The hepatitis C virus persistence: how to evade the immune system?

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Hepatitis C virus (HCV) is an emerging virus of medical importance. A majority of HCV infections become chronic and lead to chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. HCV usually induces robust immune responses, but it frequently escapes the immune defense to establish persistent infection. The fact that HCV exists as an evolving quasispecies plays an important role in the selection of escape mutants. Furthermore, several viral proteins interfere with cellular functions, in particular, those involved in the immune response of the host. Several HCV proteins also modulate cell signalling through interaction with different effectors involved in cell proliferation and apoptosis, or in the interferon-signalling pathway. In addition, HCV infects immune cells such as B and T cells, and thus affects their normal functions. These various strategies used by HCV to counter the immune response of the host are reviewed here. A better understanding of these mechanisms would help design new therapeutic targets.

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1. Introduction

Hepatitis C virus (HCV) is an emerging virus. It was first identified in 1989 (Choo *et al* 1989). Before then, it was classified as a non-A non-B hepatitis virus transmitted mainly through blood products, or via other intravenous routes as well. Acute infection is often insidious, but, in a majority of cases, the virus establishes chronic infection. The persistent HCV infection results in liver damages: for example: fibrosis, cirrhosis and hepatocellular carcinoma (HCC) (Colombo 1998). Today, HCV infection is the most common cause for liver transplantation in the United States.

The mechanism by which HCV leads, at a high frequency, to persistent infection is not fully understood. The lack of efficient *in vitro* or *in vivo* systems for HCV replication, except in chimpanzee, renders such studies difficult. Data obtained from infected patients, or from experimental infection of chimpanzees, or from transgenic mice for HCV protein(s), or from cells transiently or stably expressing one or several HCV proteins suggests that viral persistency is a multifactorial mechanism; and HCV has developed several strategies to evade the immune system and persist. The high genetic variability of HCV allows the virus to passively evade the immune system. In addition, several viral genes impair cellular functions involved in immune response, or in cell proliferation, or cause apoptosis. Furthermore, HCV does not only infect hepatocytes but infect B and T cells as well. Thus, infection of cells of the immune system impairs their functions.

Currently, the only approved therapy for HCV is by alpha interferon (IFN-α) or pegylated-IFN-α in monotherapy or in combination with ribavirin – a nucleoside

Keywords. Cell signalling; hepatitis C virus; immune system

Abbreviations used: CTL, Cytotoxic T lymphocytes; DCs, dendritic cells; HCV, hepatitis C virus; HVR1, hypervariable region 1; IRES, internal ribsomal entry site; LDLR, LDL receptor; LTβR, lymphotoxin β-receptor; MC, mixed cryoglobulinemia; MHC-I, major histocompatibility complex class-I; NK, natural killer; NOB, neutralizing of binding; nt, nucleotides; ORF, open reading frame; PBMC, peripheral blood mononuclear cells; PTB, polypyrimidine-tract-binding-proein; RdRp, RNA-dependent-RNA-polymerase; TNF, tumour necrosis factor.

analogue (McHutchison *et al* 1998; Poynard *et al* 1998). However, a sustained response to treatment is observed only in a limited number of cases. It is particularly inefficacious for the most prevalent genotype 1b (Liang *et al* 2000). The resistance to IFN therapy appears to be at least partially associated with the interactions of viral proteins with effectors of the IFN-α pathway.

In this review, we will present the latest findings on the multiple strategies developed by HCV to evade the immune system and persist.

1.1 *Molecular biology of HCV*

HCV belongs to the *Flaviviridae* family and is the only member of the Hepacivirus genus. This family includes two other genera, Flavivirus and Pestivirus and an unclassified virus GBV-B. They all share the same genomic organization and perhaps, have similar structural characteristics too. They are enveloped viruses, and their genome is composed of a single-stranded RNA of positive polarity.

1.1a *HCV genomic organization*: The HCV genome contains a long open reading frame (ORF) of approximately 9000 nucleotides (nt), flanked by untranslated regions at its 5′ and 3′ extremities (Choo *et al* 1989) (figure 1). The 5′-UTR is 341 nt long, has a complex structure (stem-loops and pseudoknots), and contains an internal ribosomal entry site (IRES) (Brown *et al* 1992) which mediates the cap-independent translation of the ORF. It also contains RNA elements implicated in the genome replication (Boyer and Haenni 1994). These sequences and structures are very conserved and interact with multiple cellular factors. The IRES contains four stem-loops which recruit translation initiation factors such as the eukaryotic initiation factor 3 (eIF3), the eIF2-GTP-initiator tRNA complex, the 40S ribosome subunit and other noncanonical factors, viz. La antigen and polypyrimidine-tract-binding-protein (PTB) (Ali and Siddiqui 1995, 1997; Pestova *et al* 1998; Sizova *et al* 1998; Kruger *et al* 2000; Shi and Lai 2001).

The 3′-UTR is 200 to 235 nt long and can be divided into three regions. First (from the 5′-end) is a region of variable sequence of length from 27 to 70 nt, followed by a poly-U/UC stretch, and finally a very conserved and structured 98 nt X-region at the end of the 3′-UTR (Kolykhalov *et al* 1996). The role of the variable region (VR) is not clear. It has been shown that VR is not required for viral replication in chimpanzees after intrahepatic inoculation with an HCV RNA transcript (Kolykhalov *et al* 1996; Yanagi *et al* 1999b). The poly-U/UC region, on the other hand, is essential for replication *in vivo*. It interacts *in vitro* with several cellular proteins (PTB, La antigen and GAPDH) which perhaps, regulate viral replication (Gontarek *et al* 1999; Luo 1999). The very conserved X-region interacts specifically with recombinant HCV RNA polymerase and PTB *in vitro*. It is also required for viral replication (Cheng *et al* 1999; Oh *et al* 2000).

The ORF encodes a long polyprotein of 3022 amino acids. Concomitantly with its translation, it is cleaved by cellular and viral proteases into ten different products (Major and Feinstone 1997) (figure 1). The N-terminal part encodes three to four structural proteins, and the rest are the non-structural proteins. Their organization and functions are described below.

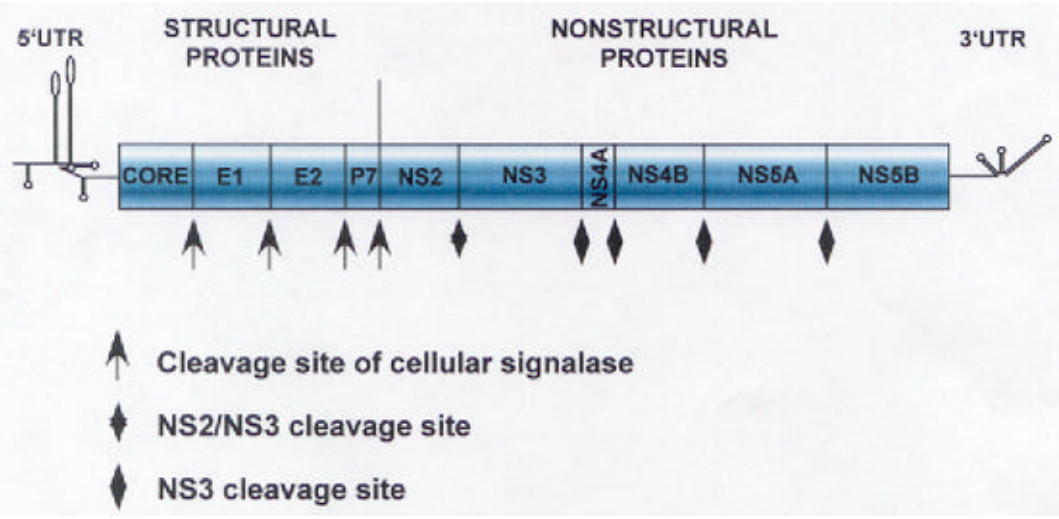

Figure 1. Genomic organization of HCV. The different types of cleavage sites of the polyprotein are indicated with different sets of arrows.

