates representing different genotypes (1a, 1b, 2a, 2b, 3a, 4a, and the novel type 5a) were analyzed. 5' UTR sequences as well as partial sequences from the core region (nucleotides 1 to 326) were determined. 5' UTR sequence analysis confirmed LiPA results, including several aberrant sequences. Nucleotide distances among N-terminal core regions were 0.0265 $\pm$ 0.0136 within each subtype, 0.0867 $\pm$ 0.0162 between different subtypes, and 0.1695 $\pm$ 0.0296 between types. Genotype 2 sequences showed more intratypic heterogeneity compared to reported Japanese isolates. Discrimination between HCV genotypes by 5' UTR analysis was completely justified by phylogenetic analysis of the core region and indicated maintenance of genotypic variation throughout the viral genome. However, HCV genotypes 2a and 4a, as identified by 5' UTR analysis, may be further subdivided by analysis of the core region and other coding regions. Comparison of the deduced amino acid sequences of all N-terminal core regions revealed the presence of several type-specific amino acids and a heterogeneous region between residues 68 and 78.

Introduction

Hepatitis C virus (HCV) is the major etiological agent of parenterally transmitted non-A, non-B hepatitis (Choo et al., 1989, Kuo et al., 1989). Based on physico-chemical properties and genomic organization, the virus is classified as a separate genus within the Flaviviridae (Choo et al., 1991, Miller and Purcell, 1990, Houghton et al., 1991). Since the discovery of HCV, an increasing amount of sequence data became available of isolates obtained from numerous geographical areas (e.g., Okamoto et al., 1992a, Kato et al., 1990, Takamizawa et al., 1991, Chen et al., 1992). Sequence comparisons revealed the existence of multiple HCV strains or genotypes. Initial attempts to classify the different isolates were based on limited data from different regions of the genome (Mori et al., 1992, Okamoto et al., 1992b, Cha et al., 1992) and associated nomenclature was confusing. A more useful classification system was proposed recently (Chan et al., 1992, Simmonds et al., 1993, Stuyver et al., 1993a) and is expected to

be generally accepted. This system differentiates between types and subtypes. Overall sequence homology is higher than 88% within each subtype, approximately 79% between subtypes and about 68% between different types (Okamoto et al., 1992a, Stuyver et al., 1993b).

Phylogenetic studies suggested that different regions of the genome could be used to distinguish HCV genotypes (Chan et al., 1992). Genotyping of HCV isolates has been described, employing either the core region (Okamoto et al., 1992b), the NS5 region (Tsukiyama-Kohara et al., 1991, Enomoto et al., 1990, Chayama et al., 1993) or the 5' UTR (Stuyver et al., 1993a, Simmonds et al., 1993).

A reverse hybridization Line Probe Assay (LiPA) has been developed for genotyping of HCV isolates by 5' UTR analysis (Stuyver et al., 1993a) and evaluated by direct sequencing (van Doorn et al., 1994b). The 5' UTR is highly conserved, and shows limited sequence variation. Two small regions (nt -132 to -117 and nt -170 to -155) were identified as (sub)type-specific sequence 'motifs' (Kleter et al., 1994, van Doorn et al., 1994a). These motifs are partially complementary and are able to form a stable RNA stem-loop structure, which is considered to be functionally important for the initiation of translation (Brown et al., 1992, Yoo et al., 1992, Wang et al., 1993, Simmonds et al., 1993). Genotyping by 5' UTR analysis would be convenient, since this region is generally used to determine HCV viraemia by the reverse transcriptase polymerase chain reaction (RT-PCR). Moreover, the higher level of variability in several coding regions can hamper the use of type-specific motifs for genotyping.

In this study, 31 isolates, representing at least seven different (sub)types, were randomly selected from a large cohort of patients, that had been genotyped by LiPA. Selected isolates were subjected to sequence analysis of the 5' UTR and N-terminal core region, in order to substantiate the classification by 5' UTR analysis and to confirm such classification between (sub)types in a coding region.

Materials and methods

HCV isolates

HCV isolates described in this study were obtained from participants in the trial for treatment with interferon-α, organized by the Benelux Study Group on the treatment of hepatitis C (Brouwer et al., 1993). The relationship between HCV genotypes and biochemical, epidemiological, serological, demographical and clinical parameters within this cohort of patients will be described in detail elsewhere (Kleter et al., manuscript in preparation). Plasma or serum samples were obtained by venipuncture, aliquotted and stored at -70°C. Informed consent was given by all patients.

RNA isolation and reverse transcriptase PCR (RT-PCR)

HCV RNA was isolated by a modified version of the guanidiniumthiocyanate method (Chomczynski and Sacchi, 1987, Kleter et al., 1993). A small number of heparanized plasma samples were analyzed by an HCV RNA capture method as described earlier (van Doorn et al., 1994a) as these samples could not be analyzed by conventional methods (Willems et al., 1993). Detection of HCV RNA was performed by RT-PCR with primers HCV35 (sense, pos -318 to -296; 5'-TTGGCGGCCGCACTCCACCATG AATCACTCCCC-3'; underlined sequence is non-HCV specific) and HCV19 (antisense, pos. -1 to -20; 5'-GTGCACGGTCTACGAGACCT-3'). Core analysis by RT-PCR was carried out with primers HCV 983 (antisense, pos. 963 to 983; 5'-GGiGACCAGTTCATCATCAT-3'; i=inosine) and biotinylated LD58C (sense, pos -57 to -34; 5'-Bio-GGTACTGCCTGATAGGGTGCTTGC 3'). cDNA synthesis was primed with antisense PCR primers.

The PCR program for 5' UTR amplification consisted of 40 cycles of 1 min. 95°C, 2 min. 48°C, and 3 min. 72°C. The PCR program for the core region comprised 40 cycles of 1 min. 95°C, 2 min. 45°C and 5 min. 72°C.

Genotyping by the Line Probe Assay (Inno-LiPA)

Amplification products from the 5' UTR were subjected to nested PCR with primers HC3 (sense: -264 to -238: 5'-TCTAGCCATGGCGTTAGTRYGAGTGT-3') and HC4 (antisense: -29 to -54: 5'-CACTCGCAAGCACCCTATCAGGCAGT-3') in the presence of biotinylated ^{11}dUTP. Biotinylated nested

PCR products were used as target for the probes on LiPA strips (prototype version of Inno-LiPA, Innogenetics, Gent, Belgium, see also figure 1) as described previously (Stuyver et al., 1993a). Briefly, biotinylated DNA was denatured by alkaline treatment. After hybridization and stringent wash in the presence of TMACl (Tetramethyl ammoniumchloride), alkaline phosphatase conjugated streptavidin was added. Results of the LiPA were determined by scoring the presence or absence of signal at each probe line. (This prototype version still contained probed 17 and 18, which are no longer present in the first generation Inno-LiPA.)

Direct sequencing

First round PCR products from 5' UTR (HCV35-HCV19) were reamplified in a nested PCR with primers NCR3 (sense, pos -314 to -288; 5'-GGGGCGGCCGCCAACCATARRA TCACTCCCCTGTGAGG-3'; R= A or G) and LD58B (antisense, pos -35 to -64; 5'Bio-GGCCGGGGCGGCCGCCAAGCACCCTA-TCAGGCAGTACCACAAGGC-3'). First round PCR products from the core region were reamplified with LD58C and 186c (antisense, pos 410 to 391; 5'-ATiTACCCCATGAGiTCGGC-3') in a semi-nested PCR. In order to obtain single stranded DNA for sequencing, biotinylated PCR products were bound onto paramagnetic streptavidin-coated beads (Dynabeads M280, Dynal, Oslo, Norway) as described earlier (Kleter et al., 1994, van Doorn et al., 1994b). Briefly, the PCR product was mixed with paramagnetic beads and strands were separated by alkaline treatment, resulting in the release of the unbiotinylated strand in the supernatant. The biotinylated strand remained bound to the beads. Separate strands were subjected to standard dideoxy-termination sequencing. 5' UTR fragments were sequenced with NCR3 and NCR4 (antisense, -66 to -47; 5'-CACTCT CGAGCACCCTATCAGGCAGTACC 3'). Core fragments were sequenced with 186c and LD58s (sense, pos. -51 to -34; 5'-GCCTGATAGGGTGCTTGC-3').

Phylogenetic analysis

Sequences were read manually from autoradiograms and analyzed by the PC-Gene software (Intelligenetics, Moutain View, CA, USA).

Phylogenetic trees were constructed by a number of computerprograms from the PHYLIP package (version 3.5; Felsenstein, 1993). Nucleotide distances between individual isolates were determined and represented in unrooted phylogenetic trees. To assess the reliability of specific sequence clusters, 100 bootstrap steps were performed.

Results

5' UTR analysis

A cohort of 278 HCV infected patients were genotyped by LiPA. Thirty-one isolates including randomly selected representatives for each (sub)type, as well as those showing an aberrant, but interpretable LiPA pattern, were further analysed. Typical LiPA results are shown in figure 1. The LiPA has at least 2 probe lines for recognition of types 1 to 3 as well as several probe lines for subtypes 1a, 1b, 2a, and 2b (see figure 1). Genotype 4 sequences hybridize with probes 19, 20 and sometimes probe 7. In a number of isolates the signals with probes 6 and 9, used to recognize type 1 and 2 respectively, were very weak or absent. Since there are at least 2 probe lines to identify these types, classification was not hampered in these cases.

