CHAPTER 1
General Introduction
Origins of virology

"Virus (Latin - poison) - any of a group of submicroscopic entities consisting of a single nucleic acid surrounded by a protein coat and capable of replication only within the cells of animals and plants: many are pathogenic". http://www.wordreference.com

"Enzyme (from Medieval Greek enzume, leavened, from Greek EN_2 + zume leaven) - any of a group of [...] proteins that are produced by living cells and act as catalysts in specific biochemical reactions". http://www.wordreference.com

History of discovery of viruses in a nutshell

Not visible in a light microscope, unable to grow on any kind of media by themselves, viruses were elusive for a long time after the discovery of bacteria. The first virus to be discovered was not from humans. In 1887, the Russian scientist Dimitri Ivanovski, investigating a case of tobacco mosaic disease in tobacco plants, provided the first operational definition of viruses by showing that "the sap of leaves infected with tobacco mosaic disease retained its infectious properties even after filtration through Chamber land filter candles". Thus, viruses have been defined as "filterable agents" for several decades after Ivanovski's discovery. Another important step was taken by the Dutch microbiologist Martinus Beijerinck in 1898. He showed that the filterable agent of tobacco mosaic disease regained its infectious "nature" after dilution only in the presence of tobacco plant cells. Viruses thus became defined as filterable infectious agents that were invisible in a microscope and only multiplied in living cells. Guided by this definition, the finding of other viruses was only a matter of time and the first human filterable agent, yellow fever virus, was soon (1901) discovered by Walter Reed and co-workers. Invention of the plaque assay, culturing of viruses in living animals and cell cultures, nucleotide sequencing, etc. had resulted in the accumulation of an enormous amount of knowledge about viruses by the 20th century. The discovery of molecular techniques like PCR paved the way for the identification of the virus that is the main subject of the present thesis. In 1989 hepatitis C virus (HCV) became the first virus to be explored by molecular cloning (Choo et al., 1989).

History of Hepatitis C virus

By the middle of the seventies of the 20th century it was already known that hepatitis A and B viruses were not the only causative agents of viral hepatitis in humans
(Barker et al., 1976, Feinstone et al., 1975). A number of patients with mild hepatitis did not fit into the clinical picture of hepatitis A or B infections and their blood serum was negative for immunological markers of the two known hepatitis viruses; this disease was called as what it was not: non-A non-B hepatitis (NANBH). By the end of the 1970, the clinical picture of the disease was well established (Wyke et al., 1980), but the etiological factor was still unknown and laboratory tests for identification of the disease were not available. No specific antibody was found against A & B viruses in the blood of diseased individuals and infectivity was only shown with chimpanzees (this was the first direct proof of existence of NANBH), which, of course, was not suitable for broad screening. However, it was possible to observe virus-like particles in liver tissue from both infected humans and chimpanzees. The morphology of the particles raised speculations about the existence of more than one virus (Maugh TH 2nd, 1980). In the eighties, the number of research centers that were dealing with NANBH and the number of publications on the matter had exploded. But quantity had not grown into quality and at the end of the eighties no etiological agent had been identified and no reliable diagnostic test had been discovered (Choksi et al., 1989). Amidst growing anxiety, a long awaited discovery was made by Choo et al. (Choo et al., 1989) at Chiron Corporation in 1989. Using an extensive set of random primers, they were able to pick up a polynucleotide sequence from the plasma of HCV -infected chimpanzees that did not belong to humans or chimpanzees. Peptides encoded by this sequence did bind to antibodies from the plasma of individuals infected with NANBH, but not of healthy individuals. Hepatitis C virus (HCV) was thus discovered. The procedure used in the experiment was extended as a ready-to-use diagnostic tool (Kuo et al., 1989). As blood screening began, the extent of the hepatitis C problem was soon unveiled. Thus NANBH virus has been renamed as hepatitis C virus (Fig. 1-1).
Fig. 1.1 Model of Hepatitis C virus
Hepatitis C virus infection is a leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide. The acute phase of infection is associated with mild symptoms, most patients fail to clear the virus and in 80% of cases, infection with HCV results in chronic hepatitis, possibly leading to cirrhosis and hepatocellular carcinoma (HCC). In the present work we briefly reviewed current concepts of the epidemiology, molecular virology, pathogenesis, natural history, diagnosis, therapy and prevention of hepatitis C.

Epidemiology and Transmission

Globally, about 300 million persons were estimated to be chronically infected with HCV, and 3 to 4 million persons are newly infected each year (Brown and Gaglio 2003). It is estimated more than 20 million people in India are already carrying this virus (Khaja et al., 2002). More than 50% of the infected individuals will develop chronic hepatitis. Approximately 1/3rd of these patients develop cirrhosis of liver or liver cancer. With the introduction of anti HCV screening of blood and blood products in 1990 new cases of post transfusion hepatitis C have reduced to minimum in many countries, but their number still remains high, making HCV one of the most common chronic infections in the world.

The prevalence rate of HCV infection differs significantly among different regions of the world (Fig.1-2). The lowest prevalence rates are observed in the United Kingdom and Scandinavia (0.01 - 0.1 %), followed by South and North America, Western Europe, Australia and South Africa (0.2 - 0.5%), Brazil, Eastern Europe, the Mediterranean, the Mideast and the Indian subcontinent (1 - 5%). The highest rate is observed in Egypt (17 - 20%) (Wasley et al., 2000).
Fig. 1.2 Global prevalence of HCV
Current estimates indicate that approximately 1.8-2.5% of Indian population is presently infected by HCV (Arankalle et al., 1992; Thyagarajan et al., 1998; Madhavi et al., 2003; Khaja et al., 2005). The general prevalence in our country is 1.5 to 1.8% (Khaja et al., 2002). A study by Chowdhury et al. (2003) from eastern India shows a prevalence of 0.87%. In South India the over all prevalence of HCV infection in blood donors is 2.4% (Khaja et al., 2005). Another study published by Panigrahi et al. (1997) from north India shows a prevalence of 1.85%. There is a relatively low prevalence of HCV antibodies in blood donors from developed countries like USA, UK, Europe is < 1%, while in developing countries like Nigeria, Ukraine and in the Central African countries, as well as Egypt the prevalence ranging from 6 to 28%. (Tanaka et al., 1992; Farrell et al., 1993; Halim et al., 2000; Delaporte et al., 1993; Kowo et al., 1995). In CRF patients, HCV prevalence averages between 10 – 40% (Sato et al., 1994). Several studies have shown that 10-30% of haemodialysis patients are infected with HCV prior to transplantation (Triolo et al., 1992: Umlanft et al., 1992) while about 10% of the uninfected patients become infected after transplantation due to contaminated blood or hospital equipment (Vuitton et al., 1996). In India the prevalence of HCV in CRF’s has been observed to be 63% (Madhavi et al., 2004; Khaja et al., 2005).

