Innate immunity and hepatitis C virus: eluding the host cell defense

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1. ABSTRACT

Interferon-alpha (IFN-alpha) mono-therapy is largely ineffective for most of the hepatitis C virus (HCV)-infected patients that receive it. The addition of ribavirin to IFN therapy has increased the response rate dramatically. While many factors are implicated in determining the success rate for IFN therapy, viral genotype seems to play a crucial role. Examining differences in viral gene sequences has and will continue to advance our understanding as to how HCV and other viruses circumvent the IFN response. Here we review the different ways that HCV evades the immune response elicited by IFN.

2. INTRODUCTION

A cascade of events leads to expression of cellular genes as part of the immune response to viral infection. Triggers, such as the intracellular presence of double-stranded (ds) RNA, initiate the Type I interferon (IFN) pathways and induce the synthesis of IFN-beta, leading to the production of interferon stimulated genes (ISGs), including IFN-alpha and beta and pro-inflammatory cytokines, needed to establish cell-mediated immunity (Figure 1).

Toll-like receptors (TLRs) are the first line of defense for the cell [for review see (1)]. These molecules
Figure 1. Interferon signaling. Type I, II and III IFNs bind to their cognate receptor and then recruit kinases leading to movement of phosphorylated transcription factors to the nucleus where binding to the enhancer region of the IFN promoter occurs. Type I and III IFNs lead to the activation and nuclear transport of the IFN-stimulated gene factor 3 (ISGF3) and induction of the IFN-stimulated response element (ISRE) to produce IFN-stimulated genes (ISGs). Type II IFN leads to activation of the gamma activated sequence (GAS) through GAS-element binding by the STAT1 homodimer and subsequent induction of the IFN gamma-activated genes (IAGs).
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Figure 2. Interferon-beta is the gate-keeper of innate immunity. dsRNA and viruses activate the toll-like receptor 3, (TLR3) leading to the induction of the IFN-beta promoter. IFN-beta then is translated in the cytoplasm, secreted from the cell and binds to Type I IFN receptors on nearby cells. This leads to the activation of Type I receptors and the induction of IFN-alpha and IFN-stimulated genes (ISGs), thus amplifying the pathway.

antigen presentation to the immune system (13). The well-studied dsRNA-activated protein kinase, PKR, has many cellular roles including proapoptotic functions, growth control and differentiation activity in addition to its antiviral role as a translational inhibitor.

3.1. The IFN-induced dsRNA-activated protein kinase, PKR
PKR is found in most mammalian cells and tissues at low levels in an inactive state. The active form of PKR is a key regulator of the host antiviral response. IFN triggers the synthesis of PKR mRNA. dsRNA (produced during viral replication and transcription), binds to PKR and activates the kinase by inducing autophosphorylation [Figure 3; (14)]. Active PKR phosphorylates its substrate, eukaryotic initiation factor 2 (eIF2), on serine 51 of its alpha subunit (15). eIF2-alpha phosphorylation entraps the guanosine nucleotide exchange factor (eIF2B) and all cellular and viral translation then ceases (16). In addition to its antiviral functions, PKR has several cellular functions. PKR exhibits anti-proliferative actions that result in apoptosis, tumor suppression, and growth control [for review, see (17-18)]. Several viruses contain genes that overcome the effects of PKR. Adenoviruses encode a small partially dsRNA, termed adenovirus-associated RNA1 (VA RNA1). This RNA is highly structured and binds to PKR in a manner that prevents activation of the enzyme, thereby permitting translation to proceed [for review, see (19)]. Other viruses, such as vaccinia, encode proteins that sequester viral dsRNA, thus blocking activation of the kinase. Vaccinia virus encodes a second protein that mimics the eIF2-alpha substrate recognition sequence, thus, inhibiting PKR activity [for review, see (20)].