Figure 1.

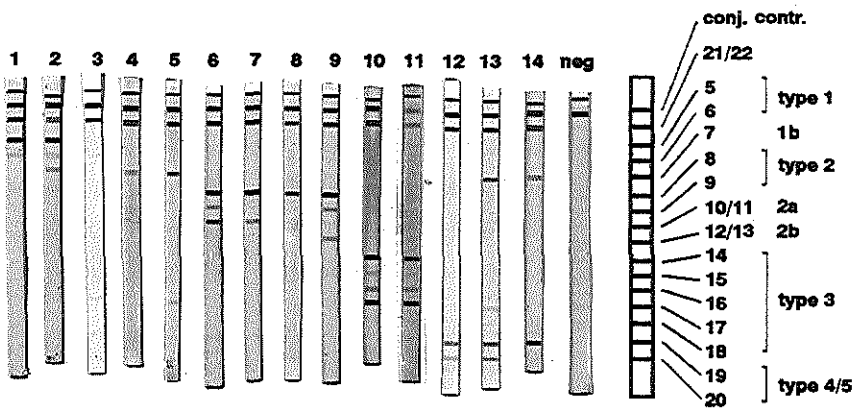


Figure 1. Representative LiPA results for each HCV (sub)type, and several aberrant results. Isolates numbers are: 1= HC1-n1, 2= HC1-N6 , 3= HC1-N7, 4= HC1-N8, 5= HC1-N10, 6= HC2-N1, 7= HC2-N2, 8= HC2-N6, 9= HC2-N4, 10= HC3-N3, 11= HC3-N2, 12= HC4-N4, 13= HC4-N2, 14= HC5-N2, neg= negative control, showing positive conjugate control line. Positions of LiPA probes are indicated in figure 2.

The 5' UTRs from 31 isolates, representative for a (sub)type or showing an aberrant LiPA pattern, were investigated by direct sequencing. Obtained sequences and the positions of the LiPA probes are shown in figure 2.

Genotype 1 is recognized by hybridization to probes 5 and 6. Subtype 1b sequences also hybridize to probe 7, whereas this is absent for subtype 1a. A number of isolates (HC1-N8, HC1-N10, and HC1-N11) are classified as genotype 1b by probe 7 but hybridization to probe 5 is absent, caused by a T at -159. Isolate HC1-N7 also contained this T mutation and, like isolate HC1-N9, a C at -94, which prevents hybridization to probe 7. Therefore, these two isolates were provisionally classified as type "1", but could not be subtyped by LiPA (figure 2).

Genotype 2 sequences are identified by hybridization to probes 8 and 9. Subtype 2a and 2b are recognized by hybridization to probes 10+11 and 12+13, respectively. Only two subtype 2b isolates were identified in the entire cohort of patients. PCR products from isolate HC2-N2, one of the 18 isolates classified as type 2a by hybridization to probes 8 and 10+11, failed to hybridize to probe 9, due to a A/G transition at pos. -127. Isolate HC2-N6, classified as type 2 by hybridization to probe 8, did not hybridize to any of the other type 2 probes. Sequence analysis showed that this LiPA result is caused by novel covariant mutations at positions -163 and -122. Therefore HC2-N6 was provisionally classified as subtype "2?".

Genotype 3 isolates can be identified by hybridization with probes 14 to 18. Isolate HC3-N2 shows only very weak hybridization with probe 15. This was confirmed by the presence of a C at position -118 in motif 2. Isolate HC3-N4 did not hybridize to probe 16, which was again confirmed by the absence of a G at -99.

Nineteen isolates, hybridizing to probe 19 and/or 20 were subjected to 5' UTR analysis, in order to investigate discrimination between genotypes 4 and 5a (Kleter et al., 1994, van Doorn et al., 1994a, Bukh et al., 1993, Stuyver et al., et al., 1994). Twelve type 4 isolates were recognized by probes 19 and 20, all containing a T at -238 and sometimes an A at -235. HC4-N5 also contains an insertion at -138. Sequence motifs 1 and 2 were completely conserved within this type (not all sequence data shown). The remaining seven sequences closely resembled SA-1 (Bukh et al., 1992) and BE95 (Stuyver et al., 1994) and are classified as type 5a. This genotype can be identified by the LiPA with probes 19 and 7, in the absence of reactivity to probe 20. All investigated type 5a isolates contained a double A motif at positions -236 and -235.

Figure 2. 5' UTR nucleotide sequence alignment of HCV (sub)types.

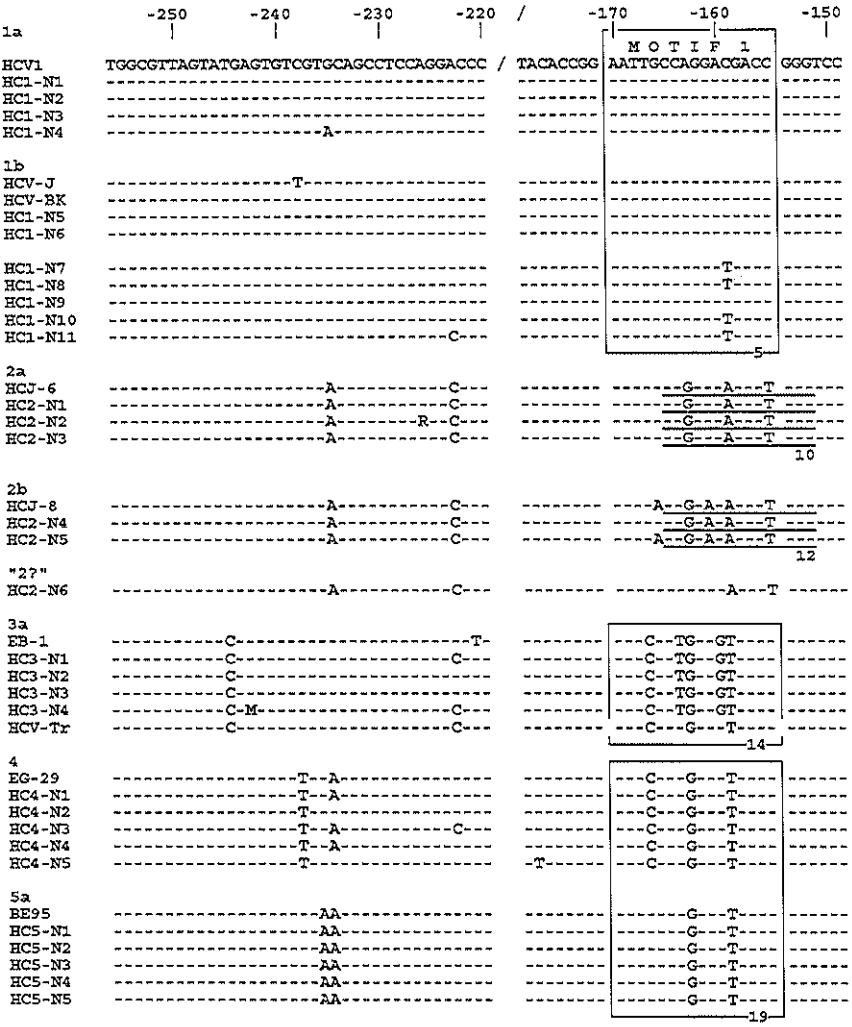


Figure 2. (continued)