Transmission of HCV from infected patients to healthcare workers has been documented, and molecular evolutionary analysis has confirmed this mode of transmission (Seeff 1991; Suzuki et al., 1994). Hollow-bore needle-stick exposures are the main cause of HCV transmission from patient to health care worker (Thomas 2000). Prospective studies have shown that the average risk of infection after a needle-stick injury involving HCV-positive blood may be as high as 3% with a range between 0.013% and 10% in different studies (Puro et al., 1995; Grande et al., 2001; Sermoneta et al., 2001; Ross et al., 2002). On the other hand, mucous membrane or skin contamination has not been associated with an increased risk of HCV infection. Prevalence among general health-care workers in India 2.7% (Khaja et al., 2005), UK has ranged between 0.3% and 07% (Thomas et al., 1993) 0.8% in Germany (Weber et al., 1995), 1.8% in Spain (Perez et al., 1992), 1.97-2% in Italy (Sulotto et al., 2002), and 2.7% in Hungary (Mihaly et al., 2001).
Lichen planus has been linked to HCV infection, with studies demonstrating a higher prevalence of anti-HCV antibody titers in patients with cutaneous and oral lichen planus, compared with control subjects (Sanchez-Perez et al., 1996; Imhof et al., 1997; Mignogna et al., 1998; Chuang et al., 1999; Bellman et al., 1995; Nagao et al., 1995). One study reported that, in Indianapolis, a region with a low endemic prevalence of HCV, tests for HCV antibody were positive in 3.5 percent of patients with lichen planus (Chuang et al., 1999). Two studies of patients with lichen planus that did not exclude patients with normal liver function reported a positive HCV antibody in 23 percent of patients tested in Miami and 60 percent of patients tested in a region of Japan with a high endemic HCV prevalence (Bellman et al., 1995; Nagao et al., 1995). In India the prevalence of HCV among Lichen planus patients has been reported as 44% (Khaja et al., 2005). A prevalence of 5.5% (anti HCV antibodies) was reported in the Bagia tribe from central India, while the corresponding figure for other tribes from western India is 0.5% (Reddy et al., 1995). A relatively high prevalence of 7.89% was reported from the Lisu community of Arunachal Pradesh (north-easten India) (Pukhan et al., 2001). In South India a community called lambada the prevalence of HCV was 1.9% (Madhavi et al., 2003).

Transmission of HCV occurs via direct blood contact. This can occur in different situations:

*Injection drug use*: Use of contaminated injection material or needles for injections is the cause of the high prevalence (70 to 90%) of HCV among drug users (Thorpe et al., 2000).

*Blood transfusions and use of blood products*: Multiple cases of non-A, non-B hepatitis after blood transfusions were the principal motivation of the search for HCV in the 70s. Accurate statistical data on blood-transfusion-associated cases of HCV during that time are not available since the screening for potential contamination of donor blood had begun in the beginning of the 1980s, before discovery of the virus and the availability of a reliable detection method. The risk of HCV infection in connection with blood transfusion became as low as 0.001% per unit of transfused blood by the beginning of the 1990s (Donahue et al., 1992). Before vapor treatment of factors VIII and IX became standard, up to 95% of patients with hemophilia were sero-positive for HCV (Feinstone et al., 1975; Blanchette et al., 1991).
Chronic hemodialysis: The current risk of HCV infection after hemodialysis is around 10% per year (Fabrizi et al., 2001). The main risk factors for HCV infections in dialysis units include blood transfusion, kidney transplantation and treatment within a dialysis unit.

Organ transplantation: Patients receiving organ transplants are at a higher risk of acquiring HCV infection. Post transplant acquisition of HCV has been shown to be 50-100% in recipients of kidneys, liver or heart from anti-HCV positive organ (Wreghitt et al., 1994). The reasons for differential rates are not clear.

Occupational: Health care workers are obvious risk group for HCV. In the early 1990s, the prevalence of HCV among health care workers was 3 times higher than in the rest of the population (Lanphear et al., 1994).

Sexual transmission (Fletcher 2003): The overall rate of anti-HCV positivity in spouses of HCV infected individuals is shown to be about 5% (Kao et al., 2000).

Vertical transmission (mother to child): Infection of a child during birth and breastfeeding is possible (Tajiri et al., 2001).

Intrafamilial: In the uncontrolled studies, additional domestic factors could be identified. These included sharing razors, nail scissors, reuse of syringes and common tooth brush.

Miscellaneous: tattooing, body piercing, etc.

Taxonomy

HCV belongs to the Hepacivirus genus of the Flaviviridae family. It is an enveloped, single-strand positive-sense RNA-containing virus. Like other Flaviviruses, the RNA of HCV propagates by using an RNA-dependent RNA polymerase (RdRp), which lacks a proofreading mechanism. This gives rise to great heterogeneity in the HCV genome. Based on sequence analysis of the 5'-non-coding region (Bukh et al. 1992), genes coding for different structural and non-structural proteins (Bukh et al., 1993; Bukh et al., 1994; Simmonds et al., 1993) or the whole open reading frame (ORF) (Bukh et al., 1995), six major genotypes (I-VI) and 120 subtypes of HCV have been originally identified.
Despite difficulties in classifying some HCV isolates into any of the six major genotypes, the classification is still in use. Due to its high mutational rate, HCV circulates as a population of related, but slightly different genomes, the so-called quasispecies (Martell et al., 1992) and identification of a viral strain infecting a particular patient may be quite complicated by that fact.

**Molecular Virology**

The HCV genome is about 9.5 kb long, slightly shorter than the genomic RNAs of pestiviruses and flaviviruses. It encodes a large polyprotein of 3010-3033 amino acids (aa) (Choo et al., 1989; Kato et al., 1990), which is cleaved by both host- and virus-specific proteases into at least ten structural and nonstructural proteins (Fig.1-3) (Table.1-1). The N-terminal quarter of the polyprotein contains the virion structural proteins, the core protein, and two envelope proteins, E1 and E2, all of which are released from the viral polyprotein by host signal peptidases. The core protein constitutes the viral nucleocapsid and also possesses other activities that can modulate various cellular functions, including potential oncogenic properties (Lai et al., 2000). The E1 and E2 proteins are type 1 transmembrane glycoproteins, which are localized predominantly to the endoplasmic reticulum (ER) in cells. They form a noncovalently linked heterodimer, which is presumed to be the functional subunit of the viral envelope (Lanford et al., 1993; Ralston et al., 1993; Dubuisson et al., 1994). As the virion envelope proteins, E1 and E2 are thought to be responsible for virus binding to target cells. Indeed, some E2-specific antibodies exhibit virus-neutralizing activity (Rosa et al., 1996). The E2 protein has also been shown to inhibit double-stranded (ds) RNA-activated protein kinase (PKR) (Taylor et al., 1999), a key mediator of the antiviral and antiproliferative effects of Interferon (IFN) (Barber et al., 1995). The remainder of the polyprotein is processed by viral proteases into the viral nonstructural proteins, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (De Francesc o et al., 2000), although a structural role has been implicated for p7 (Lin et al., 1994; Mizushima et al., 1994). NS2 and NS3 are the viral proteases responsible for the cleavages at different boundaries between the different NS proteins. Cleavage at the NS2/NS3 junction is accomplished by a zinc-dependent metalloprotease encoded within NS2 and the N terminus of NS3 (Grakouri et al., 1993; Hijikata et al., 1993; Reed et al., 1995). The remaining cleavages downstream from this site are carried out by a serine protease
Fig. 1-3 HCV genome organization
Fig. 1-4: Schematic diagram of HCV genome organization showing the location of HCV genes and proposed functions of the gene
<table>
<thead>
<tr>
<th>Cleavage product</th>
<th>Size (kDa)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core</td>
<td>21</td>
<td>Structural, forms the viral nucleocapsid; Oncogenic?</td>
</tr>
<tr>
<td>E1</td>
<td>31</td>
<td>Structural, forms heterodimer with E2 gp; forms envelope of the virus</td>
</tr>
<tr>
<td>E2</td>
<td>70</td>
<td>Structural, forms heterodimer with E1 gp; forms envelope of the virus; mutations in this region determines the response to IFN</td>
</tr>
<tr>
<td>P7</td>
<td>7</td>
<td>Unknown</td>
</tr>
<tr>
<td>NS2</td>
<td>23</td>
<td>Forms autoprotease with NS3</td>
</tr>
<tr>
<td>NS3</td>
<td>70</td>
<td>Serine protease, Oncoprotein(?)</td>
</tr>
<tr>
<td>NS4a</td>
<td>8</td>
<td>Protease cofactor</td>
</tr>
<tr>
<td>NS4b</td>
<td>27</td>
<td>Unknown</td>
</tr>
<tr>
<td>NS5a</td>
<td>56</td>
<td>IFN sensitivity determining region</td>
</tr>
<tr>
<td>NS5b</td>
<td>68</td>
<td>RNA dependent RNA polymerase</td>
</tr>
</tbody>
</table>
Contained within the N-terminal region of NS3 (Bartenschlager et al., 1994; Tanji et al., 1994; Han et al., 1995). Besides its protease function, NS3 also contains RNA helicase and RNA-stimulated NTPase activities at its C terminus, which are likely involved in HCV RNA replication (Kim et al., 1995; Failla et al., 1995). NS4A forms a heterodimeric complex with NS3 and serves as an essential cofactor for efficient proteolytic processing by the NS3 protease (Failla et al., 1995; Kim et al., 1996). The function of the hydrophobic NS4B has not been defined, but it is required for the hyperphosphorylation of NS5A (Koch et al., 1999; Neddermann et al., 1999). A recent study has reported that NS4B is capable of transforming NIH3T3 cells in cooperation with the Ha-ras oncogene (Park et al., 2000).

