Hepatitis C: Current Status and Future Directions for Antiviral Therapy

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Introduction

Hepatitis C virus (HCV) was first identified and shown to be the cause of almost all cases of non-B posttransfusion hepatitis in the late 1980s [1,2]. The virus, now classified within the genus Hepacivirus of the family Flaviviridae, is unique in its ability to establish persistent infection in the great majority of infected persons, many of whom develop evidence of chronic inflammatory liver disease [3]. These chronically infected persons are at risk for cirrhosis and, to a lesser extent, hepatocellular carcinoma [4,5]. Such serious complications of HCV infection usually develop over several decades, although in exceptional cases life-threatening liver disease can become evident within 10 years or less of infection. Despite this, most infected individuals appear to reach a relatively healthy equilibrium with this infection, and the majority of infected persons will probably die of causes completely unrelated to hepatitis C. Little is known of the reasons why certain infected patients do well, while others get into trouble with this infection. This lack of understanding extends to an absence of prognostic tests that are capable of predicting disease outcome in individual patients.

Another unexplained mystery is that most American patients who do succumb to liver disease related to chronic hepatitis C die as a result of cirrhosis and liver failure, while most HCV-related deaths in Japan are due to hepatocellular carcinoma [6]. The factors that may account for this apparent difference in the natural history of the infection are not known. However, these may include genetic differences in the populations of the two countries, dietary or other environmental factors, differences in the

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infecting strains of HCV, differences in the age-specific distribution of HCV infections in the two countries, or other yet unrecognized factors.

Epidemiology of Hepatitis C: Contrasting Patterns in the US and Japan

HCV infection accounts for under 20% of acute viral hepatitis cases within the United States. However, it is by far the leading cause of chronic viral hepatitis and is present in 40%–55% of persons who succumb to chronic liver disease of any type. The Centers for Disease Control and Prevention (Atlanta, GA, USA) recently estimated that HCV infection contributes to as many as 8000–10 000 deaths annually due to chronic liver disease within the United States [7]. A recent serologic survey of over 20 000 Americans has been carried out by this government agency using sera that were collected by a stratified, randomized sampling of the population. The results of this study indicate that, overall, approximately 1.8% of all Americans are positive for HCV antibody, and thus most likely persistently infected with the virus. Strikingly higher prevalences of infection were documented in African Americans and Mexican Americans, compared with Caucasian Americans. Most importantly, the highest prevalences of infection were noted in persons aged 30 to 50 years, with substantially lower antibody prevalences found in older persons of any racial background.

This pattern of the age-related seroprevalence of HCV infection strongly suggests that there has been an upsurge in new HCV infections among young adults over the past 2–3 decades. This is almost certainly related to increases in illicit injection drug use in the US since the early 1960s. Since HCV infection persists for many decades and progresses only slowly to serious liver disease, it is likely that this relatively recent epidemic spread of HCV will be reflected in substantial increases in liver-specific mortality during the next two decades. By the year 2010, it is predicted that the number of HCV-related deaths will increase to 24 000–30 000 per year [8]. This would represent a tripling of the present estimated death rate due to hepatitis C, and an annual HCV-related death rate comparable to the present number of AIDS-related deaths in the US.

These predictions are made credible by increases in the death rate due to HCV-associated hepatocellular carcinoma that have been observed over the past 20 years in Japan [9,10]. During this period, the rate of death due to liver cancer tripled among Japanese men, and this increase in

cancer deaths was related exclusively to HCV-associated tumors. Interestingly, there has been little increase in the incidence of liver tumors among Japanese women during the same period, despite the fact that the seroprevalence of HCV is roughly similar in both genders. Equally striking, however, is the age-related seroprevalence curve in Japan [11]. In contrast to the US, where a major peak is present in the 30–50-year-old age group, seroprevalence increases continuously with advancing age in Japan. This reflects the widespread dissemination of HCV infection within the Japanese population in the early post-WWII era, probably due to a combination of injection needle practices and frequent blood transfusions related to endemic tuberculosis.

Thus, both countries have experienced a cohort-effect with respect to HCV infection and disease during the last half of this century, with the Japanese experience preceding the American experience by 2–3 decades. This epidemiologic view is supported by phylogenetic analyses of HCV strains in the two countries, and is very sobering from the American perspective. It accentuates the need for a better understanding of the pathobiology of this infection, and the accelerated development of better therapies.

Mechanisms of HCV Replication: Ample Targets for Novel Antivirals

The HCV Polyprotein

Like other flaviviruses, HCV is a positive-strand RNA virus [12,13]. The virus particles possess an extensively glycosylated envelope within which the 9.7 kb single-stranded RNA genome is packaged in association with a highly basic, nucleocapsid (core) protein. As with many other positive-strand viruses, HCV expresses its protein complement in the form of a single giant polyprotein that is encoded by a large open reading frame extending through much of the length of the genomic RNA (Fig. 1). Unlike yellow fever virus and other members of the genus Flavivirus, HCV and other Flaviviridae classified with the genus Pestivirus initiate translation of this polyprotein via internal entry of the 40S ribosome subunit on the virion RNA. This process is controlled by a highly structured RNA sequence located within the 342 nucleotide-long 5′ nontranslated RNA (NTR), which is called the internal ribosome entry site (or IRES) [14]. The precise structure of the 5′ end of the genomic RNA is not known, although it is presumed not to possess a typical 5′ cap structure.