3.2. Adenosine deaminase that acts on dsRNA, ADAR1
Adenosine deaminase that acts on dsRNA (ADAR1) is also an IFN induced protein and catalyzes the deamination of adenosine residues in dsRNA [for review, see (21)], resulting in inosine substitution (Figure 3). Inosine residues are not abundantly found in cellular mRNAs, but when present are transcribed and translated as guanosine residues, which may lead to mutations (22). An RNase that specifically degrades inosine-containing RNA has been described and was proposed to be part of a putative antiviral pathway (23). An IFN-induced RNase, ISG20, has also been suggested to play a role in the antiviral activity that has been attributed to ADAR1 (24). The cytidine deaminases, known as apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3 (APOBEC3), are also inducible by IFN and may limit hepatitis B and human immunodeficiency viruses (25, 26).

4. MOLECULAR BIOLOGY OF HEPATITIS C VIRUS, HCV
HCV is a member of the Flaviviridae family and the sole member of the hepaciviruses. Variability in the genome subdivides the virus into 6 major genotypes (27).
Figure 3. dsRNA-mediated IFN-induced antiviral pathways. Virus infection leads to the synthesis of dsRNA that activates three major IFN-induced antiviral genes. The protein kinase that is activated by dsRNA (PKR) is activated by autophosphorylation after binding to dsRNA. Activated PKR then phosphorylates the alpha subunit of eIF2, which entraps eIF2B and blocking translation. dsRNA activates the 2', 5' oligoadenylate synthetase (2',5'OAS) to make 2',5' oligoadenylates (2',5'A) that activate the ribonuclease (RNaseL). The IFN-induced adenosine deaminase that acts on dsRNA (ADAR1) leads to RNA editing of adenosine monophosphates (AMP) in dsRNA. The deaminase results in inosine monophosphate (IMP) integrated into viral RNA. This leads to viral mutations and RNA degradation.

Figure 4. HCV genome. HCV proteins are translated from a ~9.5 KB RNA driven by an internal ribosome entry site (IRES). The genome encodes a polyprotein that contains structural genes in the amino terminus and nonstructural (NS) genes in the carboxyl region. The Core has been shown to modulate the effects of PKR. The E2 envelope protein has been shown to inhibit PKR and PERK kinases and to be involved in immune escape. NS3 and NS4A inhibit the RIG-I and TLR3 pathways to IFN-beta synthesis. NS5A inhibits both PKR and ERK1/2 pathways.

The viral genome is a single-stranded, positive-sense RNA of approximately 9.5 kilobases encoding a large polyprotein that is processed into structural proteins (core and envelope proteins E1 and E2) and nonstructural (NS) proteins (p7, NS2, NS3, NS4A and B and NS5A and B) [Figure 4, (28)]. The p7 protein is thought to multimerize and form ion channels in the cell with importance for infectivity (29-31). The structural proteins are processed cotranslationally by cellular proteases whereas the nonstructural proteins are processed by the viral proteases NS2 and NS3 [for review, see (32)]. NS4A is a cofactor for NS3 and is required for its proteolytic cleavage activity (33). The functions of NS4B and NS5A are not yet fully understood. In addition to its protease domain, NS3 contains a helicase domain and is required for replication. NS5B serves as the viral RNA-dependent RNA polymerase.
5.1. NS3/4A and evasion of IFN-beta induction

Innate immunity is initially triggered by the binding of viruses or viral dsRNA to Toll-like receptor 3 (TLR3) or to intracellular helicases, such as the retinoic acid inducible gene, RIG-I (38). This leads to a cascade of events resulting in the induction of IFN-beta and pro-inflammatory cytokines [Figure 2; for review, see (39)]. TLR3 requires an adaptor protein, TIR-domain-containing adapter-inducing interferon-β (TRIF), while RIG-I needs an adaptor known as CARDIF (also named MAVS, IPS-1 or VISA). The NS3/4a protease has been found to cleave each of these proteins, leading to reduced expression of inflammatory cytokines and IFN-beta (Figure 4). While the in vitro effects suggest that innate immunity should be impaired by the presence of these HCV proteins, the clinical significance is yet to be elucidated. Additionally, the 3' end of HCV has been shown to activate RIG-I (40), complicating the issue even further.