NS5A is a phosphoprotein, which appears to possess multiple functions. In particular, it was the first HCV protein identified to bind and inhibit PKR (Gale et al., 1998) and may be one of the determinants of the degree of response of HCV patients to IFN-α treatment (Enomoto et al., 1995; Taylor et al., 2000). The N-terminus-truncated NS5A possesses a transactivation activity (Kato et al., 1997; Tanimoto et al., 1997). More recently, it has also been implicated in viral RNA replication (Blight et al., 2000). NS5B, a membrane-associated RNA-dependent RNA polymerase (RdRp), is the key enzyme responsible for HCV RNA replication.

**Envelope Proteins E1 and E2 (HVR1)**

HCV is characterized by a high degree of nucleotide sequence variability. Overall, the heterogeneity of the viral genome ranges from 30 to 35% among different genotypes, although, it varies with the specific region of the genome, and it has been used to define three types of regions nearly highly conserved regions (e.g. 5'UTR), variable regions (envelope E1 and non structural NS5b) and hyper variable regions (HVR) (e.g. HVR1 and HVR2 in E2) (Simmonds 1995).

The major viral structural envelope proteins are glycoproteins, E1 and E2, which are released from the viral protein by the action of host cell signal peptidases. Apart from the interest in understanding the biological process of assembly and morphogenesis, both E1 and E2 have been extensively studied in terms of antigenic variation and mechanisms of persistence and are obviously major components of prototype vaccine studies for HCV. E2 presumably represents most variable region of the HCV genome. This variation is
assumed to be caused by random mutation and selection of mutants capable of escaping from neutralizing antibodies produced in the host. Furthermore, antibodies against E2 correlate with protection from HCV challenge in Chimpanzees (Choo et al., 1994).

The E1 and E2 proteins of different HCV isolates exhibit a high degree of genetic heterogeneity. In particular, the N terminus of the E2 protein is extremely variable and has been designated as ‘Hyper variable region 1’ (HVR1) (aa 384-410). In genotype 1b isolates, a second hyper variable region (HVR2) within the envelope was identified downstream of HVR1. The data indicating that HVR1 is on the surface of the folded envelope protein and it represents a neutralization epitope for humoral immunity. The HVR1 changes rapidly in HCV infected patients, the mutation rate is accelerated in patients subjected to immunostimulation but is sharply decreased in agammaglobulinemic patients. In addition, mutations in the genome seem to emerge in pattern consistent with selective escape from a previous immune response, appearance of antibodies against the HVR1 of the predominant sequence was followed by the emergence of new variants, against which antibodies were not detectable. Finally antibodies (rabbit hyper immune serum) directed against the HVR1 had neutralizing activity (Sullender et al., 2000; Zeuzem et al., 2000). Thus, the HVR 1 is believed to play an important role in the persistence of HCV in the majority of acutely infected patients.

Many evidences are suggesting that HVR1 plays a functional role in the attachment and entry of HCV into target cells. HVR1 is mutated during the natural course of HCV infection in untreated immunocompetant individuals. The mutation rate has been estimated to 0.1 to 0.2 nucleotide substitutes per genome per site. N-terminus of E2 sequence has the regions of extreme hyper variability (HVR), which have been the focus of more detailed study; one of such specific domain is known as HVR-I. Several neutralization antibodies in humans have seen raised against HVR-1. The possible way to escape the humoral response is to have a large diversity of epitopes that cannot be neutralized by antibodies. Several studies have shown that during HCV infection, the HVR1 sequence of E2 became progressively heterogeneous, suggesting that it is a target of selection by antiviral antibodies (Ray et al., 1999; Lechner et al., 2000). Variants have been isolated during HCV infection, which escape the neutralization. In HCV patients with impaired humoral
immune response, the HVR1 has a lower mutation rate compared to immunocompetent individuals, suggesting that mutations in the HVR1 region are the result of selective pressure and that HVR1 contains dominant B epitopes. Variation in HVR-1 sequences during acute stage of infection can also predicts the outcome of the infection.

**Interferon sensitive determinant region (ISDR)**

Interferon is most successful therapeutic agent for the treatment of chronic hepatitis C virus, although less than half of the patients treated with IFN show sustained response with eradication of the virus. Viral load, genotype and/or quasispecies complexity and sequence diversity of particular regions of HCV genome may predict the effectiveness of IFN therapy (Fried and Hadziyannis 2004). Lower pre-treatment serum viral load HCV-RNA load has been shown to be associated with a better response to IFN therapy. Patients infected with genotype 1b tend to exhibit poor IFN responsiveness compared with other genotypes (Wolf et al., 2005; Zeuzem et al., 2005). Enomoto et al (1995) reported that amino acid mutations of the non-structural protein NS5A of HCV 1b in a region between residues 2209 and 2248 were associated with improved responsiveness to IFN in Japanese patients, and region has therefore been designated as the IFN- sensitivity-determining region (ISDR) (Nakano et al., 1999). Other research groups mostly in Japan subsequently confirmed this observation. However, several reports from Europe and United states (Hofgartner et al., 1997) failed to show the correlation between ISDR mutations and IFN responsiveness, challenging the ISDR hypothesis.

**Pathogenesis**

Chronic hepatitis is a pattern of liver injury characterized by immune-mediated hepatocellular destruction accompanied by progressive scarring and alteration of architecture that ends as cirrhosis. The mechanisms responsible for liver injury in acute and chronic HCV infection are poorly understood (Cerny et al., 1999). In primary HCV infection, liver cell damage coincides with the development of the host immune response and not with infection and viral replication. In addition, persistent viral replication often occurs without evidence of liver cell damage, suggesting that HCV is not directly cytopathic. The immune response against HCV, therefore, plays a central role in HCV
pathogenesis. HCV infection does not induce protective immunity. Re- and super infection
with homologous and heterologous isolates has been shown in both experimental and
clinical studies (Farci et al., 1992; Lai et al., 1994). The role of antibodies in
immunopathology has not been studied in much detail so far. HCV-specific major
histocompatibility complex (MHC) class I-restricted CD8+ cytotoxic T lymphocyte (CTL)
(Cerny et al., 1995; Lechner et al., 2000) and MHC class II-restricted CD4+ helper T cell
responses (Diepolder et al., 1995; Missale et al., 1996) have been identified in patients
with acute and chronic HCV infection. CTL-mediated lysis of virus-infected host cells may
lead to clearance of the virus or, if incomplete, to viral persistence and eventually chronic
hepatitis. Based on these observations and parallels in other viral diseases, viral persistence
and immunologically mediated liver cell injury are important mechanisms leading to
chronic hepatitis C (Cerny et al., 1999).