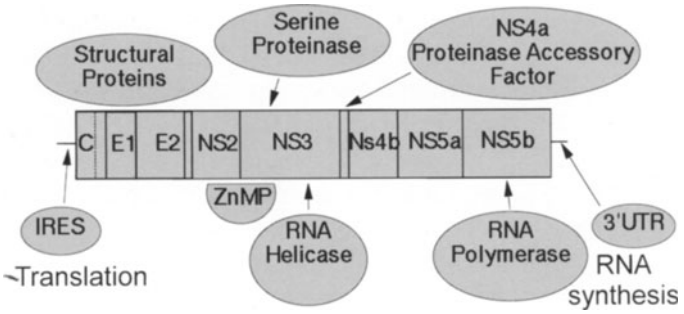

Fig. 1. Diagram depicting the genetic organization of the hepatitis C virus (HCV) genome. Major protein products derived from proteolytic processing of the polyprotein are indicated. *E1* and *E2*, secreted glycoproteins that form the envelope of the virus

The 3′ nontranslated RNA appears also to be relatively structured [15,16], but it also contains a lengthy pyrimidine-rich tract that is largely poly-(U) in composition. The function of this tract is unknown.

The polyprotein of HCV is comprised of approximately 3000 amino acid residues. It undergoes cotranslational and posttranslational cleavages that are directed by both host cell and virus-encoded proteinases (Fig. 1) [17–19]. Signal sequences located within the amino terminal third of the polyprotein direct its secretion into the endoplasmic reticulum (ER). There, several cleavages directed by host cell signalase produce a series of structural proteins. These include the nucleocapsid protein (otherwise known as the core protein), two envelope glycoproteins, E1 and E2, and a small membrane-associated protein, p7 (or NS2A) that plays an uncertain role in viral assembly. The nucleocapsid protein remains within the cytoplasm and may possibly undergo further proteolytic processing. In contrast, E1 and E2 are secreted, and extensively glycosylated within the ER and Golgi [20,21]. Their subsequent trafficking within the cell remains uncertain. However, there is little evidence that these glycoproteins reach the cell surface, and it is possible that viral assembly involves budding of virus particles into internal membranous structures within the cell.

The remaining segment of the polyprotein is processed entirely under the control of two viral proteinases. What may be a primary cleavage occurs at the NS2/NS3 junction, under control of a *cis*-acting zinc-

dependent metalloproteinase that spans this junction [18,19]. Further processing of the polyprotein is controlled by the viral serine proteinase located within the amino terminal third of the NS3 protein, and is likely to involve both *cis*- and *trans*-active cleavages. A small polypeptide, NS4A, which appears to be cleaved from the residual amino end of the nascent polypeptide following the release of NS3, assembles noncovalently with the NS3 molecule to form a fully active proteinase [22,23]. Although the temporal sequence of the processing cascade remains poorly documented, this NS3/NS4A complex cleaves at several different sites within the polyprotein, resulting in several additional proteins (NS4B, NS5A, NS5B). While NS5B is an RNA-dependent RNA polymerase, the functional roles of these additional proteins are not well understood. Nonetheless, it is likely that each of the resulting cleavage products has a specific function related to replication of the RNA. In addition to its proteinase activities, NS3 has NTPase and RNA helicase activities mapping to its carboxy two-thirds [24,25]. NS4A plays a role in controlling the phosphorylation of NS5A [26], although the function of the latter protein remains uncertain.

Molecular Events in HCV Replication

Replication of the viral RNA proceeds through a negative-strand intermediate, and is likely to occur in association with membranous replication complexes in the cellular cytoplasm. These complexes almost certainly include each of the nonstructural proteins of the virus [27]. Initiation of negative-strand synthesis is likely to be dependent upon specific recognition of the 3′NTR RNA structure by this replicase complex. Among the HCV proteins, RNA binding activities are present within the core protein as well as NS3 and NS5B.

Similarly, the initiation of positive-strand RNA synthesis from newly synthesized negative-stranded RNA is also likely to require specific recognition of the 3′ terminal negative-strand structure by the RNA replicase. This latter structure is of course formed by sequence complementary to the 5′NTR of the virion RNA. The extent to which recognition of the 3′ and 5′ ends of the RNA by the replicase complex include common RNA-binding activities is not known. However, it is noteworthy that there is no recognizable structural homology between the 3′ ends of these RNA molecules. Replication of the RNAs appears to occur asymmetrically, with positive-strand synthesis exceeding negative-strand synthesis.

Remarkably, however, most studies indicate that infected liver contains less than 10-fold more copies of the positive-strand than the negative-strand [28]. This is very different from the situation with the picornavirus, hepatitis A virus (HAV), where the abundance of positive-strand copies exceeds the negative-strand intermediate by a factor of 100 or more [29].

Thus, although HCV is a relatively simple virus that expresses less than a dozen separate proteins, its replication is dependent upon a considerable number of virus-specified enzymatic activities or highly specific macromolecular interactions. Each of these is a potential target for development of new antiviral drugs. It is remarkable that a such a large amount of information has been gathered concerning a number of these viral activities, despite the fact that the virus is unable to replicate with any degree of efficiency in cell culture.

Potential Mechanisms Underlying Viral Persistence

The morbidity and mortality associated with HCV infection is due largely to its unique propensity to cause persistent infection in most persons, a feature that distinguishes this virus from other known hepatitis viruses [3]. Persistent infections with hepatitis B virus (HBV), for example, generally occur only in individuals who are immunocompromised, or who are infected at birth, while truly persistent infections with HAV occur rarely if ever. In sharp contrast, recent data from a cohort of injection drug users that has been studied prospectively in Baltimore by Thomas and colleagues suggest that persistent infection develops in about 85% of adults who undergo acute HCV infection with seroconversion to the virus [6]. Although the specific mechanisms underlying the persistence of HCV are not known, the available evidence suggests several possibilities.