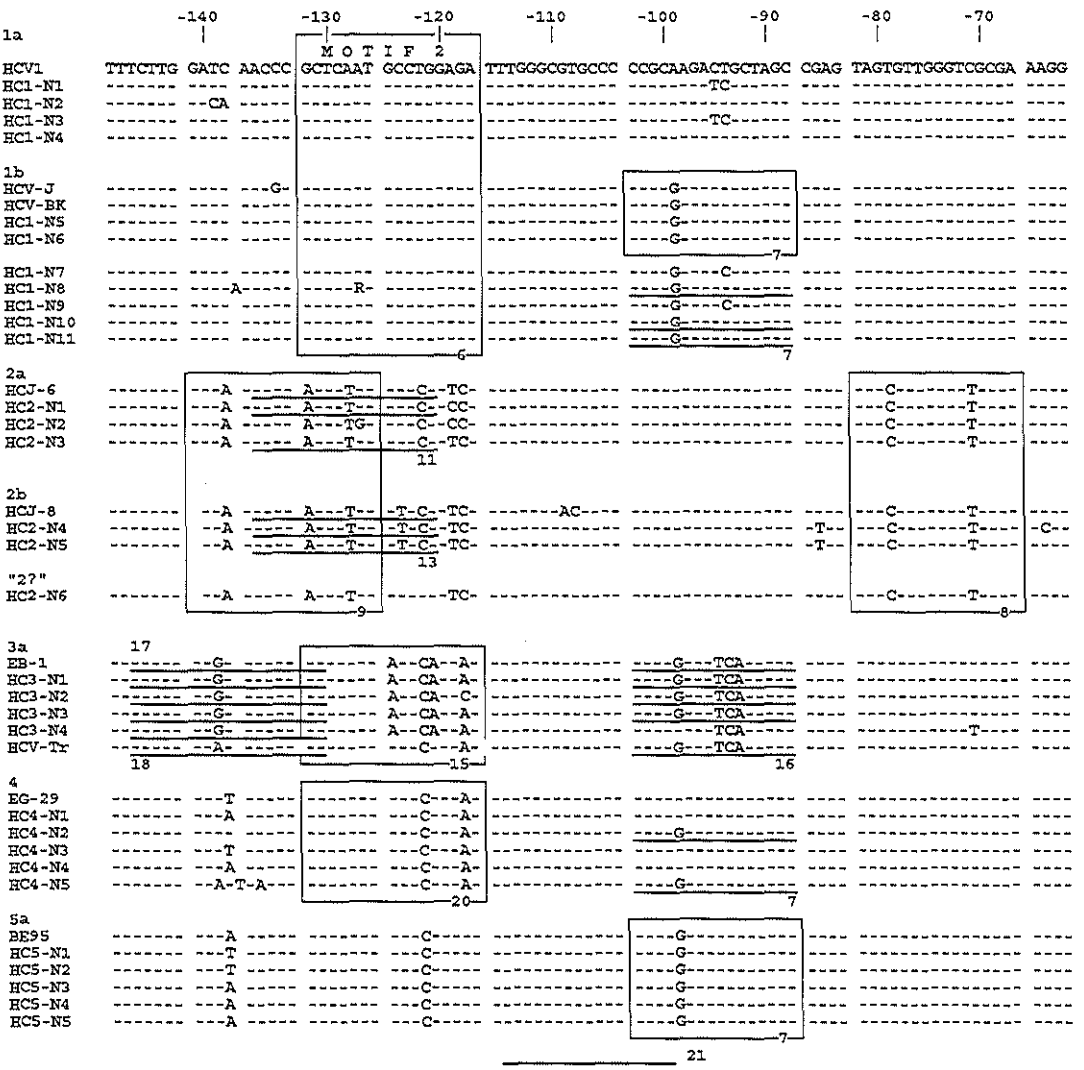


Figure 2. Nucleotide sequences alignment from the 5' UTR (positions -256 to -63). Sequences between positions 219 and -179 are completely identical to HCV1 sequences among all isolates and are not shown. Boxed and underlined nucleotides indicate the positions of corresponding probes on the LiPA strip (see also figure 1). R= G or A, Y = A or C, Y = C or T, N= not determined. Universal probe 22 is located between positions -195 and -180. The origins of the reported isolates are: HCV1 (Choo, 1991); HCV-J (Kato, 1990); HCV-BK (Takamizawa, 1991), HCJ-6 (Okamoto, 1991); HCJ-8 (Okamoto, 1992) EB-1 (Chan 1992); HCV-Tr (Chayama 1993); EG-29 (Simmonds 1993); BE95 (Stuyver 1993c).

Core sequences

The 31 isolates, selected from the patient cohort based on 5' UTR analysis were also subjected to sequencing of the N-terminal core region (nt 1-326). Nucleotide and amino acid sequences are shown in figure 3 and 4, respectively (Classification in these figures was performed according to initial 5' UTR analysis). Nucleotide distances among the defined (sub)types are presented in Table I. Based on the bootstrap results, 5 different groups could be confidently distinguished, corresponding to genotypes 1a, 1b, 2b, 3a, and 5a. Within these subtypes, nucleotide distances are 0.0265 $\pm$ 0.0136, whereas in genotypes 2a and 4a, these distances are 0.0813 $\pm$ 0.0123 and 0.0815 $\pm$ 0.0189, respectively.

Nucleotide sequences from the two type "1" isolates (HC1-N7 and 9) are highly homologous to subtype 1b sequences and can be classified accordingly. These isolates indeed exhibit the subtype 1b-specific G at position -99 in the 5' UTR.

Nucleotide sequences from isolate HC2-N6, provisionally classified as subtype "2?", resembles genotype 2a (0.0688 $\pm$ 0.0135) sequences more than 2b (0.1006 $\pm$ 0.0066).

Phylogenetic analysis of core sequences is represented as an unrooted tree in figure 5. A higher degree of heterogeneity among genotypes 2a and 4a isolates can be observed compared to other subtypes, of which the isolates can be clustered by bootstrapping analysis.

Amino acid homologies among the core sequences (figure 4) range from approximately 90% between types to almost 100% within a subtype. Several type-specific amino acid residues can be identified, e.g., Val at position 36 in type 3, and Asn at position 106 in type 5 isolates. The region between residue 68 and 78 seems the most variable part of the N-terminus of the core protein.

Figure 3. N-terminal core region nucleotide sequence alignment HCV genotypes 1-5.

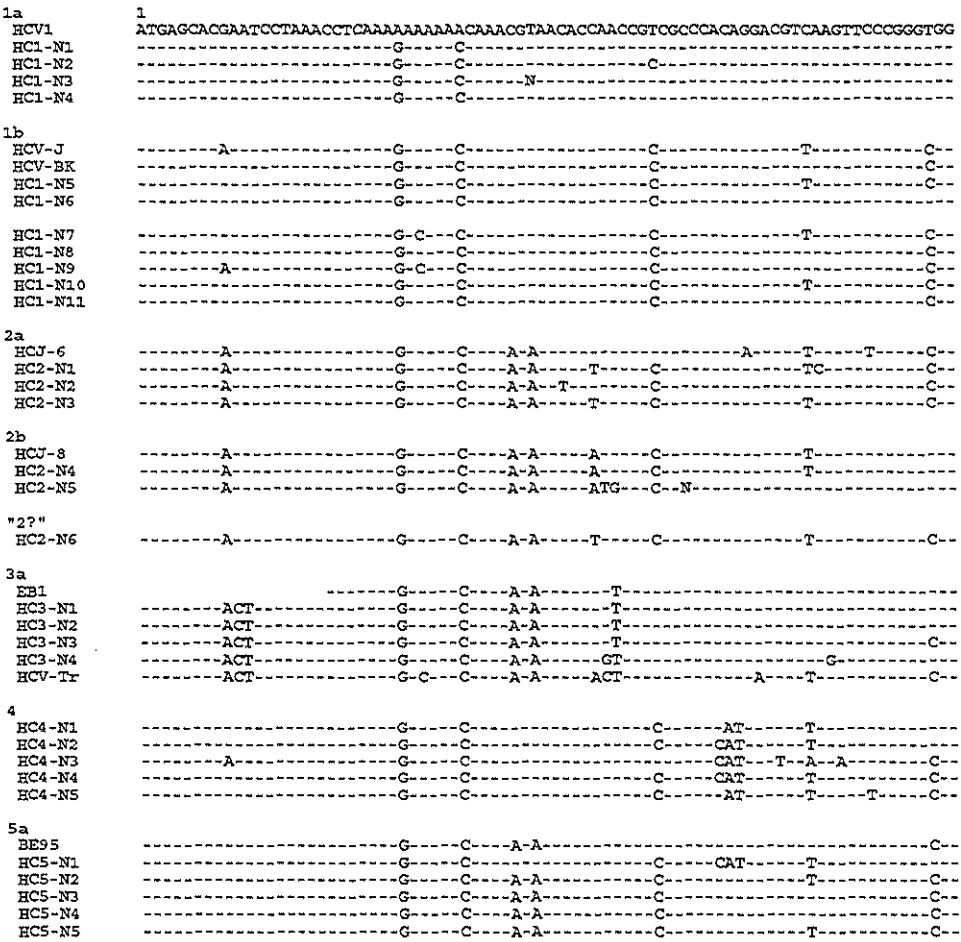


Figure 3 (continued 1)

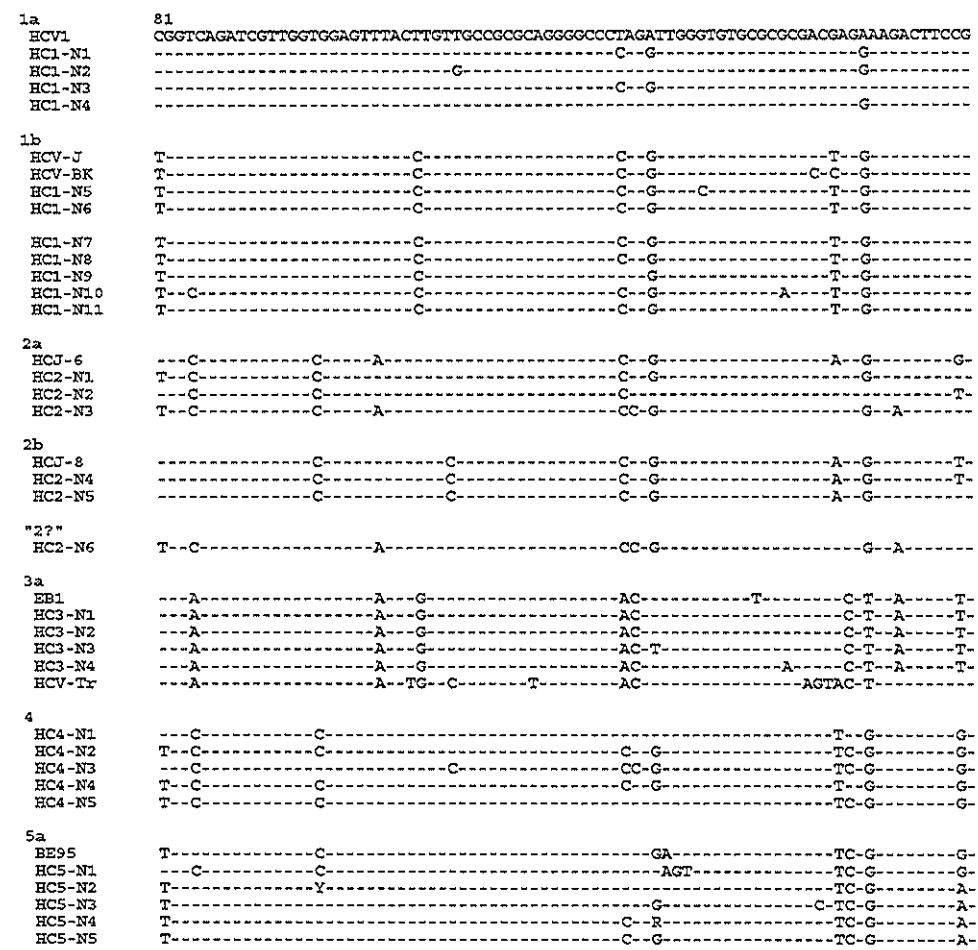


Figure 3 (continued 2)