Patients who clear HCV infection have a more vigorous CD4+ (Diepolder et al.,
1995; Missale et al., 1996) and CD8+ T cell response early on (Lechner et al., 2000).
Despite the presence of an immune response, however, HCV is rarely eliminated. For a
noncytopathic virus to persist it must overwhelm, not induce, or evade an antiviral immune
response. Perhaps the simplest explanation is quantitative, based on the kinetics of
infection relative to the induction of a CTL response during the early phase of an infection
(Zinkernagel et al., 1996). According to this model, viral persistence would be predicted if
the size of the inoculum or the replication rate of the virus exceeds the kinetics of the
immune response. However, to be consistent with the repeated observation that the CTL
response is less vigorous in chronically infected patients than it is during acute self-limited
infection, additional mechanisms must be involved. These may include the induction of
peripheral tolerance or exhaustion of the T cell response, infection of immunologically
privileged sites, inhibition of antigen presentation, down regulation of viral gene
expression and viral mutations that abrogate, energize or antagonize antigen recognition by
virus-specific T cells (Cerny et al., 1999). There is some evidence that privileged sites may
play a role since HCV may infect extra hepatic cells and tissues. Inhibition of antigen
presentation is for some viruses a mechanism to establish a persistent infection. Thus far,
however, there is no evidence for these processes in HCV infection (Moradpour et al.,
2001). HCV, however, may interfere with the IFN system (Gale et al., 1998; Taylor et al., 1999). As mentioned above, the role of viral escape mutations and the quasispecies nature of HCV as a cause of viral persistence have attracted considerable interest. Molecular mimicry represents another potential mechanism of viral persistence and pathogenesis. In this context, we have recently found that CTL induced by an HCV core-derived synthetic peptide in some cases also recognized cytochrome P450; an observation that lends support for the hypothesis that HCV infection may be causally linked to autoimmune hepatitis in some patients (Kammer et al., 1999).

Natural History

Hepatitis C is the most common blood-borne infection and characterized by onset that is largely silent because of the paucity or even total absence of clinical manifestations during the acute infection (Fig 1.5). Acute HCV infection is characterized by the appearance of HCV RNA in the blood beginning 1-3 weeks after exposure. Symptoms can include jaundice, malaise, weakness and anorexia. However, symptoms are not observed in many individuals, even if liver enzymes in serum (e.g. alanine aminotransferase, ALT) are elevated. In most patients, symptoms resolve, but only about 23% truly recover, as defined by HCV RNA becoming undetectable in their serum (Seeff et al., 2001). HCV infections evolve into chronic hepatitis in 60-85% of acutely infected patients. The definition of chronic hepatitis C is generally accepted as the detection of HCV RNA in serum for a period of at least 6 months. Most chronic HCV infections are life-long in duration without effective therapy. Spontaneous clearance of HCV occurs in patients with
Fig 1.5 Natural History of Hepatitis C infection
chronic HCV infections at a rate of 0.5% per year (Watanabe et al., 2003). The existence of chronic HCV infection may first be noted when elevated ALT levels are found on routine screening. As a result, many chronic HCV infections are not recognized until very late in the course. Many patients with chronic HCV infection will develop fibrosis of the liver and some will progress to cirrhosis. The task of predicting who among them will develop cirrhosis is daunting and new approaches are still being developed. About 70% of individuals with chronic HCV infection are asymptomatic. ALT levels can also be normal in some asymptomatic patients. Among all individuals with chronic HCV infection, 30% have persistently normal ALT levels, and another 40% have minimal or intermittent minimal elevations of ALT levels. Most of these individuals with normal or minimally elevated ALT levels are believed not to be at risk for progression to cirrhosis or HCC. However, careful observation is advisable because of the theoretical possibility that some could progress to cirrhosis or HCC (Puoti 2003). Most patients with HCV RNA in their serum for a period of years and normal ALT levels have been shown to have persistently normal liver histology in liver biopsies obtained 2-5 years later (Martinot-Peignox et al., 2001). Life-long immunity to HCV appears to develop in almost all patients in whom spontaneous resolution occurs (i.e. loss of detectable HCV RNA). This combination of resolution and lasting immunity apparently requires the presence of an effective CD4 T-helper cell response. Conversely, the absence of an effective CD4 response has been shown to be associated with persistent viraemia and the emergence of escape mutants (Grakouri et al., 2003). If the CD4 response is adequate but is not accompanied by adequate interferon (IFN)-γ production, chronicity of HCV infection and development of HCC may be more likely to occur (Sugimoto et al., 2003). From 4% to 24% of persons with chronic HCV infection develop cirrhosis. The rate depends on the population, with lower rates (4-7%) being found in community based and blood donor cohorts, and higher rates (>20%) being found in populations of HCV-infected patients followed in liver clinics (Freeman et al., 2000; Minola et al., 2002). Risk factors for development of cirrhosis include acquisition of HCV infection after age 30 years and heavy ingestion of alcohol. Approximately 70-80% of HCC patients have cirrhosis in the adjacent non-tumorous liver. The high rate of coexisting cirrhosis in HCC patients and the emergence of HCC in prospectively followed cirrhosis patients have led to the assumption that pre-existing
cirrhosis is an important prerequisite for hepaticarcinogenesis, although some HCCs do arise in the absence of cirrhosis. It is generally believed that the majority of HCCs develop in a progression from acute hepatitis through various stages of chronic hepatitis, to cirrhosis, to HCC. In HCV-associated cirrhosis, the cumulative development of HCC increases steadily with time, reaching as high as 75% at 15 years in one study (Ikeda et al., 1993). Higher rates of HCC have been reported in cirrhosis inpatient studies (Kato et al., 1999). There is an even greater risk of a patient with cirrhosis developing HCV-associated but not HBV-associated HCC if alcoholism is present in addition (Yamauchi et al., 1993). Once cirrhosis is established the rate of HCC development is 1-4% per year. HCV infection appears to be responsible for a substantial increase in HCC incidence and mortality (El-Serg et al., 2000). Although recent experimental evidence raises the possibility that HCV might operate through direct pathways in promoting malignant transformation of hepatocytes, it is generally believed that HCC associated with chronic hepatitis C develops through a general pathway of increased liver cell turnover, induced by chronic liver injury and regeneration, resulting in multiple and stepwise genetic alterations (Moradpour et al., 2001). The highly variable natural course of chronic hepatitis C may depend on viral (e.g., inoculum size, genotype, and quasispecies complexity) and host related factors (e.g., age at the time of infection, gender, MHC haplotype, confection with HBV or HIV, and, probably most important, alcohol consumption).

Chronic HCV infection has been associated with a number of extra hepatic manifestations, including mixed cryoglobulinemia, glomerulonephritis, lichen planus, and porphyria cutaneatarda (Zignego 1999). Cryoglobulins are detectable in up to one third of patients with chronic hepatitis C, while the clinical syndrome of mixed cryoglobulinemia occurs in only 1-2% of patients. Recently, chronic hepatitis C has been associated with non-Hodgkin's lymphoma and type 2 diabetes mellitus (Bahtiyar et al., 2004). However, these associations are still debated.

**Diagnostic Perspectives of HCV**

The use of serological and virological tests has become essential in the management of hepatitis C virus infection in order to diagnose infection, guide treatment decisions and assess the virological response to antiviral therapy. The detection of anti-
HCV antibodies in plasma or serum is based on the use of third-generation EIAs, that detect mixture of antibodies directed against various HCV epitopes. The third generation EIAs, approved by the Food and Drug administration for blood donor screening. (Ortho HCV 3.0 ELISA, Ortho clinical systems, Raritan, NJ; MONNOLISA HCV, Sanofi, Diagnostics Pausteur, Marnesola-coquette, France; and Abbott HCV 3.0 EIA, Abbott laboratories, North Chicago, IL). Anti-HCV tests are less sensitive, however, in immunocompromised individuals or hemodialysis patients, for these patients, RT-PCR should be performed. The recombinant immunoblot assay (RIBA) is a supplemental assay that can be used to confirm a positive ELISA, particularly in low-risk populations. No routine serological test for a viral antigen is available as yet. However, immunoassays based on the use of high-affinity monoclonal antibodies against core protein to detect and quantitate HCV in serum are currently being evaluated.