5.2. NS5A and IFN resistance

Much attention has been focused on the nonstructural 5A (NS5A) gene of HCV and its potential role in IFN-alpha resistance (Figure 4). The presence of an IFN-sensitivity determining region (ISDR) in NS5A was first reported by Enomoto and coworkers (37) wherein four or more amino acid mutations were observed in viruses from patients that responded to IFN therapy. Many groups outside of Japan have not seen this correlation [for review, see 41]. Consequently, the presence and importance of this region is still under debate. The Katze laboratory first reported that the ISDR and the region carboxy-terminal to it were important for PKR binding and inhibition (42, 43). Further work in the Katze lab by Tan and coworkers (44) demonstrated that stably transfected NS5A bound to growth factor receptor-bound protein 2 (Grb2) and resulted in decreased extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) phosphorylation. Grb2 binds to the proline-rich sequences in the C-terminal region of NS5A in a Src-homology 3 (SH3) ligand-dependent manner. During viral infection or growth factor treatment, Grb2 mediates signaling from the plasma membrane to ERK1/2. When ERK1/2 are activated they phosphorylate the signal transducers of activated transcription (STATs), which leads to IFN induction (45). Alteration in ERK phosphorylation by NS5A may trigger down regulation of IFN gene expression.

A region of variability in NS5A (which has, for the most part, been overlooked), termed V3, was found by Duverlie and coworkers (46). The V3 region contains mutations in IFN-alpha-sensitive strains of HCV. Sequences in the V3 region are much more conserved in IFN-alpha-resistant strains, suggesting that this portion of NS5A plays a role in IFN sensitivity, but to date, no mechanism has been proposed.

Podevin et al. (47) engineered stable hepatocytic cell lines expressing NS5A proteins to examine its role in IFN resistance. They used NS5A genes, obtained from a patient that responded to IFN therapy and two nonresponders, and examined NS5A’s role in conferring IFN resistance to EMCV and VSV (IFN-sensitive viruses). NS5A from the responder (R1; which should be IFN-sensitive) and one of the nonresponders (NR2) had wild-type ISDR sequences. However, the ISDR sequence from another nonresponder (NR1) contained 8 amino acid mutations. They found that NS5A from NR1 rescued VSV and EMCV replication in the presence of IFN-alpha. Surprisingly, cell lines made from NR2 and R1, also rescued viral replication, albeit to a lesser extent. This study demonstrated that NS5A plays a role in evading the IFN response, but independently of the ISDR since the number of mutations in this region did not affect its ability to confer IFN resistance. Polyak and coworkers (48) showed that a deletion mutant, lacking the ISDR, rescued VSV replication as competently as wild-type NS5A from the 1a and 1b genotypes, suggesting that the ISDR is not required for NS5A to confer IFN resistance. Furthermore, they observed by confocal microscopy that while PKR and NS5A are both located in the perinuclear compartment of the cell, they do not appear to colocalize. The authors also showed that NS5A does not co-immunoprecipitate with PKR and that PKR is fully IFN inducible and activatable by poly-inosinic acid:poly-cytidylic acid (polyI:polyC) treatment of these NS5A-expressing cell lines. The level of
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phosphorylation of eIF2-alpha remained the same for NS5A-expressing cells as for control cells, indicating that in Huh7 hepatoma cells PKR is not inhibited even by high concentrations of NS5A. The Podevin study was the first in which PKR inhibition by NS5A was examined in a liver cell line, while previous reports of PKR inhibition by NS5A have used yeast Saccharomyces cerevisiae (43, 49) and NIH 3T3 cells (50).