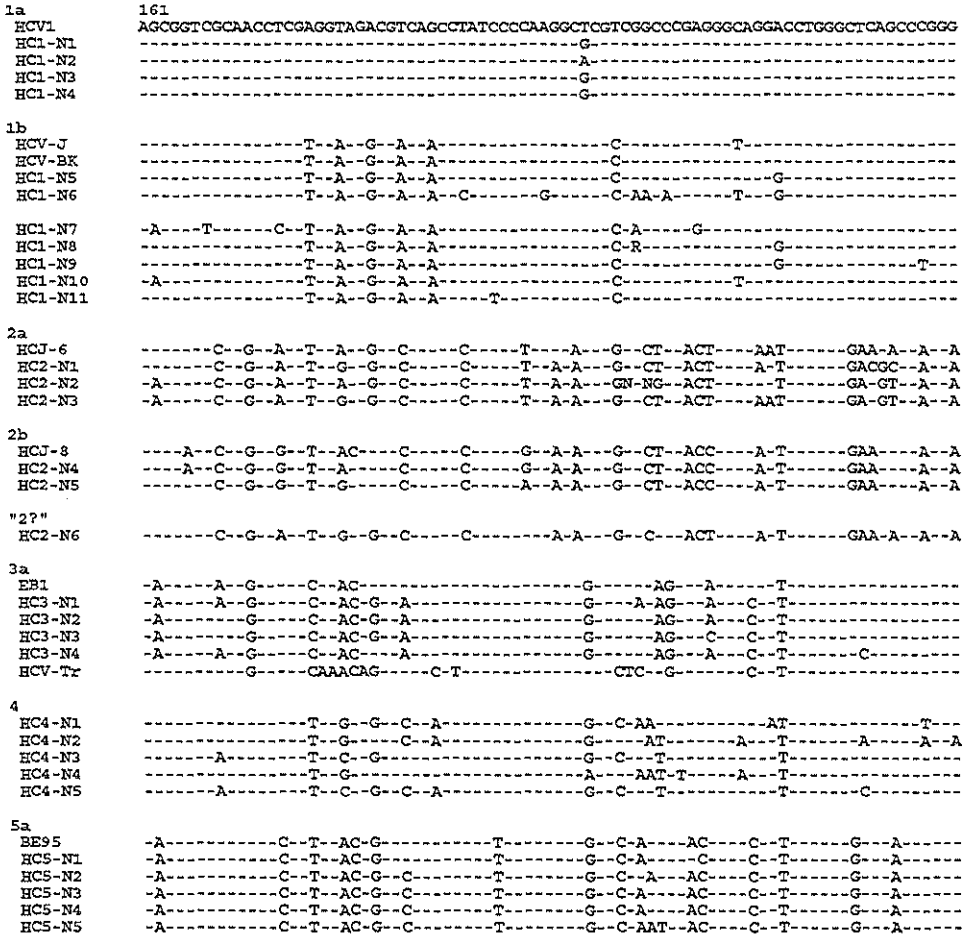


Figure 3 (continued 3)

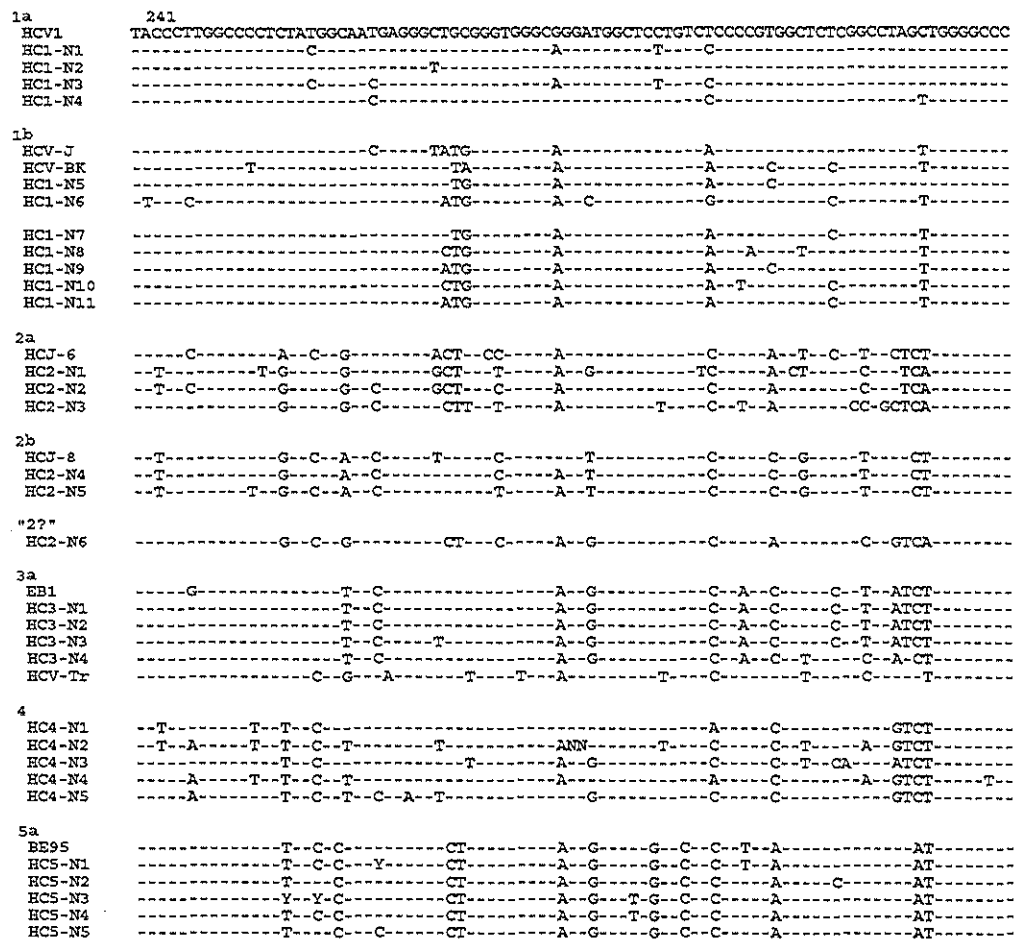
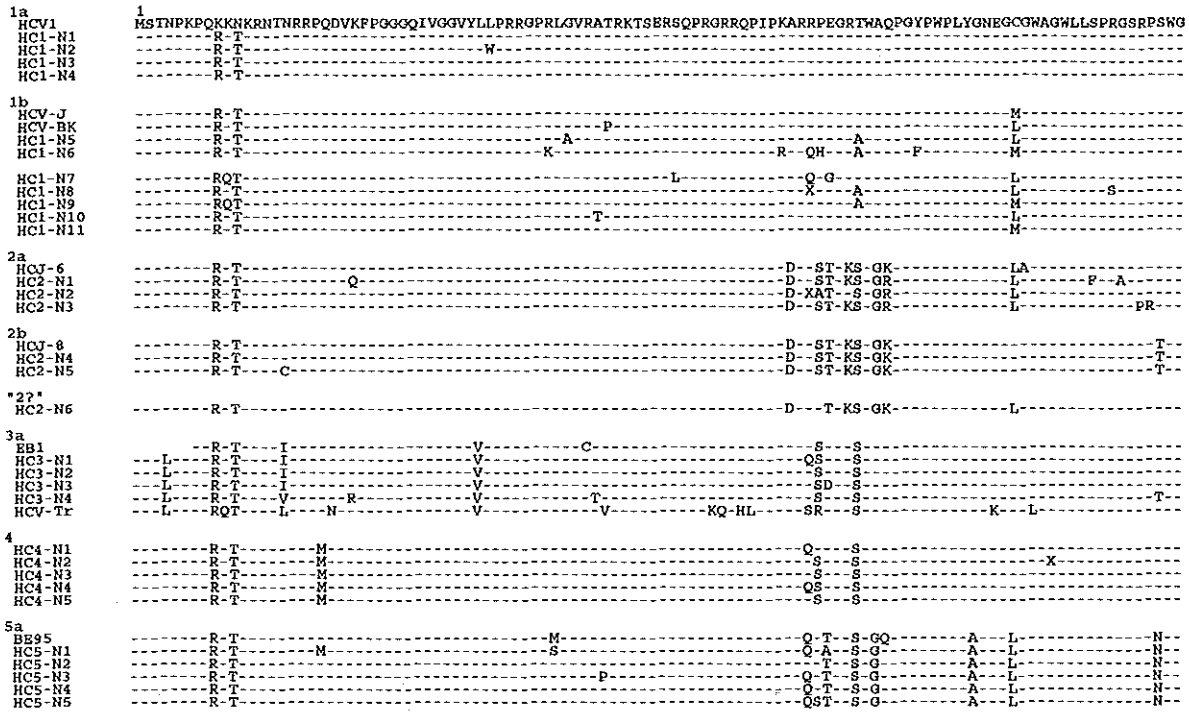


Figure 3. Core region nucleotide sequence alignment of the analyzed fragment (nt 1 to 326). For designation and origin of reported isolates see legend figure 2.