Regulated Replication of HCV

Among potential mechanisms contributing to viral persistence is the possibility that the replication of HCV may be specifically regulated by a mechanism that minimizes the expression of viral proteins. From a teleological point of view, this would offer several survival advantages to the virus. First, it would contribute to the lack of cytopathic effect that appears to mark this flavivirus relatively uniquely. Second, it could conceivably lower the profile of the infection as seen by the host immune system,

rendering an infected cell less likely to be recognized by virus-specific cytotoxic T lymphocytes (CTLs). There is no solid evidence for specific regulation of the HCV replication cycle. However, the hepaciviruses differ significantly from the flaviviruses and pestiviruses (other members of the family Flaviviridae) in that they lack the capacity for vigorous replication such as that observed with yellow fever virus or bovine viral diarrhea virus both in animals and in cell culture. It is noteworthy that HCV titers in the blood are never very high, even during acute infections before the evolution of virus-suppressive CTLs, despite the fact that the liver contains an enormous mass of apparently permissive hepatocytes.

Furthermore, it is curious that, while HCV does apparently undergo replication in cultured lymphoblastoid cells, it never adapts to growth in these cells [30–32]. HAV readily adapts to growth in monkey kidney cells over the course of 10–20 consecutive passages [33]. However, there is no significant increase in the replicative capacity of HCV, even after continuous passage for up to a year in lymphoblastoid cells. The infectious titer of the virus present in harvests of these cells never exceeds 2–3 $\log_{10}$/ml. This is puzzling for a positive-strand RNA virus that is known to undergo relatively frequent mutation during the course of human infection [34]. These observations suggest an intrinsic restriction on replication of the virus, at least in these cells.

A possible molecular mechanism for such regulation of virus replication is suggested by studies of the HCV IRES. Several lines of evidence suggest that the 40S ribosome subunit forms an important primary contact with the viral RNA directly at the site of the initiator AUG codon at nt 343 of the genome [35–37]. There is no scanning of the ribosome subunit on the RNA prior to its being positioned over the initiator AUG. Interestingly, the initiator AUG is located within the single-stranded RNA segment of what appears to be a small stem-loop (Fig. 2) [35]. Preservation of this structure is not required for IRES activity in synthetic RNA transcripts, and mutations which enhance its stability have a notable negative effect on virus translation. Yet, this RNA structure appears to be present in all strains of HCV that have been studied to date. One possible explanation for these findings is that the stem-loop might act as a regulator of translation, were it the target site of a viral RNA-binding protein that was capable of stabilizing this RNA structure through a protein–RNA interaction [35]. If this were the case, the viral protein could effectively compete with the 40S ribosome subunit for the viral RNA, providing a convenient mechanism for autoregulation of viral gene expression

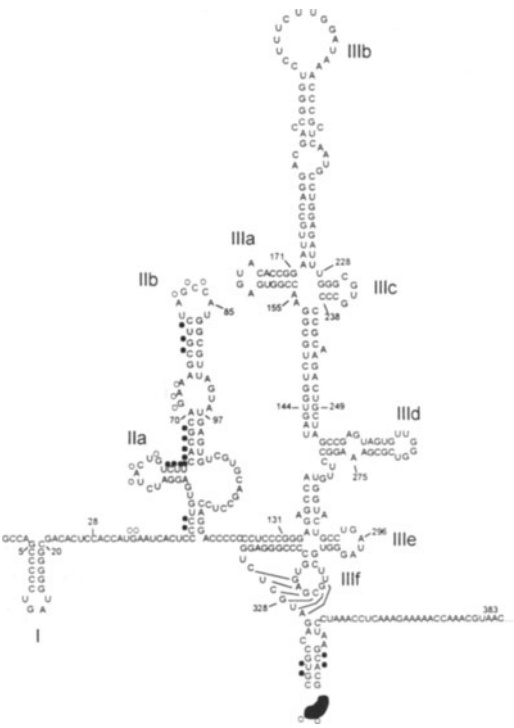

Fig. 2. Secondary and tertiary RNA structures that are present within the 5′ nontranslated RNA (5′NTR) of HCV and which control cap-independent translation of the virus by a process of internal ribosome entry. A primary contact is formed between the 40S subunit and the viral RNA near the site of the initiator AUG (*shaded box*), which is located within the single-stranded sequence of a small stem-loop [35]. Modified from Lemon and Honda [14]

through translational repression. There are as yet no hard data that support the autoregulation of HCV translation by an HCV gene product, but very similar regulatory mechanisms have been demonstrated to exist among some prokaryotic viruses.

Quasispecies Variation

Like the human immunodeficiency virus (HIV-1), HCV demonstrates extensive nucleotide sequence variation within individual patients. The virus is present in blood as a diversity of genetically related but individu-

ally distinct sequences (i.e., a quasispecies "swarm") [34]. The extent of quasispecies variation is dependent on two major factors: the extent of ongoing viral replication, and the presence of selective forces which favor the amplification of certain quasispecies over others. Viral replication is essential to this phenomenon, because it is the machine that allows the virus to explore "sequence space" (i.e., all possible virus sequences). The RNA polymerase, like all viral RNA-dependent RNA polymerases, lacks a proofreading capability and makes relatively frequent errors during RNA transcription. On average, this is likely to amount to more than one misincorporated base in every progeny RNA molecule. The primary selective forces that shape the process of quasispecies variation include the replication capacity of the variant RNA, and immunologic pressure applied by CTL activity and neutralizing antibodies. These latter forces lead to the continued evolution of both B- and T-cell escape mutants in chronically infected persons [38–40].

The role played by this quasispecies variation in the establishment of persistent infection has been extensively debated. Although genetic variation is extensive, it is far from clear that it is a cause of virus persistence. It seems more likely that quasispecies variation occurs as a result of persistence, with continuous replication providing the virus with the opportunity to explore sequence space. According to this hypothesis, the evolution of escape mutants is then a reflection of the presence of an active yet ultimately ineffective immune response to this infection.