HCV becomes positive by RT-PCR as early as 1-2 weeks after infection and 4-6 weeks before anti-HCV seroconversion. The determination of HCV RNA is, in principal, important for the selection of patients for antiviral therapy and assessment of its efficacy (European association for the study of the Liver. Consensus statement. 1999). More over it is now well documented that the commercially available third generation EIA cannot be used to detect viral infection in Indian patients owing to genetic variations (Poduri et al., 1998 and 2000). It may be noted at this stage that the commercial third generation EIA is based on the genotype 1 and that the genotype specific antibody response in this virus is now documented (Bhattacherjee et al., 1995). To overcome this problem, sensitive and cost effective diagnostic peptide-based enzyme immunoassays are designed to detect HCV infection in Indian patients has been developed (Panigrahi et al., 1998). Presently, an Indigenous peptide based HCV EIA kit is available in India. (Xcyton™ Banglore, India).

In clinical practice, the usual approach is to test initially for antibodies to HCV antigens (anti-HCV), then to use HCV RNA to document viremia. Because many HCV infected persons have HCV RNA levels in the range of the quantitative assays and the quantity of HCVRNA is useful to know before providing and monitoring HCV treatment (McHutchison et al., 1998). Many experts routinely obtain quantitative rather than
qualitative HCV RNA tests to confirm the presence of viremia (Carithers et al., 2000). However, quantitative HCV RNA tests are generally not very sensitive; therefore, some experts prefer a qualitative HCV RNA test either as the primary test to confirm a positive HCV antibody result in patients with a negative result by quantitative assay (Pawlotsky et al., 1998; 2002). A negative sensitive RNA test in a person with HCV antibodies most likely indicates that the HCV infection has resolved. Other interpretations are that the anti-HCV immunoassay is falsely positive, the HCV RNA test is falsely negative, or rarely that a person has intermittent or low-level viremia.

Qualitative assay used to detect HCV RNA in the blood using amplification techniques, such as polymerize chain reaction (PCR) or transcription-mediated amplification (TMA) (Pawlotsky 2003). The Food and Drug Administration (FDA) has approved 2 PCR-based tests for qualitative detection of HCV RNA (1) Amplicor Hepatitis C Virus Test, version 2.0, and (2) COBAS Amplicor Hepatitis C Virus Test, version 2.0 (Roche Molecular Systems, Branchburg, NJ), which have lower limits of detection of approximately 50 IU/ml. Some diagnostic laboratories use other commercially available nonapproved assays.

Quantitative HCV RNA assays ascertain the quantity of HCV RNA in blood using either target amplification (PCR, TMA) or signal amplification (branched DNA assay). The level of HCV RNA in blood helps in predicting the likelihood of response to treatment and the change in the level of HCV RNA during treatment. The only quantitative test that has currently received FDA approval is COBAS amplicor monitor HCV version (Detection limit 600-5,00,000 IU/L) (Pawlotsky 2002).

Discrimination of genotype 1 from genotypes 2 and 3 as well as quantitative determination of viremia levels has become important for the selection of the optimal treatment regimen (Hadziyannis et al., 2004). In general, however, genotyping and quantitative RT-PCR tests should be used only in the context of a defined therapy protocol and not for the initial diagnosis of HCV infection. Two commercial ones are widely used for clinical purpose. 1. The Trugene HCV 5'NC Genotyping kit (Visible Genetics, Toronto, Canada), which is based on direct sequencing followed by comparison with a
reference sequence database, and 2. Line-probe assay (Inno LiPA HCV II, Innogenetics, Ghent, Belgium), which is based on reverse hybridization of PCR amplicons on a nitrocellulose strip coated with genotype specific oligonucleotide probes (Stuyver et al., 1996; Ansaldi et al., 2001).

Liver biopsy allows determining the necro-inflammatory activity (grading) and the degree of fibrosis (staging), as well as to recognize or exclude coexisting liver pathology (Such as alcoholic liver disease or hemochromatosis). It is strongly recommended before the initiation of antiviral therapy (Fontaine et al., 2001).

**HCV GENOTYPES**

HCV being an RNA virus exhibits a remarkable degree of heterogeneity throughout the length of its nucleic acid (Okamoto et al., 1992). Traditionally, this heterogeneity has been classified as i. Quasi-species and ii. Genotypes (Martell et al., 1992). This heterogeneity arises as a consequence of lack of proof-reading activity of RNA-dependent RNA polymerase during replication of the virus. Over a period of time these mutations get accumulated at specific regions, leading to what are known as genotypes.

HCV is the one of the most suitable virus models for studying genomic variability. Its classification into six genotypes has been accepted with intrasubtype and intrasubject heterogeneity. The development of an effective vaccine to protect humans from HCV infection and chronic liver disease is public health priority, yet differences in antigenic epitopes in different genotypes, subtypes, and quasispecies could make cross-protection unlikely. This genotypic variation is most obvious in studies that addressed in liver disease progression or response to IFN therapy. The severity of liver disease was based on histological activity in some cases and on the development of cirrhosis or HCC in others.

HCV exhibits a high genetic variability, enabling its classification into four hierarchical strata, clades, genotypes, subtypes and isolates. (Robertson et al., 1998). A high mutation rate is considered to be a major factor behind its genetic diversity. (Farci and Purcell 2000). As a result, extensive genetic heterogeneity of HCV exists in infected individuals, and HCV isolates are found as either group of isolates with very closely related genomes referred to as quasispecies or genetically distinct groups called genotypes (Table. 1.2).
Geographic Distribution of HCV Genotypes

At least six major genotypes of HCV, each comprising multiple sub types, have been identified worldwide (Zein and Persing 1996) (Table.1.3). Substantial regional differences appear to exist in the distribution of HCV genotypes. Although HCV genotypes 1, 2, and 3 appear to have a worldwide distribution, their relative prevalence varies from one geographic area to another.

HCV subtypes 1a and 1b are the most common genotypes in the United States (Zein et al., 1995). These subtypes are also predominant in Europe (Dusheiko et al., 1994; McOmish et al., 1994; Nousbaum et al., 1995). In Japan, subtype 1b is responsible for nearly 73% of cases of HCV infection (Takada et al., 1993). HCV subtypes 2a and 2b are relatively common in North America, Europe, and Japan, subtype 2c is found commonly in Northern Italy. HCV genotype 3a is particularly prevalent in intravenous drug abusers in Europe and United States (Pawlotsky et al., 1995). HCV genotype 4 appears to be prevalent in North Africa and the Middle East (Abdulkarim et al., 1998; Chamberlain et al., 1997a and 1997b) and genotypes 5 and 6 seem to be confined to South Africa and Hong Kong, respectively (Cha et al., 1992; Simmonds et al., 1993). HCV genotypes 7, 8, and 9 have been identified only in Vietnamese patients (Tokita et al., 1994a and 1994b) and
### Table 1.2

Terminology commonly used in studies related to HCV genomic heterogeneity

<table>
<thead>
<tr>
<th>Terminology</th>
<th>Definition</th>
<th>% nucleotide similarity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>Genetic heterogeneity among different HCV isolates</td>
<td>65.7 – 68.9</td>
</tr>
<tr>
<td>Sub type</td>
<td>Closely related isolates within each of the major genotypes</td>
<td>76.9 – 80.1</td>
</tr>
<tr>
<td>Quasispecies</td>
<td>Complex of genetic variants within individual isolates</td>
<td>90.8 – 99.0</td>
</tr>
</tbody>
</table>