Figure 4. Deduced amino acid sequences from the N-terminal core region genotypes 1-5.



Discussion

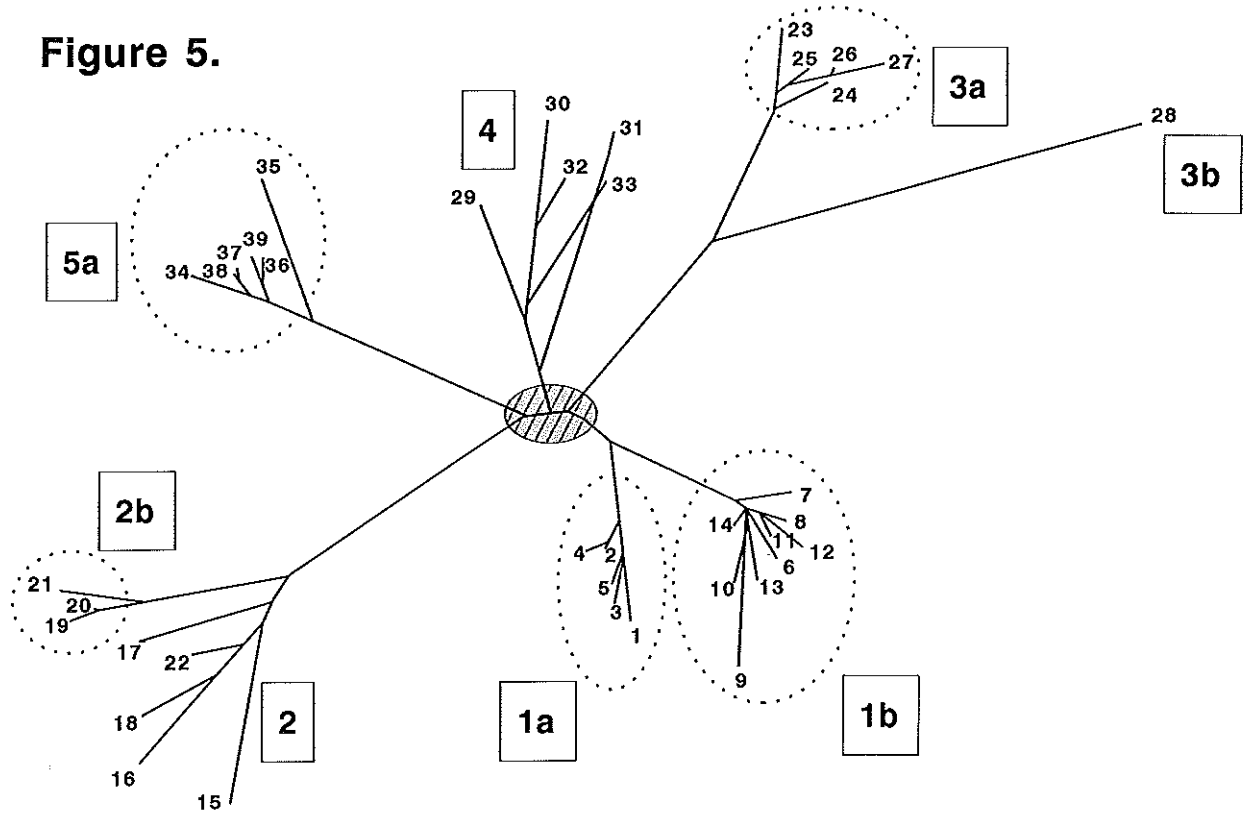
Hepatitis C virus isolates from a cohort of 278 patients with a chronic HCV infection were analysed. Since phylogenetic studies have suggested that comparison of different regions from the HCV RNA genome resulted in similar classifications (Chan et al., 1992), and since genotype-specific motifs can be observed in the 5' UTR (Stuyver et al., 1993a), analysis of this region should allow consistent genotyping. This paper describes the correlation between 5' UTR (sub)type-specific sequence variation and sequences from the core region. Although the overall sequence conservation within the 5' UTR is very high, there is significant sequence variation in two limited regions (motif 1 and 2 figure 2; Stuyver et al., 1993a, Simmonds et al., 1993, Kleter et al., 1994, van Doorn et al., 1994b). Genotyping of the patient isolates was performed with the Inno-LiPA. The specificity of this assay has been described earlier (Stuyver et al., 1993a, van Doorn et al., 1994b). The LiPA contains probes against (sub)type-specific regions in the 5' UTR and almost all (>98%) of the 278 isolates could be genotyped and displayed a consistent hybridization pattern on the strip (Kleter et al., manuscript in preparation).

A very limited number of isolates failed to produce a clear signal at the probe 6 (type 1b isolates HC1-N8, HC1-N10) or probe 9 (type "2?" isolate HC2-N6) lines, while hybridization is expected based on the sequencing results. Degeneration at pos -127 in isolate HC1-N8 can only partly explain such result. Although errors during PCR and sequencing cannot be excluded, the reason for these aberrant results remains unclear. The great majority of LiPA results, including some exceptional aberrant patterns (HC3-N2, HC2-N2, see figure 1) were in complete accordance with the determined sequences.

Several isolates from each (sub)type, as classified with LiPA, were selected randomly for further analysis and were subjected to direct sequencing of the 5' UTR. The core region is less conserved than the 5' UTR and sequence comparisons should clearly distinguish between (sub)types (Chan et al., 1992).

Figure 4. Deduced amino acid sequences from the analyzed N-terminal core region (residues 1-108). X indicates that the amino acid could not be specified, due to nucleotide sequence ambiguity. For origin of reported isolates see legend figure 2.

Figure 5.


23
25
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27
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3a
28
3b
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33
29
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5a
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39
34
38
36
2b
21
20
19
17
22
18
16
15
2
4
2
5
3
1
1a
7
14
8
11
12
6
10
13
9
1b

Whereas the 5' UTR is a non-coding region containing a highly conserved, presumably functional, element such as an internal ribosomal entry site (IRES, Yoo et al., 1992, Wang et al., 1993), the core gene encodes the putative nucleocapsid protein, and might be under different selective pressure than the 5' UTR. Since the mutations are not randomly distributed along the 5' UTR (figure 2), it is not useful to calculate the percentage of homology and standard deviation. Nucleotide mutations along the N-terminal core region are much more evenly distributed. Nucleotide distances within subtypes 1a, 1b, 2b, 3a, and 5a are all below 0.050. Within types 1 and 2, distances are between 0.08 and 0.11. Nucleotide distances between different types can increase to 0.2237 (between genotypes 2a and 3a, see table 1).

Five isolates (HC1-N7, HC1-N8, HC1-N10, HC1-N11, and HC1-N9;) showing a slightly aberrant LiPA pattern, were further investigated because of the presence of a T at -159 and/or a C at -94. These mutations were shown not to be involved in covariance. Analysis of nucleotide sequences from the core region (table 1, figure 5) indicate that these isolates are indistinguishable from subtype 1b. Conclusively, these HCV isolates should all be classified as subtype 1b. This underscores the importance of the G at position -99 in the 5' UTR to identify subtype 1b isolates.

Isolate HC1-N6 was classified as subtype 1b by 5' UTR analysis, and this was confirmed by core analysis, despite the greater nucleotide distance to other 1b isolates in the core region (figure 5). Nevertheless, considering the amino acid sequence variation (figure 4), this isolate is an interesting candidate for further analysis of other coding regions.

Discrimination between subtypes 2a and 2b is based on covariance at positions -123 and -160 in the two 5' UTR complementary sequence motifs (Simmonds et al., 1993, Kleter et al., 1994, Stuyver et al., 1993a). Nucleotide distances in the core region among isolates classified as subtype 2a by LiPA (0.0813 ± 0.0123) are remarkably higher than within other subtypes, except genotype 4a. Isolates classified as subtype 2a by LiPA did not reach the confidence level by bootstrap analysis.

Figure 5. Phylogenetic unrooted tree of nucleotide sequences (nt 1-326) in the core region. Individual sequences are numbered according to figures 2 and 3. Circled sequences indicate clusters that were confirmed by bootstrap analysis. Isolate codes are: **Type 1a**:1=HCV1, 2=HC1-N1, 3=HC1-N2, 4=HC1-N3, 5=HC1-N4. **Type 1b**:6=HCV-J, 7=HCV-BK, 8=HC1-N5, 9=HC1-N6, 10=HC1-N7, 11=HC1-N8, 12=HC1-N9, 13=HC1-N10, 14=HC1-N11. **Type 2a**:15=HCJ-6, 16=HC2-N1, 17=HC2-N2, 18=HC2-N3. **Type 2b**:19=HCJ-8, 20=HC2-N4, 21=HC2-N5. **Type "2?"**:22=HC2-N6. **Type 3a**:23=EB1, 24=HC3-N1, 25=HC3-N2, 26=HC3-N3, 27=HC3-N4. **Type 3a**:28=HCV-Tr. **Type 4**:29=HC4-N1, 30=HC4-N2, 31=HC4-N3, 32=HC4-N4, 33=HC4-N5. **Type 5a**:34=BE95, 35=HC5-N1, 36=HC5-N2, 37=HC5-N3, 38=HC5-N4, 39=HC5-N5. Hatched region represents uncertainty limit in bootstrapping analysis.