Potential Role of the Envelope Glycosylation in Viral Persistence

The envelope proteins of HCV are glycosylated to an unusual extent, as the addition of microsomal membrane preparations to cell-free translation reactions results in an approximate doubling of the molecular masses of both E1 and E2 [17]. This is reminiscent of the extensive glycosylation of the envelope protein of HIV. The glycosylation of the HCV envelope proteins likely reduces the ability of the immune system to respond effectively to the presence of these proteins. On the whole, there is relatively little evidence for neutralizing antibodies that are capable of significantly reducing viral infectivity. The absence of an effective neutralizing antibody response is reflected in generally high levels of anti-E2 antibodies in individuals who are viremic with HCV [41]. Another measure of the lack of an effective neutralization response is the high frequency of second infections observed in chimpanzees on reexposure to

an HCV inoculum that was previously used to infect the animal [42]. However, more detailed studies of the neutralizing antibody response to HCV are not possible due to the absence of an effective cell culture system for propagation of the virus. This has precluded the development of classical viral neutralization assays.

Despite these indications of a poor overall neutralizing antibody response to HCV, the E2 protein contains a highly variable domain near its amino terminus (HVR-1 domain). This domain appears to form an immunogenic loop on the surface of the virion and it is suspected to interact with neutralizing antibodies [38,43]. The HVR-1 domain is a prime site for the evolution of quasispecies mutations. What specific role it might play in the pathobiology of this infection is unclear.

Could HCV Specifically Disarm the Immune System?

Despite the above speculations, it seems likely that HCV, like many other viruses, may have evolved specific mechanisms for evasion of the host immune system. There are two suggestive lines of evidence for this. First, the core protein of HCV has been shown to interact directly with the cytoplasmic domain of the lymphocytotoxin beta receptor and other members of the tumor necrosis factor (TNF) receptor family [44]. The evidence suggests that this interaction occurs close to the "death domain" through which an apoptotic signal is transduced to the nucleus following the binding of ligand to the receptor. Although the molecular evidence for this interaction is strong, its biologic significance remains uncertain. Initially suspected of potentially blocking signal transduction (and thereby protecting the infected cell from attack by CTLs), recent evidence suggests that the expression of core protein may actually sensitize some cells to apoptosis following the binding of ligand [45–47]. While this makes little sense in the context of infected hepatocytes, there is evidence that suggests that HCV may also infect lymphoid cells [31,48]. In this case, early apoptosis may prevent an infected lymphoid cell from participating in the activation of CTLs or the production of antibody. Clearly, more work is needed in this area, but efforts will be slowed by the continued absence of a small animal model for hepatitis C.

A second possible way in which HCV could interfere with the host immune response concerns the cellular interferon-inducible kinase, PKR. Phosphorylation of this kinase is induced by the presence of double-stranded RNA, and phosphorylated PKR acts to inhibit host cell transla-

tion through phosphorylation of the cellular translation initiation factor, eIF-2. This results in both an antiviral and antiproliferative state in the cell. Following discovery of an "interferon sensitivity determining region" (ISDR) within the NS5A protein [49,50], it was shown recently that NS5A interacts directly with the catalytic domain of PKR [51]. This suggests that the presence of NS5A might create a cellular environment within which interferon-mediated antiviral responses are relatively ineffectual. This is an attractive hypothesis, because the virus clearly is able to survive the endogenous host interferon response, despite its suppression by exogenous interferon administered in large doses. However, as with the core–TNF receptor interaction, the biologic significance of the NS5A-PKR interaction remains to be proven.

Careful consideration of the mechanisms underlying viral persistence will be important to gaining an understanding of the pathobiology of hepatitis C. It may even lead to novel strategies for intervention. However, it is important to remember that there may be no single underlying cause for HCV persistence. Instead, persistent infections may result from a combination of factors, including both those that are described above as well as others that are waiting to be discovered.

Vaccines Versus Antiviral Agents in the Control of Hepatitis C

In addition to quasispecies variation, there is considerable genetic diversity among different strains of HCV [34]. Although classical neutralization assays have not been developed and data are thus sparse, it is likely that these different HCV genotypes also vary substantially in their antigenicity. Pairwise comparisons of the nucleotide sequences of NS5B from different HCV genotypes reveal a magnitude of difference that is far greater than that which exists between the RNA polymerase (3Dpol) sequences of types 1 and 2 poliovirus. This suggests that different HCV genotypes may well be different serotypes in that they may stimulate little if any cross-protective immunity to each other. However, as indicated above, we lack the tools (neutralization assays and small animal models) that would be required to test this point.

Thus, genetic, and likely antigenic, variation poses major problems for the development of vaccines against HCV. To a large extent, the technical hurdles in HCV vaccine development mirror those that have been encountered in HIV vaccine development. Although a candidate vaccine comprised of recombinant envelope proteins was shown to protect

chimpanzees against a low-dose homologous challenge [52], the ability of such a vaccine to protect against other HCV strains is doubtful at best. It is an important point to test, however, and it would be premature to consider dropping such a conventional approach to vaccine development. In the long run, however, it seems likely that the extent of antigenic variation among the envelope glycoproteins may ultimately point this field in the direction of T cell vaccines. Such a vaccine has been shown capable of protecting mice from infection with lymphocytic choriomeningitis virus [53].

In the absence of any clearly feasible strategy that might allow development of effective immunization against hepatitis C, only three general approaches offer any hope of decreasing the disease burden due to HCV infection. First, new infections may be prevented in the absence of effective vaccines by screening blood products that pose risks for transmission of HCV. Although it is likely that a small number of transfusion-related cases of hepatitis C continue to occur, this approach has been spectacularly successful in reducing the frequency of transfusion-related hepatitis C since 1991 in many countries [7,54,55]. However, the impact of blood screening is of course limited by the extent to which other mechanisms of transmission (e.g., injection drug use) contribute proportionately to new infections.