5.3. E2 and IFN resistance

E2 is the outer protein of the viral envelope and most probably participates in virus binding to the target cells (51). It is the most variable region of the entire viral genome among different HCV isolates and this variability may result in resistance to IFN-alpha therapy (Figure 4). The variability of E2 may allow the virus to escape from neutralizing antibodies (51, 52). Most of the sequence variability in E2 is located in two regions, termed hypervariable region 1 (HVR1) and HVR2 (53). Different quasispecies can be found in each patient. After IFN-alpha treatment, some quasispecies disappear, suggesting that sensitivity to IFN-alpha is different among quasispecies (54). Viruses from responders show low sequence complexity (55, 56) and little sequence diversity in the HVRs, whereas isolates from those patients that fail to respond to IFN show a high degree of heterogeneity in this region (36, 57, 58). The E2 envelope protein is retained at the endoplasmic reticulum (ER) membrane through a carboxy-terminal transmembrane domain. One study showed that those patients, who responded to IFN therapy, also had antibodies directed to the HVRs of E2 (59). Perhaps those patients that are infected with viruses that contain high sequence variability in the HVRs fail to mount an adequate humoral response to potentially important epitopes, and thus, viral titer increases beyond the range of IFN-alpha’s effectiveness.

E2 HVRs are characterized by nonsynonymous nucleotide substitutions that arise during IFN-alpha therapy (54). HCV isolated from patients who responded to IFN-alpha therapy showed little sequence diversity in the E2 HVRs, suggesting that these patients were infected with a relatively homogeneous pool of HCV variants (57). These findings suggest a crucial role for the E2 protein in the development of chronic hepatitis and that mutant forms of the E2 gene may allow the virus to escape the adaptive immune response.

HCV E2 also inhibits PKR and shares sequence homology with PKR and the PKR substrate, eIF2-α. E2 and PKR contain eight amino acids of identity, four of which are autophosphorylation sites in PKR (60). Adjacent to this PKR homology sequence is a site that contains four amino acids of identity with eIF2-α, including serine 51. This region is known as the PKR-eIF2α phosphorylation site homology domain (PePHD). These sequences are more homologous in the HCV-alpha-resistant genotypes (1a and 1b) than in corresponding sequences found in the IFN-alpha-sensitive genotypes (2 and 3). E2, from a relatively IFN-alpha-resistant genotype (HCV-1a), inhibits PKR activation in vitro, in yeast and in human cells (49). E2 has been shown to act as a pseudosubstrate and this interaction occurs before PKR is activated (60). E2 colocalizes with the PKR although it may be only an unglycosylated, cytoplasmic form of the envelope protein that interacts (61). These findings only explain the relative resistance of genotype 1 (vs. 2 and 3) to IFN-alpha and do not explain the differences in IFN resistance among individual isolates within each genotype. Thus, the E2 protein may help to enable HCV to evade the antiviral effects of IFN by modulating the activities of PKR and stimulating viral translation. A secondary effect of PKR inhibition may be stimulation of cell proliferation which could contribute to the development of HCV-associated hepatocellular carcinoma.

While NS5A has been shown to block the antiviral effects of IFN, sequence differences in the ISDR may not be responsible for these activities. However, NS5A from both IFN responders and nonresponders seem to display similar activities in vitro, thus raising more questions (47). This opens up the possibility that other viral genes or host factors are responsible for determining IFN resistance. We know that sequences within E2 from IFN-resistant genotypes confer IFN resistance through PKR inhibition but sequences in this region are very conserved within each genotype (49). Therefore, E2 also probably provides a basal level of IFN resistance, but only for the IFN-resistant genotypes.

5.4. The HCV IRES and evasion of IFN

Initiation of protein synthesis is mediated by the HCV IRES and is independent of the cap-binding proteins (eIF4E) but was thought to be dependent on the eIF2 ternary complex. The IRES has been shown to bind and inhibit PKR (62), thus enabling eIF2-dependent initiation. However, the HCV IRES can initiate translation whereby preformed 80S ribosomes can assemble independent of initiation factors (63). This suggests that upregulation of PKR was part of the IFN response and that subsequent eIF2-alpha phosphorylation can be tolerated by the virus and translation can proceed. Still, translation was stimulated when eIF2-alpha phosphorylation was inhibited, suggesting that the IRES prefers traditional, eIF2-directed initiation (64). Nevertheless, if PKR is activated, translation is not shut down.