Isolate HC2-N6 contained a novel combination of covariants in both 5' UTR typing motifs and was therefore classified as "2?". To avoid confusion, no letter was used to indicate a novel subtype within type 2. Based on nucleotide distances among core region sequences, this isolate was indistinguishable from the subtype 2a isolates (figure 5). Taken together, these results indicate that type 2a isolates are more heterogeneous than previously reported (Okamoto et al., 1991, 1992a, b) and may need further differentiation into several new subtypes. Due to limited sequence data from subtype 2a and the rarity of "2?" isolates, such division is not yet possible. Subtype 2b seems to be a highly homogeneous group, although only 2 isolates could be analysed. Similar small nucleotide distances within the 2b subtype have also been observed in the NS5a region (Stuyver et al., unpublished observations)

The selected type 3 isolates appear to be a homogeneous group, with intratypic nucleotide distances of only 0.0243 $\pm$ 0.0104. Therefore all these type 3 isolates can be classified within subtype 3a. Existence of subtype 3b (Chayama et al., 1993; isolate HCV-Tr) was not shown in this population. If any type 3b isolates had been present these would have been identified by hybridization to probe lines 16, 19 and 20 in LiPA (figure 2).

Among nineteen isolates that hybridized with probes 19 and/or 20, two genotypes (4 and 5a) could be recognized by LiPA. Genotype 4 sequences have been described recently (Simmonds et al., 1993, Stuyver et al., 1993a). Nucleotide distances among type 4 core region nucleotide sequences are 0.0815 $\pm$ 0.0189, and like for subtype 2a, these sequences could not confidently be assigned to a single subtype by bootstrap analysis. However, amino acid sequence homologies within type 4 (98.4 $\pm$ 0.82%) are considerably higher than within subtype 2a (94.0 $\pm$ 1.74%).

The existence of genotype 5a has been suggested previously after analysis of the 5' UTR from the South African isolate SA-1 (Bukh et al., 1992, 1993) and Belgian isolate BE95 (Stuyver et al., 1994). Among type 5a isolates described in this study, core sequences from isolate HC5-N1 are slightly different from the other type 5a sequences (figure 5), although within the limits to be classified within a single subtype. The 5' UTR sequence from HC5-N1 is indistinguishable from the remaining type 5a isolates.

Recently, an HCV typing system based on type-specific primers in the core region was described (Okamoto et al., 1992b). Different genotypes yield fragments of specific lengths, allowing classification after agarose gel electrophoresis of PCR products. Comparison of core sequences described in this study and type-specific primers as described by Okamoto (Okamoto et al., 1992b) revealed that primers for subtype 1a and 1b should perform properly. However, subtype 2a specific primer no. 134 (nt 302 to 321) had a perfect match to HC-J6 only. Other 2a sequences contained up

to 6 mismatches. Subtype 2b sequences also showed mismatches to the type-specific antisense PCR primer no 135 (nt 251 to 270). Isolate HC2-N5 showed a perfect match, whereas HC2-N4 contained 3 mismatches. Experimental results showed that isolate HC2-N5 yielded a fragment of the expected length, whereas HC2-N4 failed to produce any fragment after nested PCR (results not shown, Kleter et al., manuscript in preparation). Conclusively, the described typing system does not allow efficient genotyping of type 2 isolates with this system. Type-specific PCR primers for genotypes 3a, 4 and 5a have not yet been described and can be designed, based on this study. However, there is a considerable risk that nested PCR, in the presence of multiple different type-specific primers, and the diversity of viral genomes, may produce erroneous results.

Serologic differentiation between HCV genotypes 1 and 2 was described recently (Machida et al., 1992). Two distinct type-specific peptides comprised amino acid residues 65 to 85 and detected type-specific antibodies against the core protein. Our results (figure 4) indicate that the amino acid sequences between res. 65 to 85, even within a genotype, are more heterogeneous than described, which may account for the limited specificity and sensitivity of the method (Machida et al., 1992). Since the number of completely (sub)type-specific amino acids in this region is limited, it is unlikely that corresponding peptides will be sufficiently specific to serologically discriminate between all HCV genotypes. The E1 protein (Maertens et al., 1994) and the NS4a/b region (Stuyver et al., 1993b, Simmonds et al., 1993) may be more promising regions for serotyping.

Sequence analysis of either the 5' UTR or the core region results in similar classifications of HCV genotypes 1 to 5. This indicates that, as described earlier for types 1, 2 and 3 (Chan et al., 1992, Cha et al., 1992) the genotypic differences are indeed maintained throughout the HCV genome. Recent studies (Bukh et al., 1993) revealed that 12 genotypes can be distinguished by analysis of the E1 region. However, only 9 of those could be recognized in the 5' UTR. Our study indicates a similar situation when comparing 5' UTR with core region analysis. Most HCV genotypes can be distinguished by differences in the highly conserved 5' UTR. Further discrimination between variants is possible by analysis of the relatively well conserved core region as shown in this study. Subsequently, the more variable E1 region may reveal additional differences. Finally, the hypervariable region present at the 5' end of the E2/NS1 region allows discrimination between almost every single HCV isolate (Weiner et al., 1991). Our current knowledge of genotypic variation of HCV is still limited.

Although the number of HCV subtypes will probably increase, and the clinical significance of genotyping remains to be substantiated, 5' UTR analysis by the LiPA provides a reliable system to discriminate between genotypes of HCV.

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CHAPTER 8

Summary and general discussion

(samenvatting en discussie)

In this thesis, several aspects of a hepatitis C virus infection are studied. The first part focuses on the detection of HCV viraemia by molecular biological tools. Recent quality control studies revealed considerable variability among HCV-RNA detection results in different laboratories. This indicates the need for simple and reproducible methods that are less prone to contamination.

In chapter 2, a novel HCV RNA-capture assay is described. This method is based on the selective hybridization of the HCV genomic RNA to a complementary oligonucleotide, which is immobilized on paramagnetic beads. The captured RNA is subsequently detected by reverse transcription polymerase chain reaction (RT-PCR), aimed at the highly conserved 5' untranslated region (5' UTR). The capture technique provides a tool for rapid isolation of HCV RNA from blood, and enables specific detection of (near) full length HCV genomic RNA. HCV is selectively purified from possible inhibitory substances in blood. Furthermore, this RNA isolation method omits the use of hazardous chemicals, is less prone to contamination with PCR amplimers, and might be automated in the future. Similar RNA-capture from serum is also applied in the Chiron b-DNA assay to quantitate HCV RNA. A combination of HCV-RNA capture onto magnetic beads and the b-DNA detection technology might improve sensitivity of this quantitative assay.

Application of the HCV-RNA capture assay was shown by analysis of chimpanzee serum samples. The presence of RNA correlated very well with the presence of anti-HCV in samples from the convalescent phase of the disease. This indicates that during chronic HCV infection, both HCV RNA and anti-HCV are present, whereas after acute-resolving HCV infection, both markers become eventually undetectable. During the acute phase of infection HCV RNA detection is the only method to determine viraemia, since no anti-HCV antibodies are detectable.

The HCV RNA-capture assay was further optimized and evaluated in human samples, as described in chapter 3. Comparison of the capture assay with a highly sensitive conventional RNA isolation method revealed similar specificity (95.8%) and sensitivity (92.3%) by testing a coded panel of 50 human plasma samples. The sensitivity of the current capture method is slightly less than the standard assay. Further adaptations, such as capturing of HCV RNA at multiple conserved sites may increase the sensitivity considerably. As observed for the chimpanzee sera, also in these samples a very good correlation between the presence of HCV RNA and anti-HCV was found.

The second part of this thesis describes experimental HCV infections in the only known susceptible animal, the chimpanzee (chapter 4).

First, results indicate the efficacy of the HCV RNA-capture assay for monitoring of the infection. HCV RNA is detectable within one week after inoculation in three chimpanzees but only after more than 4 weeks in another animal. HCV RNA becomes undetectable immediately after the normalization of ALT levels in the serum. This considerable decrease in serum HCV RNA concentration after the major ALT peak indicates that in acute hepatitis C a large portion of HCV infected hepatocytes are destroyed as a result of direct viral cytopathic effect, action of the host immune system or by a combination of both. In the chronically infected chimpanzee, the decrease of the HCV RNA level in the serum is transient and returns to detectable levels.

Secondly, results from anti-HCV assays show that the antibody profiles vary considerably among different chimpanzees, although a closely related HCV inoculum was used in all animals. Anti-core antibodies were first detected after 40-54 days post-infection in 3 chimpanzees with acute resolving infections, but in one chimpanzee with a chronic infection, these antibodies were detected more than 5 years after inoculation. Acutely infected chimpanzees showed prolonged reactivity against one of the core epitopes. Whereas none of the sera from these animals reacted with NS-5 peptides, anti-NS-5 antibodies were first detected in the chronically infected animal after 5 years. This underscores the importance of the host immune system for the course and outcome of HCV infection.