1.1b *HCV proteins and functions*: The first structural protein, from the N-terminus of the polyprotein, is the core protein. It constitutes the virion nucleocapsid and most likely interacts with the viral RNA (Baumert *et al* 1998). The full-length core protein has been shown to localize in the cytoplasm on the external membrane (cytoplasmic side) of the endoplasmic reticulum, but some of its truncated forms have been found in the nucleus (Santolini *et al* 1994; Suzuki *et al* 1995). Several cleavage products of the core protein have been identified in cell culture (Lo *et al* 1994; Hussy *et al* 1996; Liu *et al* 1997). The relevance of those different forms in natural infection has not been established. The core protein has been extensively studied and appears to play multiple roles in various cellular signalling pathways, and potentially in oncongenesis (Chang *et al* 1998; Lai and Ware 2000). It can also activate various promoters, as shown using reporter genes under the control of cellular promoters, such as c-myc and c-fos, or viral promoters (retrovirus LTR, HBV) (Ray *et al* 1995, 1997, 1998).

The next two proteins are the envelope glycoproteins E1 and E2. They are believed to associate as a non-covalent heterodimer (Deleersnyder *et al* 1997) and are exposed on the virion surface. E2 mediates viral binding to the cells, as shown by a decrease of infectivity by incubation of the virus with anti-E2 antibodies (Rosa *et al* 1996), but the HCV receptor has not yet been identified. Among the potential candidates, CD81 (Pileri *et al* 1998), a ubiquitous molecule, binds to E2 but does not mediate viral entry. The other candidate is the LDL receptor (LDLR) (Agnello *et al* 1999). Since circulating HCV particles in sera of the patients are associated with lipids and lipoproteins, the nonspecific uptake of the virus through the LDLR is possible. E1 and E2 contain both an ER retention signal, which limits their intracellular localization to the ER (Cocquerel *et al* 1998, 1999). Cell-surface expression of E1 an E2 is very limited, which may explain why the infected cells can escape from the immune recognition. Their ER localization strongly suggests that, as for other *Flaviviridae* members, HCV assembles at the ER membrane. Localization of the core protein near this structure is consistent with this hypothesis. E1 and core can interact which each other, suggesting that the viral nucleocapsid is enveloped through this interaction. Beside its structural role, E2 has been shown to modulate the IFN-α response (Taylor *et al* 1999). The next protein on the polyprotein is p7, which is a membrane-associated protein, but its precise role in viral structure or replication is not yet clear (Carrere-Kremer *et al* 2002).

The nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) have various functions involved in viral RNA replication or proteolytic processing of the polyprotein (De Francesco *et al* 2000). NS2 and NS3 are the two viral proteases responsible for the cleavage of all the NS proteins (Grakoui *et al* 1993; Hijikata *et al* 1993). Furthermore, NS3 has a helicase and an NTPase activity, suggesting that it plays a role in RNA replication as well (Tai *et al* 1996). NS4A is a cofactor of NS3, with which it forms a heterodimer (Failla *et al* 1994; Kim *et al* 1996). NS4B is an integral ER membrane protein. Its function is not yet known, but it may play a role in the anchorage of the replication complex to membrane – as observed for the replication of other RNA viruses (Hugle *et al* 2001). The role of NS5A in the HCV life cycle is also not known. This protein is phosphorylated (Koch and Bartenschlager 1999) and has been shown to interfere with the IFN response (Gale *et al* 1997). Recent studies of HCV RNA replicon have shown that many adaptive mutations that enhance viral replication are localized in NS5A, suggesting that it plays an important role in viral replication (Blight *et al* 2000; Lohmann *et al* 2001). Finally, NS5B is the viral RNA-dependent RNA polymerase (RdRp). It does not show a rigid template specificity *in vitro* but can copy a full-length HCV genomic RNA (Oh *et al* 1999). NS5B has a hydrophobic domain at its C-terminus, allowing its insertion into membrane (Yamashita *et al* 1998).

The viral life cycle is initiated by binding to a receptor, and after penetration or concomitantly with it, the viral RNA is uncoated and translated into the viral proteins by the cellular machinery. The viral RNA first replicates into the negative-strand RNA, followed by positive-strand RNA, using the NS proteins. The newly synthesized positive-strand RNA is encapsidated together with the core protein. Viral budding occurs most likely in the ER-Golgi compartments. It is not known whether the viral particles are secreted through vesicles or are released after cell lysis.

1.1c *Genetic heterogeneity and quasispecies*: As common to all RdRps, NS5B does not have a proofreading activity. As a result, HCV has a high mutation rate (10^{-5} error/nt) and a large genetic heterogeneity and quasispecies (Ogata *et al* 1991). Based on genomic sequence analysis, HCV is classified into at least six different genotypes, 1(a, b, c), 2(a, b, c), 3(a, b), 4a, 5a, 6a, and 52 subtypes (Simmonds *et al* 1993, 1994; Bukh and Miller 1994; Bukh *et al* 1995). Different HCV genotypes differ from one another by at least 30% overall, while the different subtypes within a genotype may vary from one another by more than 20%. Within a subtype, there is not more than 10% of sequence variation (Simmonds 1994). HCV sequences isolated from any single patient usually consist of heterogeneous population, termed the 'quasispecies'. The consensus viral sequence is the sequence with the most common nucleotide at each position (Forns *et al* 1999). These sequence variations are concentrated in the hypervariable regions of the genome, which though are not essential for viral replication, allow a certain degree of

plasticity. The best-characterized hypervariable region of HCV is located at the N-terminal part of the envelope protein E2 (hypervariable region 1, HVR1) (Weiner *et al* 1991, 1992; Kato *et al* 1992). Another HVR, HVR2, is located slightly downstream on E2. Quasispecies constitute a pool of viral variants that can change and acquire new selective advantages in a very short time. Thus, these new variants have adaptive advantages with a modified viral tropism, host range, virulence, and drug resistance – i.e. ability to escape from the host immune response. Indeed, HVR1 of HCV is a dominant epitope (Kato *et al* 1993; Cerny *et al* 1994) and its variation can impair both humoral and cellular immune response.

1.2 *HCV tropism*

As HCV is associated with hepatitis, liver is considered to be its primary site of replication. Viral proteins and RNA replicative intermediates (negative-strand RNA) can be detected in hepatocytes of infected patients. Furthermore, HCV can infect and replicate, at low efficiency, in primary human hepatocytes *in vitro* (Fournier *et al* 1998). More recently, the use of selectable replicon has confirmed that a hepatoma cell line (Huh-7) can support HCV RNA replication (Lohmann *et al* 1999).