5.5. HCV inhibits IFN signaling

The specific mechanisms by which HCV resists the IFN response is not fully understood but viral proteins seem to play a role in evasion of the cellular defense system. In stably-transfected human osteosarcoma cells, where all of the HCV proteins were expressed under the control of a tetracycline-inducible system, ISGF3 was defective in ISRE binding (65). The failure of IFN-alpha signaling was accompanied by the reduced expression of Stat1 and p48, however, whether the decrease in protein expression was the cause of or the result of interference with IFN signaling is unclear. ISGF3 proteins were present in the nucleus of the HCV expressing cells, and neither transport nor tyrosine phosphorylation were affected by HCV. The nuclear extracts, however, were deficient in the ability to bind to the ISRE. Since all of the HCV genes...
were present in this system, it will be interesting to know which of the individual HCV genes is responsible for this block in IFN-alpha signaling. Another group found that Stat1 was degraded in HCV replicon-transfected cells and resulted in a reduced amount of phosphorylated Stat1 in the nucleus (66).

5.6. HCV and 2',5' oligoadenylate synthetase, 2',5'OAS

Several clinical studies suggest that the IFN-induced 2', 5' oligoadenylate synthetase (2',5'OAS) may be impaired in virus-infected cells. 2',5'OAS inhibits cellular and viral translation by synthesizing 2'-5' polyadenylate chains that activate RNase L. Activated RNase L then catalyzes the degradation of RNAs [Figure 3; (67, 68)]. While 2',5'OAS protein expression seems to be elevated during HCV infection (69, 70) no difference in the induction of 2',5'OAS by IFN treatment between responders and nonresponders could be observed (71, 72). Levels of induction were high before IFN treatment of chronically infected patients and did not increase during IFN therapy (71). This may suggest that chronic induction of 2',5'OAS by HCV results in decreased sensitivity to exogenously supplied IFN. In another study, the level of 2',5'OAS was shown to be higher in patients with chronic HCV than in healthy individuals, confirming that HCV is capable of mounting an IFN response (73). While Pawlotsky and colleagues noted that 50% of patients receiving IFN therapy expressed the active form of 2',5'OAS, the synthetase and RNase L enzymatic activities have yet to be examined. The possibility that HCV has a mechanism for overcoming the inhibitory effects of 2',5'OAS or RNase L is intriguing. In contrast, it is also possible that the 2',5'OAS system is intact and that HCV has developed a strategy to utilize this pathway for its own benefit. For example, no specificity of RNase L for viral RNA is apparent and so HCV may regulate this pathway and potentially modulate viral growth thus, promoting persistent infection. Alternatively, subcellular localization, or localized activation, may protect some cellular or viral RNAs from the effects of 2',5'OAS and HCV might capitalize on this feature of the system and exploit it to promote virus-specific translation.

5.7. HCV and Mx proteins

Other ISGs that may affect HCV pathogenesis include the Mx proteins. The presence of Mx RNA in peripheral blood mononuclear cells (PBMCs) should indicate that the IFN-mediated antiviral response is stimulated. MxA, a protein with activity against some RNA viruses, is a guanosine triphosphatase and is an inhibitor of viral replication (74). An increase in MxA mRNA was observed in PBMCs from HCV patients that were classified as responders (75). A single nucleotide polymorphism in the promoter of the MxA gene influences the response to IFN therapy. Because up to 90% of HCV patients can be classified as nonresponders, it may be conceivable that those isolates that are resistant to IFN may encode viral genes that can evade the effects, or downregulate the expression of Mx genes. While the effects of Mx proteins on HCV replication are unknown at this time, the presence of this IFN-induced gene may be a predictive marker for successful IFN-response.