A second approach is to foster modification of risky behaviors in certain high-risk populations (i.e., injection drug users). This may be more difficult to achieve than screening the blood supply. However, there have been significant decreases in the incidence of all forms of viral hepatitis associated with illicit injection drug use within the United States over the past decade. The basis for these decreases is far from clear, but it is likely that they reflect a reduction in risky needle-sharing behaviors related to the fear of AIDS. Whatever the reason, there has been a dramatic decrease in the incidence of new HCV infections within the United States since 1988 [7]. Nonetheless, approximately 4 million persons remain persistently infected with HCV within the United States alone. These individuals, most of whom are currently in their third and fourth decades of life as indicated above, will remain at significant risk for cirrhosis and hepatocellular carcinoma in the absence of new therapeutic modalities.

The third and last general strategy is the development of better therapeutic regimens that are capable of reducing or possibly even eliminating the liver destructive effects of HCV infection.

Interferon Treatment of Chronic Hepatitis C

A considerable number of controlled clinical trials have proven that treatment with several different formulations of interferon may be beneficial in a small proportion of patients with chronic hepatitis C [8]. This beneficial response is marked by normalization of serum alanine aminotransferase (ALT) values, elimination of viral RNA, and/or improvements in hepatic histology. A full discussion of these reports is well beyond the scope of this review. However, several points are worth emphasizing. First of all, the mechanism by which interferon exerts its therapeutic benefit is not well understood. There is a clear antiviral effect, with relatively rapid declines in HCV viremia on institution of interferon therapy. However, it is not clear whether this reflects a direct suppression of viral replication, or enhanced immunologic suppression of viral replication due to the immunomodulatory action of interferon. The latter includes the up-regulation of class I markers on the surface of hepatocytes, which would enhance the recognition of infected cells by virus-specific CTLs.

It is interesting to note, however, that improved liver histology is the measure of response to interferon that occurs with the greatest frequency in treated patients. This significantly exceeds the frequency with which ALT levels are normalized or viral RNA eliminated. In early randomized, placebo-controlled, prospective clinical trials, improvements in histology occurred in an average of about 70% of all patients with hepatitis C who were treated with interferon [56–58]. In contrast, ALT levels became normal in only about 40% of patients by the end of 6 months of therapy with 2–3 million units interferon-α thrice per week. Thus, whatever the underlying therapeutic mechanism, at least a transient reduction in hepatic inflammation frequently accompanies interferon therapy.

This suggests that interferon may have a significant impact on the outcome of the disease, but data supporting a tangible improvement in health or quality of life have been sparse. Nonetheless, one randomized, controlled, prospective study has demonstrated a reduction in the incidence of hepatocellular carcinoma in Japanese patients with well compensated cirrhosis who were given relatively high-dose interferon therapy (6 million units of interferon-α three times a week for 12–24 weeks) [59]. Hepatologists in the west have been slow to accept these results due to the extraordinarily high incidence of liver cancer that was reported in the control group in this study. This approached 40% after 2–7 years of posttreatment follow-up. However, it is likely that the high incidence of

cancer in the untreated patients may simply reflect the natural history of hepatitis C in Japan, which appears to differ from that in the US as indicated above [6,60]. At least two additional, retrospective studies (one in Japan and one in Europe) suggest similar conclusions [61]. These studies, one of which was presented at this symposium, suggest that patients who respond to interferon have a lower risk of developing hepatocellular carcinoma than interferon nonresponders.

In addition to a relatively low response rate, there are other major impediments to successful treatment of chronic hepatitis C with interferon. These include the extraordinarily high cost of the drug, its administration, and attendant patient monitoring. Moreover, interferon therapy for chronic hepatitis C is marked by a relatively high frequency of adverse side effects such as cytopenia, depression, autoimmunity, and increased frequency of bacterial infections, among others. Most notable, however, is the high rate of relapse that typically follows completion of interferon therapy. This generally occurs in about half of all treated patients whose ALT levels have been rendered normal by the end of therapy, although it may occur in a slightly smaller proportion of patients if therapy is continued for a year [8]. The low overall frequency of sustained response to interferon is a major reason for the current intensive search for better therapeutic regimens.

Approach to Antiviral Therapy—The Case for Combination Therapy

Recent successes in the treatment of HIV-1 infections with combinations of antiviral drugs argue strongly for a similar approach to the treatment of chronic HCV infection. This is particularly so since HCV infections share a number of features in common with persistent HIV-1 infection, including the capacity for substantial quasispecies variation and the potential for selection of resistant viruses in patients receiving monotherapy. Combination therapy with antimicrobial agents has a long history in the treatment of chronic infectious diseases. It has been used for decades in the treatment of tuberculosis for very similar reasons. In the case of hepatitis C, recent data strongly support a synergistic effect when ribavirin is combined with interferon, both in interferon-naïve patients as well as in the retreatment of interferon nonresponders.

Ribavirin is a synthetic, oral guanosine nucleoside analog that has been used clinically for a number of years as treatment for several different viral infections. It has demonstrated efficacy in the treatment of neonatal

respiratory syncytial virus infections, as well as in life-threatening arenavirus infections [62,63]. The compound is unusual in that it possesses a broad range of antiviral activity against viruses of vastly different type. It has been suggested to block the synthesis of functional rhabdovirus mRNAs [64], possibly by interfering with capping of the 5′ ends of the RNA (a phenomenon that is likely to be irrelevant in the case of HCV). Other data suggest that it may suppress transcription of double-stranded reovirus RNAs, possibly by interfering with viral RNA helicase activity [65]. However, its mode of broad antiviral action has never been satisfactorily explained and, as indicated below, it may have important immunomodulatory activities.