The possibility that HCV infects other cells than hepatocytes as well came from the observation that transplanted livers in HCV patients are usually very rapidly re-infected by HCV (Araya *et al* 1997; Garcia-Retortillo *et al* 2002). This observation suggests the presence of other potential reservoirs for HCV. Several studies have shown that HCV can replicate in the peripheral blood mononuclear cells (PBMC) (Lerat *et al* 1996; Bronowicki *et al* 1998). It has been shown that the pattern of circulating virus and the virus in the liver are different from those in the PBMC, suggesting that PBMC are an independent site of viral multiplication. Furthermore, HCV can infect and replicate in PBMC *in vitro*, at least to a low level (Mizutani *et al* 1996; Nakajima *et al* 1996). It is possible that some of the HCVs may have a modified tropism replicating perhaps more efficiently in monocyte/macrophage or lymphocytes. Consistent with this hypothesis, Laksus *et al* (2002) have shown that after liver transplantation, the liver graft is colonized primarily by liver-derived virus remaining in the circulation but not by those found in PBMC of the recipient, suggesting that the latter might have been adapted to PBMC and infect hepatocytes less efficiently.

B cell infection by HCV has been shown by detection of HCV negative-strand RNA in PBMC of HCV patients and *in vitro* infection of lymphocytes. Furthermore, in our laboratory, a B cell line (SB) has been established from the spleen of an HCV-patient with non-Hodgkin's lymphoma. This cell line is persistently infected with HCV and produces virus particles, which can infect another lymphoblastoid cell line, such as Raji cells (Sung *et al* 2003).

Implication of HCV infection of the cells of immune system as a mechanism to impair immune response and facilitate viral persistence will be discussed in the later sections.

HCV replication *in vitro* is very limited. Several studies have attempted to establish tissue culture models, but none of them can support an active production of HCV virion. Human primary hepatocytes and hepatocyte-derived cell lines are permissive to HCV infection, but viral production is too low for biochemical and biological studies. RNA transcripts derived from the full-length HCV cDNA clones of several isolates, including 1a, a chimeric 1a-1b and 2a, are infectious upon intrahepatic injection into chimpanzees (Yanagi *et al* 1997, 1998, 1999a). However, the disease evolution in chimpanzees results in most cases in virus clearance and mild hepatitis (Lanford *et al* 2001).

Due to the absence of good animal or tissue culture models, most HCV studies have been carried out in stable cell lines or transiently transfected cell lines expressing one or several viral proteins. Transgenic mice have been engineered with one or several viral proteins under various promoters (Lai 2000). SCID/plasminogen activator transgenic mice carrying chimeric human liver have been developed recently (Mercer *et al* 2001). These mice can be infected by HCV and produce high virus titer. However, because of the immune deficiency, this mouse system is not suitable for pathogenesis studies.

More recently, a selectable subgenomic replicon has been constructed in R Bartenschlager's laboratory. This system allows RNA replication of HCV in Huh7 cells, with high-level expression of HCV RNA and proteins (Lohmann *et al* 1999). A full-length replicon has been constructed as well, but no virus particles are detected (Pietschmann *et al* 2002). This system allows the study of some aspects of HCV replication.

1.3 *Host immune response to viral infection*

1.3a *The innate immune system*: The innate immune system is the first response to all types of pathogens prior to the appearance of the adaptive or specific response. It involves natural killer (NK) cells, complement, cytokines and apoptosis (figure 2). The NK cells are cytolytic cells that use an antigen-independent mechanism. They are activated by low level of autologous major histocompatibility complex class I (MHC-I) molecules on the surface of infected cells. Some viruses inhibit MHC-I expression to limit the specific cell-mediated immune response, but it enhances NK cell function. NK cell activity is modula-

ted by other components of the innate immune response, such as α- or β-IFN (Paul 1999).

The complement is a component of the innate system as well as the specific immune system. It is composed of soluble molecules (C1q, C3b, etc.) that can interact directly with viruses, or with virus-antibody complexes, or with receptors on cells of the immune system. The complement-binding activates a cascade of proteases that leads to lysis or the activation of cells of immune response.

IFNs and other cytokines are induced after infection and regulate the various mechanisms that inhibit virus replication, cell proliferation and apoptosis via different signal transduction pathways. They play a role in both innate and specific immunity. IFNs are classified into two types: I and II. Type I (IFN-α and IFN-β) are produced in