5.8. HCV and major histocompatibility complex (MHC) class II antigens

MHC class II antigens are IFN-induced and are expressed on immunocompetent cells, such as B cells and macrophages. They are important for antigen presentation and are effective for the interaction between immunocompetent cells. Some correlation between disease-susceptibility and human leukocyte antigen system (HLA) alleles may have relevance with respect to the IFN response and HCV infection. The MHC class II, HLA allele DRB1 *0404 was significantly higher in patients with a sustained response to IFN (76). The HCV E2 protein has been shown to induce dendritic cell maturation as detected by an increased expression of a number of cell surface markers, including MHC class II antigens (77).

5.9. HCV and the IFN-alpha receptor, IFNAR

Expression of IFNAR1 and 2 mRNA in the liver was found to be higher in responder than in nonresponder patients with chronic active hepatitis (78, 79, 80). No correlation between IFNAR mRNA levels and genotype were seen (81). IFNAR mRNA in the liver but not in PBMCs was associated with IFN response (82). Resistance to IFN may be due to low level IFNAR expression (83).

5.10. HCV and nitric oxide

Levels of nitric oxide synthetase type 2 (NOS2) expression may be associated with activation of mononuclear phagocytes. Constitutively-expressed NOS2 synthesizes nitric oxide (NO) that is toxic to the liver. However, NO that is produced by NOS2 (or iNOS, the inducible isoform) can be toxic or protective, depending on the type of infection [for review, see (84)]. IFN-treated patients had higher levels of NOS2 activity, NOS2 antigen and NOS2 mRNA, which correlated with a decrease in serum ALT levels (85). Hepatic iNOS expression was positively correlated with hepatic HCV-RNA levels and weakly with IFN-gamma expression, consistent with iNOS’s involvement in the antiviral actions of IFN (86).

Clearly there are multiple viral genes helping to evade the IFN response (Table 1). Future studies should focus on identifying the role that these and other viral genes play in IFN resistance. Further development of cell culture systems for the study of viral host interactions will facilitate these types of studies. Elucidating the mechanism of IFN resistance will have great therapeutic importance, leading to improvements in the response rate to IFN therapy or to new treatments for HCV.

6. CONCLUSIONS AND FUTURE DIRECTIONS

While several HCV genes have been linked to IFN-alpha-resistance through various ISGs, protein expression levels have been the focus of most of these studies (Table 1). In patients, the expression level of ISGs can reflect a patients’ ability to mount a response to IFN-alpha, but cannot predict the outcome of IFN-alpha therapy. For example, while a high level of 2',5' OAS reflects the patients’ ability to respond to IFN-alpha, it does not suggest that 2',5'OAS actively contributes to IFN-alpha sensitivity.
During HCV infection. When proteins are expressed during the IFN response, commonly they are enzymes that require further activation such as RNase L or PKR. Therefore, enzyme activity, and not protein expression, is the key determinant when dissecting the effects of IFN-alpha on viruses or the effects of viral genes on the IFN-alpha response. These experiments have been done in vitro for PKR and for ADAR1, but not for other IFN-induced proteins. Hopefully future studies of IFN-induced antiviral enzymes will expand on initial observations and include enzyme activities in the presence of HCV genes. Further work is needed to evaluate enzyme activities in the HCV cell culture system and through in vivo studies.

Understanding the mechanism underlying IFN resistance by HCV has tremendous therapeutic applications. Because some strains of HCV do respond to IFN, there is hope and potential for circumvention of these viral strategies. With more than 200 genes induced by IFN, new pathways will surely be revealed in the future. We have only just begun to uncover the different strategies for HCV’s evasion of the IFN response. Elucidation of the mechanism of HCV genes and how they interact with cellular antiviral response factors will enable a better understanding of the pathogenesis of HCV and how it and other viruses evade the IFN response.

7. ACKNOWLEDGMENTS

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