Thirdly, sequence analysis of the postulated hypervariable region, located in the 5' terminus of the E2/NS1 region, was performed to assess genetic drift of the particular HCV isolate used in all chimpanzees. Follow-up samples from 3 chimpanzees with a chronic infection and acute-phase samples from 3 animals with acute-resolving infection were studied. Results indicate genomic stability of the HCV genome upon transmission into another chimpanzee. However, genetic drift of the hypervariable region differed considerably during chronic infections. Two chimpanzees show considerable nucleotide and amino acid sequence variation during 6 years of follow-up, whereas one chimpanzee shows almost complete conservation of the region during more than 10 years of follow-up. Hypothetically, new sequence variants are immune-escape mutants. When the wild-type strain virions are neutralized and eradicated by specific humoral and/or

cellular immune responses, variant strains, originating from random mutations, emerge in the circulation and become predominant. If immune pressure is absent, the original strain remains predominant and new variants will presumably not reach detectable levels in the circulation.

Future research will be aimed at further elucidation of the interactions between HCV and the immune system of the host. This is of particular interest for antiviral therapy with immunomodulating agents such as interferon-α.

Genomic sequence variation between different HCV isolates to determine viral genotypes was subject of the third part of the thesis. Sequence comparisons of HCV isolates from the United States and Japan first indicated the existence of multiple strains or genotypes. Genotypes can be defined by consistent and significant differences between isolates both at the nucleotide and amino acid level. Different genotypes presumably originated from a common ancestor by sequential processes of mutation and selection. Each new mutant will be under a different selection pressure. Mutations that lead to a stop codon or frame shifts in the large open reading frame will have a dramatic impact on the function of the resulting virion, and are obviously lethal for the virus. Other mutations that are advantageous for the replication of the virus, will lead to an increase in relative abundancy of these mutants. Comparisons of complete sequences revealed that different levels of homology exist. This is reflected in the nomenclature and classification. Isolates with an overall sequence homology of $\pm$88% are defined as the same subtype. Sequences with approximate homologies of 79% belong to the same type and when homology is about 68%, isolates are classified into different types. However, it is not feasible to analyze complete viral genomes. Therefore, small regions are studied and compared between isolates.

Chapter 5 describes the use of the 5' UTR to discriminate between genotypes by RT-PCR amplification and direct sequencing. Results show that the 5' UTR sequence is highly conserved. Sequence variation is mainly restricted to two "motifs", that are part of an important secondary RNA structure. Variation in these motifs is often type-specific and can be used efficiently to differentiate (sub)types of HCV. A reverse hybridization method to detect sequence variation in the 5' UTR, the Line Probe Assay (LiPA) is described in chapter 6. LiPA results correlate very well with data from direct sequencing. Furthermore, there was a correlation between genotype and anti-HCV antibody responses against various viral epitopes, although discrimination between genotypes by serological tests is not yet possible. Initial reports had suggested that certain HCV

genotypes were restricted to a specific geographical area, e.g., the "American type", the "Japanese type", and the "South-African type". In the Netherlands, 5 genotypes with several subtypes were recognized. These results, and other recent reports, indicate that probably all HCV genotypes have a worldwide distribution, although the relative abundancy of each (sub)type may vary considerably between different geographical areas.

To substantiate the possibility of genotyping by 5' UTR analysis, a large cohort of patients was genotyped with the LiPA. Several isolates, representing the five different genotypes were randomly selected from this population and subjected to analysis of the N-terminal core region (chapter 7). Core sequence analysis revealed that this region is relatively well conserved among the HCV genotypes. Nucleotide sequence homologies within each subtype exceed 95%, although type 2 and 4 isolates were less homogeneous and may be further divided in several subtypes.

Differentiation between core sequences resulted in identical classification as the initial 5' UTR analysis. This justifies HCV genotyping by 5' UTR analysis and indicates that genotypic variation is maintained throughout the genome. Since a large amount of HCV isolates obtained worldwide have been analyzed, it is expected that the most important genotypes of HCV have been identified by now. New (sub)types may be discovered, but presumably with a very low incidence. Sequence data from different HCV genotypes will lead to the improvement of both serological and molecular biological diagnostic tools. Furthermore, this knowledge will be essential for the development of an effective vaccine.

Clinical relevance of genotyping will be subject of further studies, but it is likely that discrimination between (sub)types 1 to 5 will be sufficient in the majority of HCV cases.

An advantage of 5' UTR based genotyping is the fact that this region is used for determination of HCV viraemia by RT-PCR already in most laboratories. Therefore, 5' UTR genotyping provides a convenient complementary tool in routine settings.

Detailed knowledge of the RNA genome of HCV is required for efficient diagnosis of HCV viraemia by various molecular biological and serological methods. Investigations described in this thesis contributed to the reliable and efficient detection of HCV RNA and discrimination between various HCV genotypes. Studies on the natural course of HCV infections in chimpanzees and determination of genetic drift of the viral genome during chronic infections has increased our insight in the biology of this fascinating virus considerably.

Samenvatting en discussie

Dit proefschrift beschrijft een aantal aspecten van Hepatitis C virus (HCV) infecties. In hoofdstuk 1 wordt een samenvatting van de literatuur gegeven.

Het eerste deel van dit proefschrift handelt over de moleculair biologische detectie van HCV viremie. Gezien de verschillen tussen resultaten van HCV-RNA detectie door verschillende laboratoria is de noodzaak voor de ontwikkeling van simpele en reproduceerbare detectiemethoden die minder gevoelig zijn voor contaminatie duidelijk. In hoofdstuk 2 wordt een nieuwe HCV-RNA capture assay beschreven. Deze methode is gebaseerd op de selectieve hybridisatie van het enkelstrengs HCV RNA genoom aan een complementaire oligonucleotide, die geïmmobiliseerd wordt op paramagnetische deeltjes. Het weggevangen en gezuiverde RNA wordt vervolgens gedetecteerd door de reverse transcriptase polymerase chain reaction (RT-PCR), gericht op het zeer geconserveerde 5' onvertaalde gebied (5'UTR). De capture techniek biedt een methode voor snelle isolatie van HCV RNA uit bloed en maakt specifieke detectie van het complete, intacte genoom (full length RNA) mogelijk. HCV RNA kan bovendien selectief worden gescheiden van mogelijke storende factoren in bloed, zoals heparine. Deze nieuwe RNA isolatiemethode vergt geen extracties met fenol/chloroform en ethanolprecipitaties. Door capture van HCV RNA op sequenties die buiten het amplificatietarget liggen is de methode minder gevoelig voor contaminatie met PCR amplimeren gedurende de isolatiefase. Een soortgelijke RNA capture methode wordt toegepast in de Chiron b-DNA assay om HCV RNA te quantificeren. Een combinatie van de in dit proefschrift beschreven HCV-RNA capture techniek met behulp van paramagnetische deeltjes en het b-DNA systeem zou een gevoelige quantitatieve assay op kunnen leveren.

Het capture systeem is toegepast voor de analyse van HCV RNA in chimpansee serummonsters. De detectie van HCV RNA correleerde zeer goed met de aanwezigheid van anti-HCV antilichamen in deze sera, die minstens twee jaren na de acute fase waren afgenomen. Dit geeft aan dat tijdens een chronische infectie, HCV RNA en anti-HCV aantoonbaar blijven, terwijl bij een acute infectie beide markers uiteindelijk verdwijnen. Gedurende de acute fase van de infectie is HCV RNA detectie de enige mogelijkheid om viremie aan te tonen, omdat dan (nog) geen anti-HCV is gevormd.

De HCV RNA capture assay werd verder geoptimaliseerd en geëvalueerd in een gecodeerd

panel van humane serummonsters, zoals beschreven in hoofdstuk 3. Er werd een vergelijking gemaakt tussen de HCV capture assay en een zeer gevoelige conventionele methode. Beide methoden hebben een vergelijkbare specificiteit (95.8%) en sensitiviteit (92.3%). Evenals in de chimpansee sera werd ook hier een duidelijk verband waargenomen tussen de aanwezigheid van HCV RNA en anti-HCV. De absolute detectiegrens van de huidige versie van de RNA capture assay ligt echter ongeveer een factor 10 hoger dan die van de conventionele assay. Verdere aanpassingen, zoals simultane capture van HCV RNA op verschillende plaatsen, zouden de gevoeligheid aanzienlijk kunnen vergroten.

Het tweede deel van dit proefschrift beschrijft experimentele HCV infecties in het enige bekende infecteerbare dier, de chimpansee (hoofdstuk 4). In 4 chimpansees werden regelmatig serum monsters afgenomen voor en tijdens de HCV infectie. Daarin werden HCV RNA en anti-HCV gemeten. Met behulp van de capture assay was HCV RNA binnen 1 week detecteerbaar in drie chimpansees, maar pas na ruim 4 weken in de vierde chimpansee. Opvallend is dat direct na normalisatie van het ALT niveau in het serum, HCV RNA in alle chimpansees tot onder de detectiegrens daalde. Dit duidt op de simultane vernietiging van een groot aantal HCV geïnfecteerde hepatocyten ten gevolge van een intense immuunrespons van de gastheer. Daardoor stijgt de ALT concentratie in het serum tijdelijk sterk. Na de massale vernietiging van geïnfecteerde hepatocyten zal de produktie van HCV sterk verminderd zijn, hetgeen de daling van HCV RNA in het serum verklaart. In één chimpansee was de concentratie HCV RNA slechts tijdelijk verlaagd en steeg daarna weer tot boven de detectiegrens, hetgeen wijst op de ontwikkeling van een chronische HCV infectie. In de drie andere chimpansees daarentegen was de daling van de HCV RNA concentratie permanent, hetgeen duidt op volledige klaring van het virus.