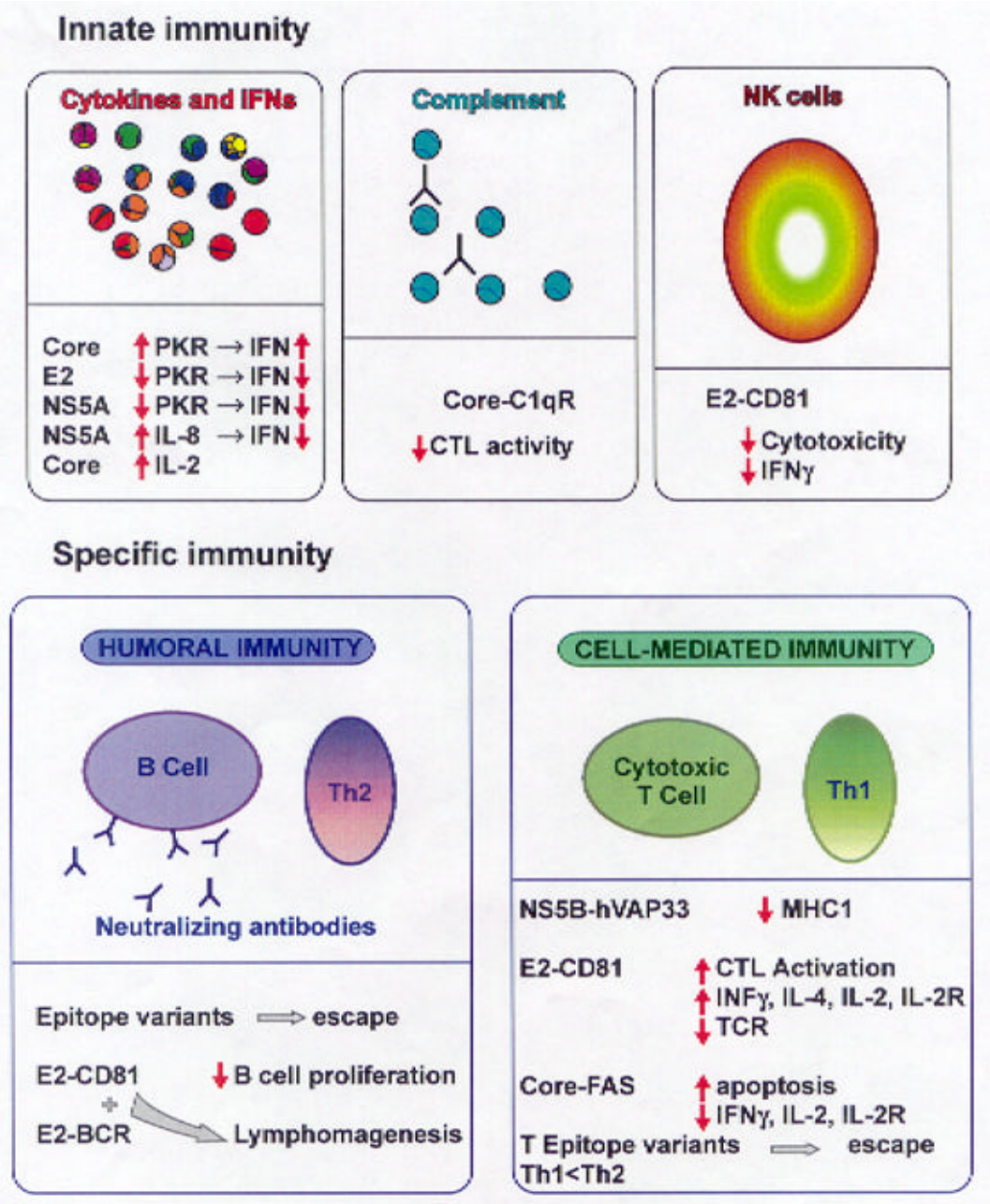

Figure 2. Interactions of HCV proteins with different effectors of the immune response. The effects of HCV proteins on the different components of the innate and specific immunity are summarized.

most cell types and are typically induced by double-stranded RNA, which is either synthesized in the course of many viral infections, or by other cytokines and growth factor, such as interleukins 1 and 2 (IL-1 and IL-2) and tumour necrosis factor (TNF). Type II IFN (IFN-γ) is synthesized mainly by T lymphocytes and is involved in the antigen-specific immune response. After binding of IFNs with their cognate receptors, a cascade of events occurs, which result in the activation of genes involved in the antiviral response such as the 2′,5′-oligoadenylate synthetase, MHC-I and the IFN-induced double-stranded RNA-activated protein kinase (PKR). PKR activation results in the phosphorylation of eIF-2α, which, in turn, inhibits translation, and other signals implicated in cell proliferation, transformation and apoptosis.

Other cytokines are also induced after viral infection; namely, TNF-α, TNF-β, IL-1, IL-2, IL-4, IL-5, IL-6, and IL-8 (Paul 1999).

Apoptosis is an innate defense to eliminate infected cells as well as a component of the specific immune response, involving the cytotoxic activity. Multiple mechanisms can lead to apoptosis: for example; some cell surface receptors (termed death receptors) such as Fas, TNF-R1 or CD40 can be activated by binding of their cognate ligands, leading to the initiation of the caspase cascade, which results in apoptosis (Paul 1999).

After HCV infection, the innate immune system is the first line of defense. Since acute HCV infection is silent in most cases, very few data are available on the acute phase in human. During the chronic phase, the complement and cytokine profiles vary with individual patients, depending on the presence or absence of IFN treatment. The complement and cytokine profiles also depend on the stage of the disease, the ethnicity of patients and the compartment studied (serum vs. liver) (Shapiro *et al* 1998; Biro *et al* 2000; Cotler *et al* 2001; Kimball *et al* 2001; Neuman *et al* 2001).

1.3b *Specific immune response*: The specific immune response is divided into two major types of effector: cellular effectors comprised of cytotoxic T lymphocytes (CTL), and humoral effectors comprised of antibodies secreted by activated B lymphocytes. In both the cases, recognition of a specific viral epitope is required. However, T and B epitopes are different: T epitopes are exclusively linear, whereas B epitopes can be linear or conformational. Both of them can target any of the viral proteins, but only some of the B epitopes which are localized on the viral envelope glycoproteins or outer viral capsid proteins, can induce the production of neutralizing antibodies that can inhibit the binding and entry of the virus. T cells recognize epitopes presented by MHC molecules; CTL requires epitope presentation by MHC-I molecules and helper T cells by class II. CTL response is responsible for the elimination of infected cells, and antibodies can bind free virus and mediate lysis of infected cell as well. CTL uses two major pathways to eliminate infected cells; the perforin-mediated cytolysis; or the Fas-mediated pathway. CTL also secretes cytokines, such as IFN-γ and TNF, which contribute to the control of viral infection by a non-cytolytic mechanism. Helper T cell (Th) secretes cytokines that are important for optimal responses of B cells and antibody production or CTL. Two types of Th are distinguished according to the type of cytokines they secrete. Th1, important for CTL activation, secretes IFN-γ, TNF-β and IL-2. Th2, important for B cell activation and secretion of antibodies, secretes IL-4, IL-5, IL-6 and IL-13. After activation by interaction with their specific antigen, B cells produce antibodies if they receive a second signal from Th2 cells. Immunoglobulin diversity is obtained by recombination of different genes (VDJ) and somatic mutations. Class switching (such as from IgM to IgG) involves different cytokines. The spleen is the first place where B cells start their maturation after antigen stimulation (Paul 1999).

Dendritic cells (DCs) are important for initiation of specific immune responses because of their competence to capture and present antigen to T cells. After antigen internalization, the DCs themselves undergo a process of maturation, migration, and relocation (Bell *et al* 1999). During maturation, DCs up-regulate MHC, adhesion, and co-stimulatory molecules. Mature DCs also secrete high levels of IL-12: a Th-1-polarizing cytokine that promotes the maturation of CTL.

During HCV infection, a robust CTL response is observed with a persistent intrahepatic Th1-associated cytokine production (IL-2, IFN-γ, TNF) (Cerny and Chisari 1999; Cucchiarini *et al* 2000; Valiante *et al* 2000). Although intrahepatic HCV-specific CTL are detected, they are in a very limited number (1% to 2%). This Th1 response is insufficient to clear the virus and is associated with a predominant nonspecific chronic inflammation, resulting in persistent liver injury (Napoli *et al* 1996; Chang *et al* 2001). A humoral response is observed as well, but does not lead to viral clearance.

2. Evasion of the immune response by quasispecies variation

HCV as a consequence of its sequence variability, is present as a pool of viruses presenting different epitopes. Modifications of both B and T epitope patterns during HCV infection have been observed and could contribute to HCV evasion from the immune system (Cerny and Chisari 1994; Cerny *et al* 1994; Weiner *et al* 1992, 1995).

2.1 *Variation of the neutralizing epitopes*

One possible way to escape the humoral response is to have a large diversity of epitopes that can not 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 (Farci *et al* 1997). Neutralization-escape variants have been isolated during HCV infection (Weiner *et al* 1992). 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 (Kumar *et al* 1994; Booth *et al* 1998). In HCV-infected patients, antibodies are produced early after infection. Farci *et al* (2000) reported that the pattern of quasispecies (in HVR1) during the acute phase predicts the outcome of the infection. If the quasispecies pattern is limited, infection is circumvented and the virus is eliminated. However, if the quasispecies pattern continues to evolve, persistent infection results. Anti-HVR1 antibodies, referred to as 'neutralizing of binding' (NOB) antibodies, are able to bind recombinant E2, HCV virus-like particles or bona fide viral particles. But, there is no definitive proof that they block viral entry (Farci *et al* 1996). A correlation has been observed between prolonged high NOB titers in patients and natural resolution of chronic hepatitis C (Ishii *et al* 1998), suggesting that they can play a role in viral clearance. However, lack of an efficient model of infection renders the validation of this hypothesis difficult.