* % nucleotide similarity refers to the nucleotide sequence, identities of the full length sequences of the HCV genome
### Table 1.3

HCV genotype distribution in different parts of the world

<table>
<thead>
<tr>
<th>Genotype (and its variance)*</th>
<th>Geographic region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>North and South America; Europe</td>
</tr>
<tr>
<td>2</td>
<td>North and South America;</td>
</tr>
<tr>
<td>3</td>
<td>Nepal, Bangladesh, Pakistan and South Asia</td>
</tr>
<tr>
<td>4</td>
<td>Egypt</td>
</tr>
<tr>
<td>5</td>
<td>Central and South America</td>
</tr>
<tr>
<td>6</td>
<td>China, Japan and Southeast Asia</td>
</tr>
</tbody>
</table>

* Nomenclature of the genotypes as proposed by Simmonds et al.
genotypes 10 and 11 were identified in patients from Indonesia (Tokita et al., 1996). There has been disagreement about the number of genotypes into which HCV isolates should be classified. Investigators have proposed that genotypes 7 through 11 should be regarded as variants of the same group and classified as a single genotype, type 6 (Simmonds et al. 1993; Mellor et al., 1996; de Lamballerie et al., 1997; Tokita et al., 1998).

HCV genotypes in India

In India genotype 1 and 3 are predominantly found (Fig.1.6). In North India genotype 3 is more prevalent followed by genotype 1b (Panigrahi et al., 1996; Kavita et al., 2003; Sompal et al., 2004). Whereas in South India genotype 1 was more prevalent followed by genotype 3 (Valliammai et al., 1995; Kavita et al., 2003; Madhavi et al., 2003; Bhattacharya et al., 2004; Khaja et al., 2005).

In North India Panigrahi et al (1996) identified genotype 1b, 3a, 3b, and 3g, another study they identified 1a, 1b, 3a, 3b, 2a, 2b, and 4a (Sompal et al., 2004). In South India khaja et al (2005) identified 1a, 1b, 1c, 3a, 3b, 4c, and 4f. In another south Indian study genotype 1a, 1b, 3a, and 3b identified (Valliammai et al., 1995; Madhavi et al., 2003; Bhattacharya et al., 2004). In Western part of India genotype 3, 4, and 5 were identified, whereas another study identified genotype 1a, 1b, 2a, 2b, 2c, 3a, 3b, 3c, 3d, 3f, and 3g (Sawant et al., 1999; Amarapurkar et al., 2001).

The geographic distribution and diversity of HCV genotypes may provide clues about the historical origin of HCV (Smith et al., 1997). The presence of numerous subtypes of each HCV genotype in some regions of the world, such as Africa and Southeast Asia, may suggest that HCV has been endemic for long time.
Fig 1.6  HCV genotype distribution in India
Hepatitis C virus Genotyping and Serotyping

The differences in geographical distribution, disease outcome, and response to therapy among HCV genotypes have been suggested, reliable methods for determining the HCV genotype may become an important clinical test (Davis et al., 1998; Poynard et al., 1998; Zein, 2000). The reference standard and most definitive method for HCV genotyping is sequencing of specific PCR amplified portion of HCV genome obtained from the patient, followed by phylogenetic analysis.

Other methods that have been reported depend mainly on the amplification of HCV RNA from clinical specimens, followed by either re amplification with type-specific primers or hybridization with type specific probes (Okamoto et al., 1992; Li et al., 1994; Qu et al., 1994; Widell et al., 1994) or by digestion of PCR amplicons with restriction endonucleases that recognize genotype specific cleavage site (Murphy et al., 1994). HCV genotyping by using type specific primers was first introduced by Okamoto et al (1992) and used primers specific for HCV core region. This method lacks sensitivity and specificity (Xavier et al., 1998), as it is able to detect subtypes 1a, 1b, 1c, 2a, 2b, and 3a only. However, modifications have been introduced to improve the sensitivity and specificity of this method (Okamoto et al., 1994; Widell et al., 1994). Several DNA hybridization assays for HCV genotyping have been described. A commercial kit (InnoLipa) for HCV genotyping has been introduced in Europe by Innogenetics (zwijndre, Belgium) and is based on hybridization of 5′ UTR amplification products with genotype specific probes (Stuyver et al., 1993). Although the initial version of InnoLipa had lower sensitivity, the newer version is capable of discriminating among HCV subtypes 1a, 1b, 2a to 2c, 3a to 3c, 4a to 4h, 5a and 6a (Maertens et al., 1997). It has been shown that genotyping methods using 5′ UTR, including InnoLipa, may not distinguish subtype 1a from 1b in 5 to 10% cases and also may not distinguish between subtypes 2a and 2c (Smith et al., 1995). Others have used restriction enzymes to determine a restriction fragment length polymorphism. In this method a PCR amplified DNA fragment is digested into fragments with different lengths by restriction endonucleases that recognize cleavage sites specific for each genotype (Xavier et al., 1998). Investigators have used different regions
of the HCV genome for restriction fragment length polymorphism, including NS5 and the 5' UTR (Nakao et al., 1991; Bukh et al., 1992; Chan et al., 1992).

All these methods are able to identify correctly the major genotypic groups, only direct nucleotide sequencing is efficient in discriminating among subtypes (Bukh 1995; Simmonds 1995). Moreover, all of these PCR-based methods have the drawbacks and advantages of PCR. These methods are expensive, time-consuming, required special facilities to ensure accurate results and are prone to contamination. Their reliability may further be compromised if viral RNA is lost in the serum or plasma through storage or improper laboratory handling or if it is absent from the circulation during the sample collection. The advantages of PCR-based methods include reliability if performed accurately and ability to obtain information relevant to molecular pathogenesis of HCV.

Serologic genotyping has several advantages, it is suitable for large epidemiological studies. More recently, investigators identified genotype-specific antibodies that could be used as indirect markers for the HCV genotype (serotyping or serologic genotyping) (Machida et al., 1992; Simmonds et al., 1993; Tsukiyama-Kohera et al., 1993; Mondelli et al., 1994). These advantages include the low risk for contamination and the simplicity of the assay. However, serological typing seems to have low specificity and sensitivity, which limits its utility.

Clinical Significance of HCV Genotypes

Although the impact of HCV heterogeneity and genotypes on the day-to-day clinical management of chronic HCV infection has not been established, its role as an epidemiological marker has been clearly shown. Furthermore, the sensitivity and specificity of serologic and virologic assays for the detection of HCV may be influenced by its heterogeneity. However, the exact role of genotypes in the progression of liver disease, the outcome of HCV infection, and the response to interferon therapy are not well understood (McHutchinson et al., 1998; Poynard et al., 1998). The study of these issues has been hampered by the long natural history of HCV infection and the lack of information about the exact time of exposure to the infection.
Hepatitis C virus infects an estimated 2 to 4% of the World's population (Di Bisceglie 2000). Chronic hepatitis C develops in 60-70% of infected individuals. Among chronic patients 10-20% develop cirrhosis and among cirrhosis cases only 1-4% develop hepatocellular carcinoma (HCC) (Khaja et al., 2002). Current antiviral therapies can cure only about 40% of the cases, they are expensive, and associated with severe toxicity (NIH 2002. Management of hepatitis C. http://consensus.nih.gov/2002/2002HepatitisC2002116html.htm). Since the discovery of HCV, considerable efforts have been devoted to defining the factors that may be important in predicting the long-term response to interferon therapy (Zien and Rakela 1995). The IFN dose, duration of treatment, viral RNA levels, and liver histology all seem to play a role in predicting response. It has been suggested that patients infected with genotypes 2 and 3, are likely to have a favourable response to interferon treatment than are those infected with genotype Ib, those infected with genotype 1a has almost no response to IFN therapy (Marcellin et al., 1997).