Early studies of ribavirin monotherapy in patients with chronic hepatitis C were prompted by its known activity against other RNA virus infections. For the most part, these studies were disappointing. Although slow improvement was noted in serum ALT values, there was very little suppression of HCV viremia even after prolonged therapy. This indicates that the antiviral effect of ribavirin against HCV, if any, is extremely limited [66–69].

Despite the early results with ribavirin monotherapy, recent clinical trials indicate that the addition of ribavirin to standard courses of interferon results in significant increases in the response to therapy. This is marked by increases both in the proportion of patients with end-of-treatment responses, as well as in the proportion of patients with sustained responses. For example, in a prospective randomized trial, Lai et al. [70] treated interferon-naïve patients with the combination of ribavirin (1200 mg four times daily) and interferon-α 2a (3 million units thrice weekly) for 24 weeks. This combination treatment regimen resulted in a complete response (normalization of ALT and elimination of detectable HCV RNA) in 76% of patients by the end of therapy, and a sustained response at 96 weeks after therapy in 43% (Fig. 3). In contrast, comparable response rates in patients receiving a similar course of interferon without ribavirin were 32% and 6%, respectively.

Similar findings were reported recently by Reichard et al. [71] in a larger prospective study examining the combination of ribavirin and interferon-α 2b (3 million units thrice weekly), given for a total of 24 weeks. Altogether, 42% of the patients had a sustained virologic response after 1 year of follow-up, compared to only 20% of patients receiving interferon alone. Importantly, a retrospective analysis of the patients included in this study indicated that the combination was beneficial only

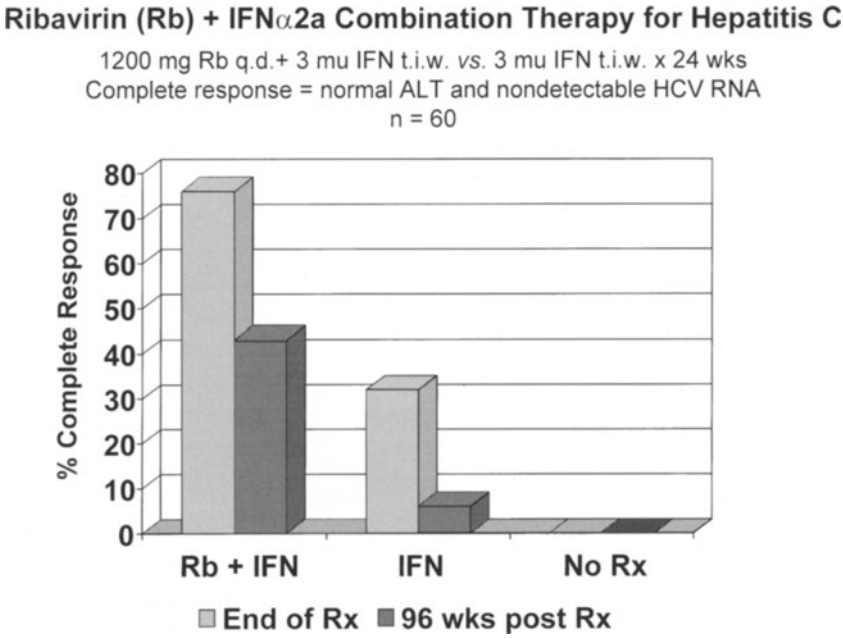

Fig. 3. Summary of results from a prospective, randomized, controlled clinical trial of ribavirin–interferon combination therapy vs interferon monotherapy, as reported by Lai et al. [70]

in patients with high levels of serum HCV RNA ($>3 \times 10^6$ genome equivalents/ml). The combination offered no advantage over interferon alone in those patients with lower levels of HCV viremia, who overall have a greater chance of a favorable response to interferon monotherapy.

Although the response to interferon–ribavirin combination therapy has not been as impressive in those patients who have previously failed interferon monotherapy, Bellobuono et al. [72] reported both greater end-of-treatment and sustained ALT responses in patients undergoing retreatment with combination therapy, compared with interferon alone. In all studies, the addition of ribavirin to interferon regimens has been relatively well tolerated, with few patients forced to withdraw from therapy due to adverse reactions. However, low-grade hemolysis has been a frequent side effect, and may lead to both anemia and increased intrahepatic iron stores [73]. The latter is a worrisome finding, inasmuch

as it could be detrimental to liver function if allowed to reach a significant level.

Although the overall sustained virologic response rates that have been reported with the combination of ribavirin and interferon are still less than satisfactory, the marked benefit that has been noted with the addition of ribavirin to previous interferon therapeutic regimens highlights the potential for combination therapy against this disease. It is particularly noteworthy that the mechanism of action of ribavirin in this setting may not be related to direct suppression of viral replication, but rather to a favorable modulation of the host immune response to the infection. This is suggested by the failure of ribavirin to suppress HCV viremia when administered as monotherapy [66,67], as well as a series of observations concerning the effects of ribavirin on various aspects of the host immune response. It is interesting that the combination of ribavirin and interferon also shows synergy in the treatment of experimental subacute sclerosing panencephalitis (SSPE) virus infections in hamsters [74]. These observations are consistent with a broad effect of ribavirin on the ability of the host immune system to control chronic viral infections.

In animal studies, the course of disease in mice experiencing fulminant hepatitis due to the coronavirus murine hepatitis virus (strain 3) can be attenuated by treatment with ribavirin [75]. This has been shown to be associated with inhibition of macrophage production of TNF and the procoagulant fgl2 prothrombinase. Ribavirin inhibited Th2 cytokine responses, but preserved Th1 cytokine production. Interleukin-6 production has also been shown to modulated by ribavirin in human pulmonary epithelial cells infected with respiratory syncytial virus [76]. The significance of these findings is unclear, however, as is their relationship to the mechanism of action of ribavirin in the setting of combination therapy of hepatitis C. Ribavirin is known to inhibit cellular IMP dehydrogenase [77], and this may influence the ability of cells either to support viral replication or to respond to appropriate immunologic stimuli.