These observations suggest that selection of viral variants that cannot be efficiently neutralized by anti-HVR1 antibodies probably contributes to the failure of elimination of HCV, leading to the establishment of a persistent infection.

2.2 *Variation of CTL epitopes*

As for B epitopes, there is a large variety of CTL epitopes on HCV proteins, including those in the HVR1 region. In resolving infection, a strong and durable CTL response targeting multiple epitopes is observed (Cucchiarini *et al* 2000; Lechner *et al* 2000). In chronic infection, a CTL response is observed as well, but there are fewer HCV-specific CTLs circulating in the peripheral blood (He *et al* 1999). CTLs are present in the liver of these patients, but they appear to be inefficient in eliminating infected cells. Studies on variation of CTL epitopes during infection have shown that the selection of viral variants early during infection may determine the outcome of the infection (Tsai *et al* 1998; Erickson *et al* 2001). HCV mutants that escape CTL recognition have been reported (Weiner *et al* 1995). Studies performed in infected patients as well as in a chimpanzee model have shown that CTL epitopes evolve during infection, confirming that there is a selective pressure against HCV quasispecies by the immune system. In both cases, an early selection of CTL escape mutants leads to chronic infection, whereas a narrow spectrum of CTL epitopes correlates with the clearance of infection (Tsai *et al* 1998; Erickson *et al* 2001). Another important phenomenon has been observed with some epitope mutants, i.e. they can function as T cell receptor antagonist and inhibit CTL activity (Tsai *et al* 1998).

As with B epitopes, HCV quasispecies imply variation of T cell epitopes during infection. An early selection of escape mutants seems to be a key event in the establishment of HCV persistent infection. In addition, a weaker CTL response (less circulating CTL or defect in CTL activity) has been observed in chronic infection.

The rapid selection of B and T epitope escape mutants early after infection probably results from selective pressure of the immune system of the host. It is not clear why, in some cases, selection of mutants is faster and leads to chronic infection, and, in others, it is not as effective and leads to elimination of the virus. It is not clear if the genotype, the viral load or the variety of quasispecies at the time of infection are important for the rapid selection of escape mutants.

Selection of escape mutants might not be the only way to escape from every possible antibodies or from all HCV-specific CTL, but if dominant epitopes are involved, it would probably contribute to the persistence of HCV (figure 2).

3. Implication of HCV proteins in the modulation of innate and specific functions

Since the selection of escape mutants to antibodies and HCV-specific CTLs does not fully explain why HCV persists with this very high frequency, many studies have been carried out to determine if viral proteins could be involved in the inhibition of cellular immune functions. Models using transient or stable expression of one or several viral proteins in different cell types as well as in transgenic mice expressing these proteins have been reported. These studies have shown that several viral proteins have potential effects on signalling pathways involved in immune response, cell proliferation or apoptosis (table 1). In this chapter, we focus on the interactions of HCV proteins with effectors of the innate and specific immune system.

3.1 *Effects of the core protein on cell signalling*

3.1a *Interaction with members of the tumour necrosis factor receptor family*: The TNF receptor family is involved in the immune system and particularly in the control of apoptosis. Studies using cell culture have shown that the core protein of HCV can interact with the cytoplasmic domain of the lymphotoxin β-receptor (LTβR), TNFR-1 (Chen *et al* 1997; Matsumoto *et al* 1997; Zhu *et al* 1998) and Fas (Hahn *et al* 2000). The interaction of core protein with LTβR occurs between the N-terminal part of core and the region of LTβR that interacts with its signalling adaptor TRAF-3 (figure 3). Thus, the core-LTβR interaction is expected to modulate the signal transduction that follows the interaction of LTβR with its ligand. Expression of core in HeLa cells increases sensitivity to apoptosis after stimulation of lymphotoxin-$\alpha\beta$ complex plus γ-interferon stimulation (Chen *et al* 1997). The core protein is also able to bind

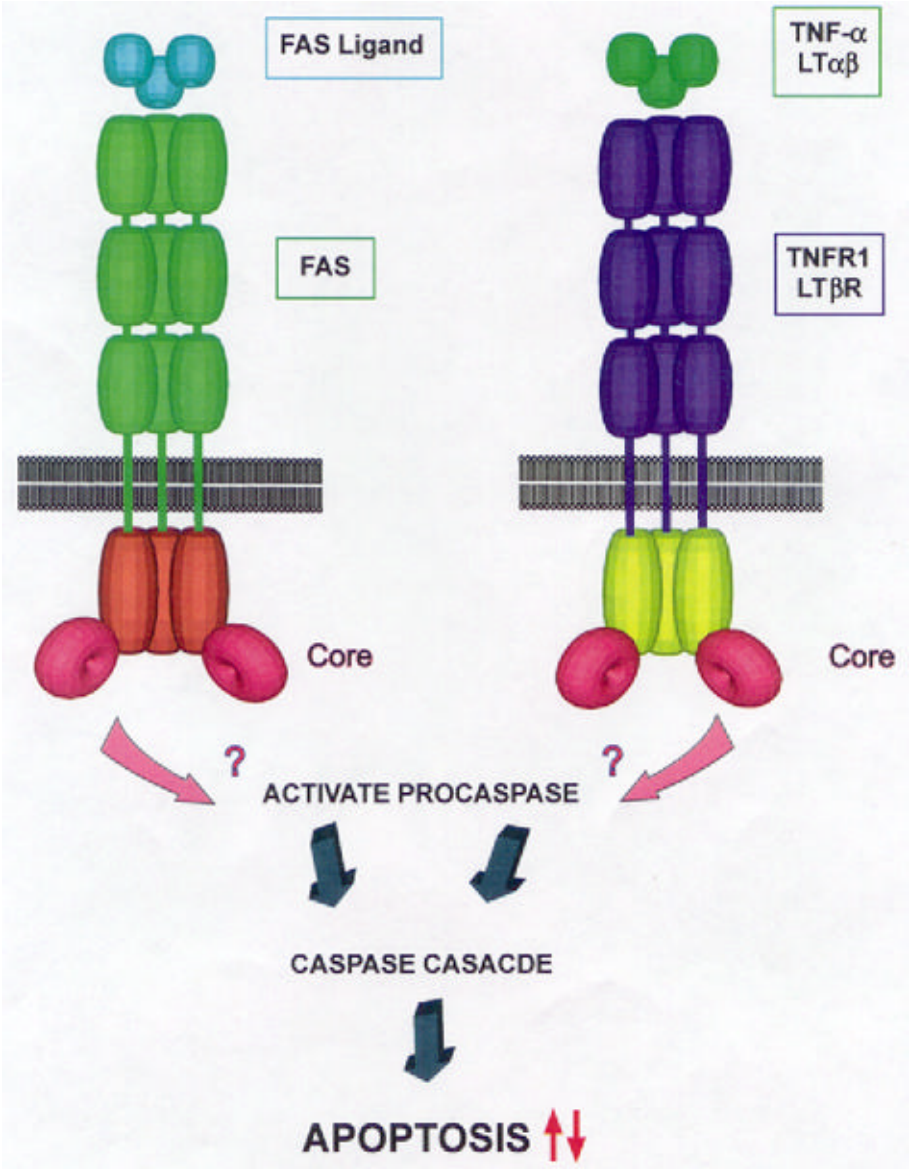

Figure 3. Interactions of the core protein with the TNFR family. The core protein binds to the cytoplasmic domain of FAS, TNFR1 and LTβR and alter their activation threshold. The final response varies with the cells.

the death domain (DD) of TNFR-1 (figure 3). This interaction reduces the binding between TNFR-1 and TRADD or TRAF-2, two molecules of the TNFR signalling pathway (Zhu *et al* 2001). Furthermore, using a mouse fibroblastic cell line, the TNF-induced NFκB activation is inhibited by the core protein (Zhu *et al* 1998).