Studies with pegylated interferon (IFN covalently attached to polyethylene glycol) have been performed in response to suggestions that the poor response rate achieved in the treatment of HCV or due to the short half-life (4 to 6 hrs) of IFN, which necessitates thrice weekly dosing, thereby leading to erratic serum concentrations and intermittent peaks and troughs (Glue et al., 2000). The covalent attachment of polyethylene glycol to the IFN molecule increases the half-life to 96 hr, thus creating high antiviral concentrations and permitting once-a-week dosing. Pegylated IFNs will replace the conventional IFNs in the near future because they are more effective and have to be injected once in a week due to their prolonged half-life.

Until the mid 1990s, interferon-2α (INF-α) was the only available treatment. The addition of ribavirin, a nucleoside analogue, substantially improved the response, however, viral genotype remains an important determinant of response rate. Although, cure remains the primary objective, the benefits of treatment are not necessarily restricted to those patients who achieved eradication of HCV. It seems that interferon treatment alone or in combination may prevent progression of, or even reverse, hepatic fibrosis in infected patients even if cure is not infected achieved. Although ribavirin alone does not seem to be
active against HCV, the combination resulted in much improved and sustained biochemical, virological, and histological response rates. Recent studies have shown that treatment with long-lasting pegylated interferon have better viral response than classic INF-α preparations in combination with ribavirin, achieved sustained viral eradication rates of 54%. In India, the percentage of sustained responders is quite high (50%-60%). Such response of INF-α therapy in chronic hepatitis C is due to a predominant prevalence of hepatitis C genotype 3.

Many treatment trails have been conducted over the past decade to determine the benefit of monotherapy and, subsequently, combination therapy in the management of HCV infection. The early studies of Interferon (IFN) monotherapy (six month duration) revealed end-of-treatment response (ETR) of approximately 50%, but a sustained response (SR), defined as continued loss of HCV RNA six months after cessation therapy, of only 15% (Ahmed and Keeffe 1999). Studies of ribavirin monotherapy were also less promising. Later two randomised controlled trials have been conducted to compare the effect of IFN monotherapy and combination therapy using IFN and ribavirin for 24 or 48-week treatment in naive patients (McHutchinson et al 1998; Poynard et al 1998). Both studies used similar patient evaluation, monitoring, and end points. Among patients receiving monotherapy, ETR was achieved in 29% regardless of duration of therapy, whereas, SR was achieved in 6% of those treated for 24 weeks and in 16% of those receiving 48 week of therapy. By contrast, patient receiving combination therapy for 24 and 48 week had ETR rates of approximately 50% and SR rates that increased from 33% in those treated for 24 week to 41% in those receiving 48 week of therapy.

Those who have been identified as being acutely infected need to be monitored. Closely for the first 12 weeks to establish whether or not they will undergo spontaneous viral clearance. Serum HCV RNA (qualitative, i.e. very sensitive) needs to be measured as close to baseline as possible, and this test should be repeated at least before 12 weeks have passed (Gerlach et al., 2003). Rapid viral decline in titer after the initial identification of viraemia may predict those who will spontaneously clear virus, although this is not 100% reliable (Hofer et al., 2003). In those who remain viraemic at 12 weeks after initial seroconversion, antiviral therapy is recommended if there are no absolute contra indica-
tions to its use. The largest uncontrolled study, which employed induction dosing with standard IFN-α-2b, indicated that 95% of treated patients achieved a sustained virological response with only 6 months of therapy (Jaeckel et al., 2001). It is unlikely that the newer forms of IFN, namely PEG-IFN-α in combination with ribavirin, are going to be much more successful.

The weekly subcutaneous PEG-IFN-α given in combination with daily oral ribavirin, but the optimal duration of treatment remains unclear. Two large phase III studies, one employing PEG-IFN-α-2b plus ribavirin (Manns et al., 2001), and the other PEG-IFNα-2a plus ribavirin (Fried et al., 2002 and 2004) - both given for 48 weeks - have demonstrated enhanced efficacy when compared with the earlier standard of care, i.e. standard IFN-α-2b given three times per week subcutaneously combined with daily oral ribavirin. SVR rates, defined as undetectable HCV RNA (using a qualitative technique) in serum 6 months after cessation of therapy were 54% and 56%, respectively, but there were some genotype-specific differences between the two forms of PEG-IFN-α (Manns et al., 2001; Fried et al., 2002 and 2004). These differences may have resulted from what has now been clearly shown to be an inadequate dose of ribavirin (800 mg daily) when given to individuals infected with genotype 1, in the PEG-IFN-α-2b plus ribavirin study. So in the 48-week trial which involved PEG-IFN-α-2b plus ribavirin, antiviral efficacy in genotype 1 in the low dose and high dose IFN regimens was 34% and 42%, respectively, whereas for genotypes 2 or 3 it was 82% and 80% (SVR for genotypes 4/5/6 were 33% and 50%).

In the 48-week phase III trial of PEG-IFN-α-2a plus ribavirin, only one dose, 180 μg/wk of IFN and 1000 - 1200 mg ribavirin, was evaluated. In those with genotype 1, high viral load (>2 x 10⁶ c/ml) overall SVR was 41% (compared with 33% for standard IFN-α-2b plus ribavirin), whereas the SVR in those infected with either genotype 2 or 3 was 76% compared with 61% in those who received standard IFN-α-2b plus ribavirin. It is likely that 24 weeks of treatment is sufficient for those infected with genotypes 2 and 3. Because of small numbers, the most appropriate duration of therapy for genotypes 4, 5 and 6 is unknown, whereas it is felt that for those infected with genotype 1 therapy for a full 48
weeks is required. Durability of SVR is excellent (Marcellin et al., 1997) and unless the patient is known to have cirrhosis pretreatment, long-term follow-up after achieving an SVR is probably unnecessary.

Adverse events occur with the use of IFN alone and with combination therapy. The most common side effects of ribavirin is a dose related haemolysis of red cells leading to severe anaemia (Bodemheimer et al., 1997). Ribavirin is excreted via the kidney, and so any renal impairment will promote high blood levels and thus its use is contraindicated in renal failure (Bruchfeld et al., 2003). The haemolysis is noted within 1 to 4 weeks of beginning therapy and can be precipitous, fatigue, shortness of breath, angina and palpitation. The most common side effect of IFN use is often a self-limited flu like syndrome (Devis et al., 1989). Although rare, IFN therapy may induce a reversible cardiac arrhythmia or cardiomyopathy (Deyton et al., 1989; Angulo et al., 1999).

Non-responders and relapsers

Several studies have shown that for those who have previously failed to respond to IFN monotherapy there is a reasonable chance of responding to combination therapy. The benefit is particularly seen in those who initially responded and then relapsed. In one study 87 of 345 patients who had relapsed following IFN monotherapy, the sustained response rate was 49% in those who received IFN and ribavirin combination therapy and only 5% in those who were retreated with IFN monotherapy (Davis et al., 1998). Response rates in previous non-responders are much lower at 15-20%. Other studies have tried other approaches such as an induction period with high dose IFN (Camma et al., 2002; Veldt et al., 2003). A pilot study of treatment with consensus IFN and ribavirin in patients who had failed to respond to standard IFN and ribavirin reported SVRs of 33% in the previous non-responders and 42% in the previous relapsers (Barbaro et al., 2002). There are limited data on PEG-IFN ribavirin regimens as retreatment, but preliminary results suggest significant benefits for those who have received IFN monotherapy and more modest gains for those who have already received IFN and ribavirin. The early virological response will hopefully also prove useful in determining the chance of an SVR in these patients, but more data are required to ascertain whether the predictors in previously treated
patients undergoing retreatment remain valid. Overall, the factors associated with a higher chance of responding to retreatment are previous relapse (rather than non-response), genotypes 2 and 3, lower levels of HCV RNA and an early virological response to treatment. Negative factors include being African-American and or cirrhotic.