Whatever the mechanism of action of ribavirin when used in combination with interferon for therapy of hepatitis C, the success of this strategy has increased the urgency of the search for additional, specific inhibitors of HCV replication. Although this search is hampered by the absence of tractable cell culture systems that would allow the development of broad-based screens for novel antiviral compounds, significant progress is being made through the use of more sophisticated biochemical screens.

Major Molecular Targets for Antiviral Drug Development—Current Status

NS3 Proteinase

The amino terminal third of the NS3 molecule contains a serine proteinase activity that is active both in *cis* and in *trans* [78,79]. This activity is responsible for the majority of cleavage events occurring within the HCV polyprotein. Thus, inhibition of this activity would have a direct effect on replication of the virus. The complete expression of NS3 proteinase activity is dependent upon the noncovalent assembly of NS3 and NS4a molecules, with the small NS4a component forming an integral part of the proteinase structure. Several pharmaceutical companies have independently solved the crystallographic structure of the proteinase domain of NS3, or the NS3–NS4a proteinase complex [23,80]. Thus, efforts at rational, structure-based drug design are ongoing, along with more conventional screening of compound libraries using biochemical assays of the NS3 proteinase activities. Thus far, however, no highly active inhibitors of this proteinase have yet been described in the literature.

NS3 Helicase

The carboxy terminal two-thirds of the NS3 molecule contains an RNA helicase activity [24,81]. This is almost certainly essential for replication of the viral RNA, although its precise role in the replication cycle is not known. The NS3 helicase domain can be expressed as an active enzyme, permitting the development of in vitro screening assays for compounds with specific inhibitory activities. Like the proteinase domain, the crystallographic structure of the helicase has been solved [25,82]. This will permit the application of structure-based, rational drug design strategies to the refinement of any lead compounds that may be identified in helicase screens.

NS5B RNA Polymerase

The NS5B molecule is the RNA-dependent RNA polymerase that is primarily responsible for the transcription of both the positive and negative strands of viral RNA during virus replication. Several research groups have expressed it in an active form from recombinant cDNA [83,84].

However, activity has been limited to nonspecific, primer-dependent transcription of a variety of RNA transcripts, or to primer-independent terminal transferase activity. No research group has yet reported the reconstruction of an active replicase complex that is capable of specific recognition and initiation of transcription of the 3′ end of either the positive- or negative-strand RNA. The reason for this is not clear, although it seems likely that a complex of several viral proteins (and possibly certain cellular proteins as well) might be required for such activity [27]. Thus, while it is possible to screen compound libraries for inhibitors of NS5b primer extension or terminal transferase activities, it is not possible to screen for specific HCV RNA transcription inhibitors. No specific inhibitors have yet been described for the former activities, although there are intense efforts to develop such compounds ongoing within both the pharmaceutical and academic communities.

Internal Ribosome Entry Site

The IRES represents an interesting and completely novel target for antiviral drug development. This segment of the viral genome is essential for the initiation of translation of the polyprotein, and thus inhibition of IRES activity would be expected to have a profound effect on replication of the virus [14]. Recent work suggests that the primary step in the initiation of cap-independent translation of the polyprotein by the IRES is the specific recognition of the 40S ribosome subunit by the IRES [85]. The ability to directly form a binary complex with the 40S subunit in the absence of any canonical or noncanonical translation initiation factor is unique among all eukaryotic RNAs. Similar activities are found only in related IRES segments from other Flaviviridae. This activity should be amenable to inhibition by small molecules which have a high affinity for the RNA structures responsible for this 40S subunit binding activity. A number of pharmaceutical companies are actively pursuing this possibility, but no specific inhibitors of the HCV IRES have yet been described.

Conclusions

Cirrhosis and hepatocellular carcinoma result from longstanding hepatic inflammation related to the persistence of HCV within the liver. Thus, this pathologic process reflects the presence of an active immune response to

the infection, yet one which is ineffectual in elimination of the virus. The reasons underlying the ability of this virus to persist in humans despite this immune response are not well understood, but are likely to involve a specific mechanism to "disarm" the immune system. Because of the extreme technical difficulties inherent in HCV vaccine development, present efforts are largely directed at the development of better therapies for chronic hepatitis C. Recent experience with the combination of ribavirin and interferon-α suggest that combination therapies will offer particular advantages in the treatment of this disease. This concept is well supported by earlier experience in the treatment of several other chronic infectious diseases. Thus, the search is on for novel, small molecule inhibitors of HCV replication. This search is complicated by the absence of tractable cell culture systems for propagation of the virus, but a number of biochemical assays have been developed which are now being used as screens for active compounds. It is highly likely that this intense research activity will result in a variety of novel therapeutic agents, and that this will open the door to a new era in the prevention of HCV-related liver disease.