These results support the hypothesis that the core protein may have a pro-apoptotic activity by affecting the TNFR signalling pathway. However, these findings are not always observed in every cell types or with all HCV core isolates (Ray *et al* 1996; Hahn *et al* 2000). Several studies, in fact, have shown that the core protein reduces sensitivity to TNF and activates NFκB, thus inhibiting apoptosis either constitutively or in response to cytokines (Marusawa *et al* 1999). These conflicting reports suggest that other, possibly cell-type specific, factors might be involved.

Modulation of Fas-mediated apoptosis by the core protein has been described in HepG2 cells (Ruggieri *et al* 1997) and Jurkat cell (Hahn *et al* 2000). In those studies, transient and/or stable expression of the core protein increases sensitivity to Fas-mediated apoptosis. The core protein binds to the cytoplasmic domain of Fas *in vitro*, and upon Fas/FasL engagement, a significant increase in caspase 3 activation is observed (Hahn *et al* 2000). Since HCV can infect T cells, decrease of apoptosis threshold by core may impair their activation and cytotoxic functions. Correspondingly, a suppression of the host immune responses has been observed in a murine model using recombinant vaccinia virus or Sindbis virus expressing HCV core protein (Large *et al* 1999). In this study, expression of the core protein increases viral titer, reduces CTL activity and reduces the level of IL-2 and IFN-γ production. These *in vivo* data confirm the modulatory effect of core on the immune system. In another cell culture study, the core protein has been shown to bind to a complement receptor (C1qR) on T cells; suppressing T cell response, and IL-2 production and IL-2 receptor expression (Yao *et al* 2001) (figure 2).

In contrast, another *in vitro* study, performed in HeLa cells, has shown an opposite effect of core expression on Fas-mediated apoptosis. In that case, an inhibition of apoptosis was observed via activation of NFκB (Marusawa *et al* 1999; Watashi *et al* 2001). Furthermore, two recent studies in mice, using either a recombinant replication-deficient adenovirus expressing the core protein or transgenic mice for the core protein, have shown no immunomodulatory effects of the core protein on virus-induced cellular immunity (Sun *et al* 2001; Liu *et al* 2002). Again, divergent effects were observed, depending on cell type, mouse strain, vector or experimental conditions, suggesting that several factors need to be considered and conclusions should be made in reference to the model used.

Implication of core protein in modulating apoptosis can have several consequences on immune response. Since HCV can infect lymphocytes, increasing their sensitivity to apoptotic stimuli affects their activation and immune functions. In chronic infection, few HCV-specific CTLs are found in the peripheral blood, which could be due to an abnormal death of activated CTL. Therefore, LTβR and TNFR1 signalling is important for the microenvironment that allows interactions of lymphocytes with antigen-presenting cells, and for B cell migration and differentiation into antibody-producing cell. Impairment of these functions may interfere with the elimination of infected cells and neutralization of virus. Thus, the HCV core protein appears to play a key role in immunomodulation and is thus one of the factors that contribute to HCV persistent infection.

3.1b *Modulation of the IFN pathway*: More recently, another potential role of the core protein has been proposed in the PKR-induced apoptosis (Delhem *et al* 2001). As mentioned earlier, PKR is induced by IFN and activated by double-stranded RNA. Activation of PKR requires its dimerization and autophosphorylation. Upon activation, PKR phosphorylates several substrates, including eIf2-α, and the inhibitor of NFκB (IκB), which lead to inhibition of translation, anti-proliferative effects and apoptosis. It has been shown that the recombinant core protein derived from HCV sequences isolated from hepatocellular carcinoma can bind and activate PKR *in vitro*. The increase of apoptosis is more pronounced with core proteins isolated from the tumour than from the non-tumour part. The increase of apoptosis is also more significant after co-stimulation with TNF-α and IFN-α – which induces PKR – than with TNF-α alone, in the presence of the core protein derived from tumour (Delhem *et al* 2001). Thus, core protein variants selected during persistent infection may have acquired new property that enhances the pro-apoptotic activity of PKR (figure 4). Promoting apoptosis could prevent viral persistence. However, depending on the cell types, particularly in immune cells, the cell death may actually favour viral persistence. Furthermore, it could induce selection of the cells resistant to apoptosis, leading to cellular transformation and carcinogenesis.

The core protein also activates multiple cellular and viral promoters. Thus, many immune-related genes or components of signalling pathways could be altered at the gene expression level by the core protein. For example, IL-2 production from T cells is enhanced by core protein (Bergqvist and Rice 2001). Also, core protein can activate 2′-5′-oligoadenylate synthetase gene, which is an IFN-inducible gene (Naganuma *et al* 2000).