Those with only mild liver damage can afford to wait for the development of new antiviral agents, but for those with more severe disease a more realistic priority may be to inhibit fibrosis and maintain liver function. Long-term maintenance regimens may need to be considered.

Evolving therapeutic strategies

The rapidly increasing knowledge of the HCV replication cycle provides opportunities for the development of innovative therapeutic and preventive strategies. As outlined above, specific inhibitors of the biochemically and structurally well characterized NS3 serine protease, the RNA helicase and the RNA-dependent RNA polymerase, are currently being developed as antiviral agents. A polymerase inhibitor, VP 50406, is now in phase II clinical trial and many more will likely follow soon (www.viropharma.com/pipeline/HepC.htm). In addition to these more classical pharmacological approaches, gene therapeutic strategies aimed at inhibiting HCV replication and gene expression are currently being explored in various experimental systems (von Weizsacker et al., 1997). These include, among others, antisense oligodeoxynucleotides and ribozymes. In this context, the first anti-HCV ribozyme has now entered phase II clinical testing (www.rpi.com/antihecv.cfm). Moreover, based on the concept that a quantitatively and qualitatively insufficient CD4+ and CD8+ T cell response may contribute to viral persistence, immunotherapeutic strategies aimed at enhancing the cellular immune response against HCV are currently being investigated.

Vaccine development

Apart from more efficient therapeutic strategies, the development and implementation of preventive measures are of paramount importance. The development of an effective recombinant vaccine has been hampered by the high genetic variability of
HCV and the lack of a suitable cell culture system or small animal model. It has been shown, however, that vaccination with recombinant envelope proteins expressed in mammalian cells can protect chimpanzees from primary infection with a homologous virus isolate (Choo et al., 1994). However, the correlates of and requirements for a broader protection and a potentially neutralizing immune response still need to be defined. The potential of DNA vaccination to induce a humoral and cellular immune response is particularly promising in this regard (Inchauspe 1999). Alternative currently pursued strategies include peptide and protein vaccines, dendritic cell-based vaccines, and virus-like particles. While sterilizing immunity will probably be difficult to achieve, the aim of inducing a state of immunity that prevents the development of chronic infection appears more realistic (Forns et al., 2000). In any case, it is likely that induction of both a humoral and a cellular immune response will be required for an effective HCV vaccine. Such a vaccine may be used also as a therapeutic vaccine. In this context, a phase II clinical trial based on a recombinant HCV E1 vaccine will soon be initiated in patients with chronic hepatitis C (www.innogenetics.be).
AIMS AND OBJECTIVES

1. To find out the HCV infection in high risk groups i.e. CRF’s, Tattoos, religious practitioners and patients with multiple blood transfusions, by using molecular and immunological methods.

2. To know the predominant genotypes in Indian sub-continent by using the most definitive method is sequencing of a specific PCR amplified fragment. To understand the genetic heterogeneity of HCV and the presence of various genotypes which have epidemiological and therapeutic implications.

There are six major known genotypes and more than 120 subtypes of HCV. Traditionally, this heterogeneity has been classified as (i) Quasi-species and (ii) genotypes. This heterogeneity arises as a consequence of lack of proof reading activity of RNA-dependent RNA polymerase during the replication of virus. Over a period of time these mutations get accumulated at specific regions, leading to what are known as the genotypes. Some investigators have reported an association between HCV genotype and interferon (IFN) responsiveness as well as disease progression. Thus it becomes important in clinical management of HCV infection.

The most commonly used nomenclature proposed by Simmonds et al (1993) is followed in this study. The genotyping data from HCV in India is very less as reviewed by Khaja et al (2002). Therefore, our second aim will carryout molecular epidemiological studies to determine the genetic variations in Indian HCV patients.

3. We aimed to analyze the possible relationship between Interferon (IFN) and Interferon Sensitivity Determining Region (ISDR) before treatment and after treatment along with genotype data as a function of response to Interferon therapy in hepatitis C virus – infected patients.

The persistence of hepatitis C virus infection and its poor susceptibility to treatment have been attributed to the high rate of genetic variability exhibited by the virus. Investigations have been carried to know the heterogeneity of the non-structural 5A protein in the role of HCV resistance to α-interferon (IFN-α). NS5A protein interacts in-vitro with protein kinase R (PKR), a mediator in
the IFN-α-induced antiviral cell state. A region with low degree of genetic diversity in NS5A protein, designated as 'IFN sensitivity-determining region' (ISDR), has been proposed to confer resistance to IFN-α treatment in Asian viral isolates. Subsequent reports, however, have not consistently confirmed these findings in HCV isolates from Caucasian patients. The role of the ISDR to interact with PKR in-vivo, and thus influences treatment outcome, is not completely established. In 1996, Enomoto et al reported that amino acid mutations of the nonstructural protein NS5A of HCV 1b in a ISDR region is associated with improved responsiveness to IFN in Japanese patients. Other research groups mostly in Japan subsequently confirmed this observation. However, several reports from Europe and United states failed to show the correlation between ISDR mutations and IFN responsiveness, challenging the ISDR hypothesis. Therefore, we intend to determine the variation among the different patients that respond or not respond to the IFN.

4. To find out genetic heterogeneity in the HVR-1 region of Indian HCV patients from different geographical regions.

The hypervariable region -1 (HVR-1) is located at the extreme 5’end of the E2 glycoprotein of HCV genome. Many evidences suggest that HVR-1 plays a functional role in the attachment and entry of HCV into target cells. HVR-1 is mutated during natural course of HCV infection in untreated immunocompetant individuals. The mutation rate has been estimated to 0.1 to 0.2 nucleotide substitute per genome per site. Thus HVR-1 is believed to play an important role. Variants may be related to differences in transmissibility, immunogenecity or pathogenecity, which can be important in developing prophylactic and therapeutic vaccine. Hence our objective was to clone, sequence and analyze the variability of the HVR-1 from Indian patients.

5. Molecular Cloning, Sequencing and characterization of Indian strain of Hepatitis C virus.

HCV continues to be a major threat to the Indian population. Many strains have been sequenced from different countries. Compared to the progress made in
western countries, research in the Indian subcontinent is going on at a slow pace. Hence, we have cloned and sequenced complete Indian isolate of hepatitis C virus. This will help us to plan a further research work.

6. **Antigenicity of recombinant HCV Core, NS3, NS5 in different genotypes of Indian HCV strains.**

Commercial HCV test based upon proteins or peptides derived solely from HCV genotype I may be less effective for the detection of antibody against HCV of other genotypes. It has become increasingly evident that further improvement HCV diagnostic test will rely on the care full selection of sequence variance of antigens capable of detecting antibodies against different HCV genotypes with equal efficiencies. Presently a third generation ELISA that incorporates antigens from the core, NS3, NS4, and NS5 proteins of HCV representing about 60% of total amino acid profile of HCV polyprotein is available in the market. This assay is fails to differentiate between active and post infection cases. In addition to this it is now well documented that the commercially available third generation EIA cannot be used to detect the viral infection in Indian patients owing to genotype variations. It may be noted that at this stage that the commercial third generation ELSA is based on genotype 1 and that genotype specific antibody response in this virus is now documented. To overcome this problem we focused on sensitive recombinant assays development and that is cost effective and designed to detect HCV infection in Indian patients.