Resultaten van de anti-HCV bepalingen toonden aan dat de humorale immuunrespons aanzienlijk kan verschillen tussen verschillende dieren, zelfs wanneer een identiek of sterk verwant inoculum wordt gebruikt. Anti-core antilichamen werden in drie chimpansees met een acute infectie 40-54 dagen na inoculatie voor het eerste gedetecteerd, maar in een chimpansee met een chronische infectie pas ruim 5 jaar na inoculatie. De dieren met een acute infectie bleven nog lange tijd antilichamen tegen één van de core epitopen behouden. Chimpansees met een acute infectie ontwikkelden geen meetbare antilichaamrespons tegen NS5 epitopen. Anti-NS5 werd wel

aangetoond in de chimpansee met een chronische infectie, hoewel pas 5 jaar na inoculatie. Deze resultaten onderstrepen het belang van de individuele immuunrespons van de gastheer voor het verloop van de HCV infectie, onafhankelijk van het virus.

Vervolgens werd binnen een groep van zeven chimpansees, die elk met een identiek of nauw verwant inoculum waren geïnfecteerd, de genetische variatie in de loop van de tijd ('genetic drift') onderzocht. Daartoe werden sequentieanalyses uitgevoerd op het zgn. hypervariabele gebied (HVR), gelegen in de N-terminus van E2/NS1. De resultaten van deze studie toonden aan dat transmissie van HCV vanuit één chimpansee naar verschillende andere geen onmiddellijke invloed had op de sequentie van het hypervariabele gebied tijdens de acute fase. De mate van variabiliteit tijdens een chronische HCV infectie in drie chimpansees verschilde echter aanmerkelijk. In twee chimpansees werden binnen een periode van 6 jaren significante sequentieverschillen waargenomen. Daarentegen bleken in een andere chimpansee gedurende een periode van 10 jaar nauwelijks sequentieverschillen op te treden. Op grond van deze resultaten is de volgende hypothese ontwikkeld. Virusgenomen met gemuteerde HVR sequenties ontstaan tijdens een chronische HCV infectie door random mutaties ten gevolge van RNA replicatiefouten. Sommige van deze mutanten kunnen ontsnappen aan de immunologische response van de gastheer die, in eerste instantie, specifiek gericht is tegen het wildtype virus. Deze 'escape mutanten' kunnen vervolgens het onderdrukte wildtype virus verdringen en daarna een nieuwe specifieke immuunrespons veroorzaken hetgeen kan leiden tot nieuwe 'escape mutanten'. Indien de immuunrespons tegen het wildtype virus echter uitblijft, vindt er geen selectie plaats en kan het wildtype virus blijven domineren. Vervolgonderzoek zal moeten uitwijzen of deze hypothese kan worden bevestigd en zal gericht zijn op de interactie tussen HCV en het immuunsysteem van de gastheer. Dit is in het bijzonder van belang voor de toepassing van antivirale therapie met immunomodulerende stoffen, zoals interferon-α.

Sequentievariatie vormde tevens het onderwerp voor het derde gedeelte van dit proefschrift. Verschillende HCV isolaten kunnen op grond van sequentieverschillen van elkaar worden onderscheiden. Eerdere onderzoeken toonden aan dat er verschillende genotypen van HCV bestaan, die kunnen worden herkend aan consistente en significante sequentieverschillen, zowel op nucleotide- als aminozuurniveau. Deze verschillende genotypen stammen waarschijnlijk via mutaties en selecties af van een gemeenschappelijk prototype virus. Mutaties kunnen belangrijke

gevolgen hebben voor de overleving van de mutant. Mutaties die leiden tot een stopcodon of verschuiving van het leesraam zullen veelal niet leiden tot levensvatbare virussen. Andere mutaties zullen daarentegen een efficiëntere virusreplicatie tot gevolg hebben en zorgen voor een sterke verspreiding van deze mutant.

Vergelijkingen tussen complete genoomsequenties toonden aan dat er verschillende mate van homologie tussen de diverse isolaten bestaat. Dit komt tot uiting in de huidige nomenclatuur en classificatie van de genotypen. Isolaten met een totale genoomhomologie van ongeveer 88% worden geclassificeerd binnen hetzelfde subtype. Sequenties met ongeveer 79% homologie behoren tot hetzelfde type maar een verschillend subtype en isolaten met een gemiddelde homologie van 68% worden als verschillende typen geclassificeerd. Het is echter onmogelijk om complete virusgenomen efficiënt te analyseren. Om isolaten te classificeren worden kleinere delen van het genoom met elkaar vergeleken. Hoofdstuk 5 beschrijft het gebruik van het 5' onvertaald gebied (5' UTR) om d.m.v. RT-PCR en directe sequentieanalyse van het amplificaat verschillende typen en subtypen te onderscheiden. Het 5' UTR is sterk geconserveerd en de beperkte sequentievariatie komt voornamelijk voor in twee 'motieven', die een onderdeel vormen van een functioneel belangrijke secundaire RNA structuur. Type-specifieke covariaties binnen deze motieven kunnen op efficiënte wijze gebruikt worden om onderscheid te maken tussen verschillende HCV genotypen. Een 'reverse hybridizatie' methode om diezelfde variaties te detecteren is de basis voor de Line Probe Assay (LiPA). De resultaten van deze test staan beschreven in hoofdstuk 6. LiPA resultaten correleerden zeer goed met de sequentiegegevens van het 5' UTR. Bovendien werd een verband gelegd tussen bepaalde HCV genotypes en de aanwezigheid van anti-HCV antilichamen tegen verschillende virale epitopen, zoals gemeten m.b.v. de LIA-2 en de RIBA. Het bleek hiermee echter niet mogelijk een consistent serologisch onderscheid tussen de verschillende genotypes te maken.

Eerder gepubliceerde onderzoeken suggereerden een sterke relatie tussen een specifiek genotype en de geografische verspreiding daarvan. Zo werden isolaten bestempeld als 'Amerikaans type' en 'Japans type'. Binnen Nederland echter werden bijna alle tot nu toe beschreven genotypes waargenomen. Deze resultaten duiden op een wereldwijde verspreiding van alle HCV genotypen, hoewel de relatieve incidentie op verschillende lokaties aanzienlijk kan verschillen.

Om verder bewijs te verzamelen dat het 5' UTR voor genotypering kan worden gebruikt,

werd een grote groep patientenisolaten met behulp van de LiPA getypeerd. Uit deze groep werd, na genotypering, een aantal representanten van elk (sub)type geselecteerd en verder geanalyseerd. Dit is beschreven in hoofdstuk 7. Allereerst werden door sequentieanalyse van het 5' UTR de LiPA resultaten bevestigd. Daarnaast werd van dezelfde isolaten een gedeelte van het core-gebied (nt 1-326) gesequenced. Deze core sequenties zijn relatief goed geconserveerd. De homologie binnen een subtype is in het algemeen meer dan 95%, hoewel types 2 en 4 iets meer intratypische variatie vertoonden. Classificatie van isolaten op grond van 5' UTR was volledig identiek aan de indeling gebaseerd op core sequenties. Dit bevestigt de resultaten van de LiPA en duidt op handhaving van genotypische variatie in verschillende delen van het HCV genoom. Wel is het waarschijnlijk mogelijk om op grond van core sequenties binnen een aantal genotypen een verfijnder indeling te maken in meerdere subtypes die op grond van 5' UTR alleen niet mogelijk is. Vergelijkbare resultaten zijn zeer recent ook gevonden bij de vergelijking van het 5' UTR met de E1 regio. Een voordeel echter van genotypering op grond van het 5' UTR is het feit dat dit deel van het HCV RNA genoom in de meeste laboratoria gebruikt wordt voor de bepaling van HCV viremie met behulp van RT-PCR. Daarom vormt de HCV genotypering op grond van 5' UTR een aanvulling op de routine diagnostiek.

Aangezien reeds een groot aantal HCV isolaten, verkregen vanuit wereldwijde verspreide regio's, zijn geanalyseerd, is de verwachting dat de belangrijkste HCV genotypes inmiddels zijn geïdentificeerd. Indien nog nieuwe (sub)types worden ontdekt, zullen die waarschijnlijk een lage incidentie hebben. Gedetailleerde kennis van de verschillende HCV genotypes zal leiden tot een verbetering van de serologische en moleculair biologische HCV diagnostiek. Bovendien zal deze kennis onontbeerlijk zijn voor de ontwikkeling van een effectief vaccin. De klinische relevantie van de verschillende HCV genotypes zal nog uitgebreider moeten worden bestudeerd.

De in dit proefschrift beschreven onderzoeken hebben bijgedragen aan de betrouwbare en efficiënte detectie van HCV RNA en het onderscheiden van de verschillende genotypes. Studies naar het natuurlijk verloop van HCV infecties in chimpansees en de genetische variatie van het HCV genoom in de loop van de tijd hebben het inzicht in de biologie van dit virus aanzienlijk vergroot.

Nawoord

Een nawoord behoort eigenlijk overbodig te zijn. Als een ieder die bij de totstandkoming van dit proefschrift betrokken was, pas op deze bladzijde mijn erkentelijkheid moet vernemen, heb ik de afgelopen jaren gefaald. De traditie wil echter dat de promovendus, na het leggen van het intellectuele ei, uitbarst in uitvoerige loftuitingen op de betreffende omstanders, vandaar toch een poging.

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Curriculum vitae

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