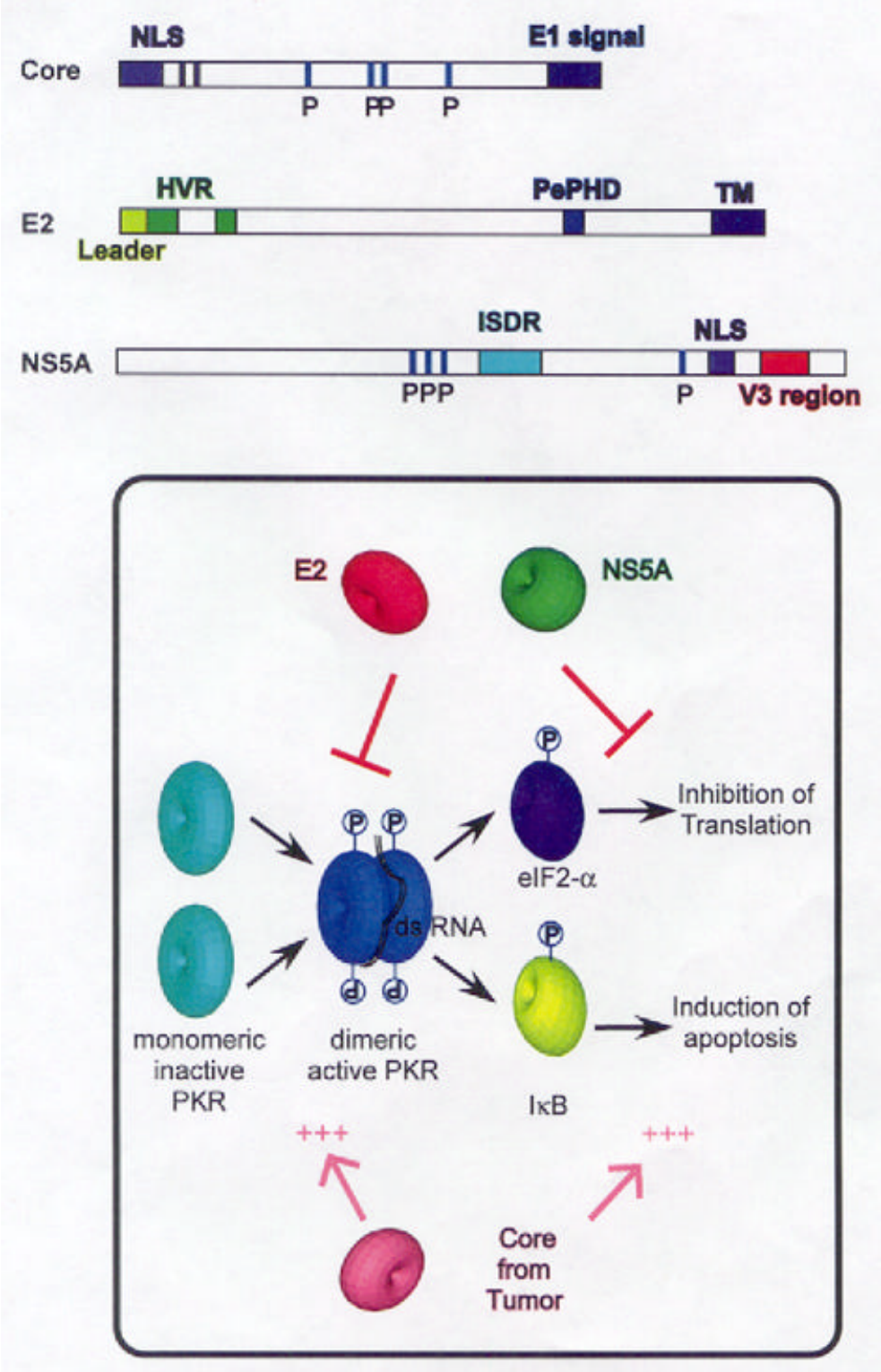

Figure 4. Structure of the core protein, E2 and NS5A and their effects of PKR cascade. E2 and NS5A inhibit PKR activity and the PKR-induced translation shut-off. Core protein isolated from tumour increases PKR phosphoryation and its pro-apoptotic activity. Abbreviations are as follows: For core: P, phosphorylation site; NLS, nuclear localization signal; E1, signal sequence. For E2, Leader: signal sequence, HVR, hypervariable region; PePHD, PKR-eIF2α phosphorylation domain; TM, transmembrane domain. For NS5A: P, phosphorylation site; ISDR, IFN-sensitivity-determining region; NLS, nuclear localization signal; V3, variable region.

3.2 *Effect of the envelope glycoprotein E2 on the IFN response*

The glycoprotein E2 of HCV contains a stretch of amino acids that share a high degree of sequence homology with the autophosphorylation sites of PKR and the phosphorylation site of its substrate eIF2α, the so-called PKR-eIF2α phosphorylation domain (PePHD) (Taylor *et al* 1999). E2 is able to inhibit PKR activation *in vitro* in a PePHD-dependent manner. This inhibition blocks the phosphorylation of eIF2α, and prevents translation shut-off mediated by IFN (figure 4). Since E2 itself is not phosphorylated, it probably serves as a pseudo-substrate of PKR. E2 is mostly glycosylated and located in the ER, but an unglycosylated form of E2 is localized in the cytoplasm and it is this form that interacts with PKR in mammalian cells (Pavio *et al* 2002). The PePHD sequence is different between the viral genotypes, and the degree of inhibition of PKR activation by various E2 sequences correlates with the degree of resistance of the different genotypes to IFN therapy. Several studies have looked for this correlation in patients and have either confirmed or invalidated this observation (Lo and Lin 2001; Puig-Basagoiti *et al* 2001). It is most likely that the E2-PKR interaction could only explain the differences in the interferon sensitivity between different viral genotypes but does not explain the differences between isolates within the same genotype.

E2 has been shown to induce an ER stress response (Liberman *et al* 1999), which may affect the expression of cell surface molecules.

Table 1. Summary of accessory functions of HCV proteins.

HCV proteins		Accessory functions
CORE	↑↓	TNFR, Gene expression
	↑	PKR, NFκB, Steatosis, Oncogenesis
	↓	CTL
E2	↓	PKR
	↑	Stress response
	↑	CD81 stimulation
NS3	↑	PKA, PKC
	↑	Oncogenesis
NS4B	↑	NFκB
	↑	Oncogenesis
NS5A	↓	PKR
	↓	Growth regulation (Grb2)
	↑	Steatosis
	↑↓	Oncogenesis
NS5B	↓	Vesicle transport

For details, see text and Sakamuro *et al* (1995), Ray *et al* (1996), Barba *et al* (1997), Borowski *et al* (1997), Gale *et al* (1999), Ghosh *et al* (1999), Tau *et al* (1999), Kato *et al* (2000), Park *et al* (2000), Arina *et al* (2001) and Shi *et al* (2002).

3.3 *Effects of the NS5A protein on IFN response and IL-8 level*

NS5A contains a region associated with IFN resistance: the IFN-sensitivity-determining region (ISDR) localized in its C-terminal part (figure 4). Mutations in this region confer sensitivity to IFN therapy (Enomoto *et al* 1996; Castelain *et al* 2002; Schiappa *et al* 2002). This correlation is mainly observed among Japanese HCV patients, but failed to be confirmed in other populations (Gerotto *et al* 2000). Since the diverse studies have been done using various cohorts of patients and using different methods, it is difficult to reach a consensus conclusion. Viral genotypes, dose of IFN used, length of the treatment and time after infection, are important parameters that need to be considered for the validation of those studies. The recent use of replicon does not support the role of NS5A (genotype 1b) in the resistance to IFN (Guo *et al* 2001). Since the role of NS5A in HCV replication is not known, it is difficult to determine why mutations in this region may confer a disadvantage to the virus during IFN therapy. Interestingly, adaptive mutations around the ISDR often contribute to the increase of replication efficiency of the HCV replicons.

The NS5A protein from IFN-resistant strains of genotypes 1a and 1b has been reported to inhibit PKR through a direct interaction between the C-terminal part of NS5A, near the ISDR, and the central part of PKR (Gale *et al* 1997). This interaction is believed to be responsible for the anti-IFN response exerted by NS5A. Again, these results are controversial depending on the experimental models used, virus-rescue experiments, transient or stable transformant, the viral genotype, etc. (Podevin *et al* 2001).

More recently, NS5A was found to induce production of IL-8, which, in turn, partially inhibits the IFN-induced antiviral response *in vitro* (Polyak *et al* 2001a). Furthermore, a study of hepatitis C patients has shown an elevation of IL-8 in their serum in association with the resistance to interferon therapy (Polyak *et al* 2001b), suggesting that NS5A has several potential ways to interfere with the immune response.

3.4 *Effects of the NS5B protein on cell surface expression of cellular proteins*

NS5B is believed to form a membrane-associated RNA replication complex with the other NS proteins. NS5B has a membrane-anchorage domain and is found in the ER or an ER-like modified compartment (Schmidt-Mende *et al* 2001). The membrane localization of NS5B allows its interaction with cellular proteins of the vesicle transport. NS5B interacts with a SNARE-like protein [soluble (N-ethylmaleimide-sensitive fusion protein) attachment

protein receptor] named hVAP33 (Tu *et al* 1999) and down-regulates the surface expression of cell surface proteins such as MHC-I (unpublished results). Thus, reduction of MHC-I on infected cells may prevent the elimination of the infected cells by CTLs. This property of NS5B may contribute to the inefficient CTL response and the persistence of HCV (figure 2).

In conclusion, several HCV proteins possess properties that can directly or indirectly affect the immune response. By interfering with the IFN pathway, E2 and NS5A of HCV genotypes 1a and 1b contribute to the viral escape from the innate immune response, and to the resistance to IFN therapy. Decrease of cell surface expression of molecules, such as MHC-I, by NS5B may contribute to the viral escape from the CTL response. This observation is also consistent with a high degree of infiltration of activated NK cells in the liver of chronically infected patients (Valiante *et al* 2000).

4. Impairment of immune functions during HCV infection

HCV can infect PBMC, in particular, B and T cells. Infection of these cells may lead to several dysfunctions.

4.1 *B cell-related pathologies associated with HCV infection*

Long-term infection with HCV is associated with immune-mediated pathologies, such as type II mixed cryoglobulinemia (MC), production of autoantibodies, the appearance of rheumatoid factors or development of B-cell non-Hodgkin's lymphomas (B-NHL).

In HCV patients, circulating immune complexes of HCV and anti-HCV antibodies with cryoprecipitating properties cause type II MC, which is associated with polyclonal or monoclonal B cell expansion. Approximately one-third of HCV-infected patients have this pathology. Although it is considered a non-neoplastic disorder, it may evolve into lymphoma in some patients (Pozzato *et al* 1994). Several studies have demonstrated a high prevalence of chronic HCV infection among patients with B-NHL (Ferri *et al* 1994; Pioltelli *et al* 1996; Zuckerman *et al* 1997). A strong expression of the anti-apoptotic bcl-2 oncogene, and a high frequency of reciprocal t(14;18) translocation, have been found in B cells from HCV patients with type II MC (Monteverde *et al* 1997; Kitay-Cohen *et al* 1999; Zignego *et al* 2000; Zuckerman *et al* 2001). In addition, a high rate of B cell oligo clonality, as detected by re-arrangement of immunoglobulin heavy chain, occurs in HCV-infected patients even in the absence of cryoglobulinemia. Thus, clonal proliferation and inhibition of apoptosis of B lymphocytes may play an important role during the multi-step process of lymphomagenesis. The role of HCV in this process has been further demonstrated by the isolation of B-NHL cells, whose B cell receptor is able to bind E2 (Quinn *et al* 2001). The authors proposed that chronic antigen stimulation in conjunction with activation though the interaction between E2 and CD81 is responsible for the lymphomagenesis. Another cell culture study has shown that interaction of E2 with CD81 on Daudi cell, a B cell line, leads to an antiproliferative effect (Flint *et al* 1999). These studies suggest that HCV can affect the immune functions by multiple ways.

4.2 *Impairment of natural killer cells functions*

Interaction of virus particle with cell surface molecules, such as CD81, may modulate cell signalling. CD81 is widely expressed and is found on NK cells. Two independent studies have shown that the cross-linking of CD81 by immobilized E2 inhibits non-MHC-restricted cytotoxicity mediated by NK cells and also IFN-γ production by NK cells following exposure to IL-2, IL-12, IL-15, or CD16 cross-linking (Crotta *et al* 2002; Tseng and Klimpel 2002). Inhibition of IFN-γ production by NK cells could alter the development of a Th1 response and favour a Th2. An imbalance in the ratio of Th1/Th2 has been shown in infected patients (Sarih *et al* 2000; Valiante *et al* 2000) (figure 2).

Inhibition of the innate immune response early after infection could confer a growth advantage to HCV that could not be controlled by the adaptive immune response. The inefficient NK cell response could allow the selection of escape variants.

4.3 *Impairment of T cell functions*

On T cells, CD81 forms a complex with several different molecules, including CD4, CD8, $\alpha 4\beta 1$ integrin, and CD82. In a recent study, using recombinant E2 as a ligand in an *in vitro* assay, it has been shown that CD81 cross-linked by E2 lowers the activation threshold of T cell (Wack *et al* 2001). E2-induced co-stimulation lowers the threshold for IL-2 receptor-α expression and IL-2 production, resulting in an increase of T cell proliferation. It also enhances the production of IFN-γ and IL-4 and causes increased T cell receptor (TCR) down-regulation (figure 2). These results suggest that, during HCV infection, even suboptimal stimuli could activate T cell and thus contribute to liver damage or autoimmune disorders associated with HCV infection.

4.4 *Impairment of dendritic cell functions*

Several viruses, such as herpes simplex virus, measles virus, and Epstein-Barr virus, have been shown to diminish DC function. It is only recently that HCV has also

been shown to affect the function of DCs. Compared to monocyte-derived DCs from healthy donors, DCs from patients with chronic HCV infection showed an impaired ability to stimulate allogeneic T cells and to produce interferon (Kanto *et al* 1999; Bain *et al* 2001). An independent study also demonstrated impaired stimulatory capacity of DCs derived from HCV-infected patients with hepatocellular carcinoma (Kakumu *et al* 2000). This impaired maturation of DCs has been correlated with persistent HCV infection (Auffermann-Gretzinger *et al* 2001). Furthermore, in patients who have cleared the virus, DC maturation is normal. It is not known yet if the virus infects DCs directly.

5. Conclusions and future directions

In conclusion, HCV has developed several strategies to counteract the immune system of the host. Combination of these different strategies probably allows HCV to establish persistence at a high frequency. HCV quasispecies play an important role in the selection of escape mutants that are not recognized by the immune system. Furthermore, viral proteins can interact with effectors of various signalling pathways involved in cell proliferation, apoptosis or transformation. These interactions can occur in the infected cells or on cell surface with circulating virus particles. Intracellular interactions have been demonstrated between the viral NS5A, E2 or core proteins with PKR, between core and several members of the TNFR family and between NS5A, NS5B and hVAP-33, a protein of the cellular transport. These interactions result in the modulation of apoptosis pathways, interference with the IFN-α pathway, or with the cellular secretion pathway and cell surface expression of MHC-I molecule. Modulation of cellular functions can also involve interaction of circulating virus particles with cell surface receptors, such as interaction between E2 and CD81 on NK cells or T cells, and sensitize these cells to apoptotic stimuli. These strategies impair both innate and specific immune responses.

Once HCV has established a persistent infection, treatment of this infection is compromized. Therefore, prevention by vaccination should be the option of choice. Unfortunately, HCV escape mutants and the various existing genotypes render the development of a protective vaccine difficult.

In summary, HCV, as an emerging virus, has evolved in such a way that it has developed many strategies to escape our immune system, resulting, first, in the difficulty to identify it and, second, in making it difficult to cure.

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