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References

1. Choo Q-L, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359–362
2. Kuo G, Choo Q-L, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miyamura T, Dienstag JL, Alter MJ, Stevens CE, Tegtmeier GE, Bonino F, Colombo M, Lee W-S, Kuo C, Berger K, Shuster JR, Overby LR, Bradley DW, Houghton M (1989) An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244:362–364
3. Alter MJ, Margolis HS, Krawczynski K, Judson FN, Mares A, Alexander WJ, Hu PY, Miller JK, Gerber MA, Sampliner RE, Meeks EL, Beach MJ (1992) The natural history of community-acquired hepatitis C in the United States. N Engl J Med 327:1899–1905
4. Jeffers LJ, Hasan F, de Medina M, Reddy R, Parker T, Silva M, Mendez L, Schiff ER, Manns M, Houghton M, Choo Q-L, Kuo G (1992) Prevalence of antibodies to hepatitis C virus among patients with cryptogenic chronic hepatitis and cirrhosis. Hepatology 15:187–190
5. Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, Watanabe Y, Koi S, Onji M, Ohta Y, Choo Q-L, Houghton M, Kuo G (1991) Hepatitis C virus

infection is associated with the development of hepatocellular carcinoma. Proc Natl Acad Sci USA 87:6547–6549
6. Lemon SM, Chisari FV, Lai MMC, Mishiro S, Nishioka K, Johnson L (1998) Meeting report: the 19th Joint U.S.-Japan Hepatitis Panel Meeting. In press
7. Alter MJ (1997) Epidemiology of hepatitis C. Hepatology 26 Suppl 1:62S–65S
8. Consensus Development Panel (1997) National Institutes of Health Consensus Development Conference Panel statement: Management of hepatitis C. Hepatology 26 Suppl 1:2S–10S
9. Hsu S-L, Lin Y-F, Chou C-K (1993) Retinoic acid biphasically regulates the gene expression of hepatitis B virus surface antigen in human hepatoma Hep3B cells. J Biol Chem 268:23093–23097
10. Kurosaki M, Enomoto N, Sakamoto N, Tanaka Y, Tang L, Hoshino Y, Izumi Y, Marumo F, Sato C (1995) Detection and analysis of replicating hepatitis C virus RNA in hepatocellular carcinoma tissues. J Hepatol 22:527–535
11. Nishioka K, Mishiro S, Yoshizawa H (1996) Hepatitis C virus infection in the general population of Japan: past and future. Viral Hepatitis Rev 2:199–203
12. Houghton M, Weiner A, Han J, Kuo G, Choo Q-L (1991) Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. Hepatology 14:381–388
13. Major ME, Feinstone SM (1997) The molecular virology of hepatitis C. Hepatology 25:1527–1538
14. Lemon SM, Honda H (1998) Internal ribosome entry sites within the RNA genomes of hepatitis C virus and other flaviviruses. Semin Virol 8:274–288
15. Kolykhalov AA, Feinstone SM, Rice CM (1996) Identification of a highly conserved sequence element at the 3′ terminus of hepatitis C virus genome RNA. J Virol 70:3363–3371
16. Tanaka T, Kato N, Cho M-J, Shimotohno K (1995) A novel sequence found at the 3′ terminus of the hepatitis C virus genome. Biochem Biophys Res Commun 215:744–749
17. Hijikata M, Kato N, Ootsuyama Y, Nakagawa M, Shimotohno K (1991) Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. Proc Natl Acad Sci USA 88:5547–5551
18. Hijikata M, Mizushima H, Akagi T, Mori S, Kakiuchi N, Kato N, Tanaka T, Kimura K, Shimotohno K (1993) Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. J Virol 67:4665–4675
19. Reed KE, Grakoui A, Rice CM (1995) Hepatitis C virus-encoded NS2–3 protease: Cleavage-site mutagenesis and requirements for bimolecular cleavage. J Virol 69:4127–4136
20. Dubuisson J, Hsu HH, Cheung RC, Greenberg HB, Russell DG, Rice CM (1994) Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. J Virol 68:6147–6160
21. Dubuisson J, Rice CM (1996) Hepatitis C virus glycoprotein folding: disulfide bond formation and association with calnexin. J Virol 70:778–786

22. Failla C, Tomei L, De Francesco R (1994) Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. J Virol 68:3753–3760
23. Kim JL, Morgenstern KA, Lin C, Fox T, Dwyer MD, Landro JA, Chambers SP, Markland W, Lepre CA, O'Malley ET, Harbeson SL, Rice CM, Murcko MA, Caron PR, Thomson JA (1996) Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. Cell 87:343–355
24. Preugschat F, Averett DR, Clarke BE, Porter DJT (1996) A steady-state and pre-steady-state kinetic analysis of the NTPase activity associated with the hepatitis C virus NS3 helicase domain. J Biol Chem 271:24449–24457
25. Yao NH, Hesson T, Cable M, Hong Z, Kwong AD, Le HV, Weber PC (1997) Structure of the hepatitis C virus RNA helicase domain. Nature Struct Biol 4:463–467
26. Asabe SI, Tanji Y, Satoh S, Kaneko T, Kimura K, Shimotohno K (1997) The N-terminal region of hepatitis C virus-encoded NS5A is important for NS4A-dependent phosphorylation. J Virol 71:790–796
27. Ishido S, Fujita T, Hotta H (1998) Complex formation of NS5B with NS3 and NS4A proteins of hepatitis C virus. Biochem Biophys Res Commun 244:35–40
28. Lanford RE, Chavez D, Chisari FV, Sureau C (1995) Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific rTth reverse transcriptase PCR. J Virol 69:8079–8083
29. Anderson DA, Ross BC, Locarnini SA (1988) Restricted replication of hepatitis A virus in cell culture: encapsidation of viral RNA depletes the pool of RNA available for replication. J Virol 62:4201–4206
30. Mizutani T, Kato N, Saito S, Ikeda M, Sugiyama K, Shimotohno S (1996) Characterization of hepatitis C virus replication in cloned cells obtained from a human T-cell leukemia virus type 1-infected cell line, MT-2. J Virol 70:7219–7223
31. Shimizu YK, Iwamoto A, Hijikata M, Purcell RH, Yoshikura H (1992) Evidence for in vitro replication of hepatitis C virus genome in a human T-cell line. Proc Natl Acad Sci USA 89:5477–5481
32. Shimizu YK, Yoshikura H (1994) Multicycle infection of hepatitis C virus in cell culture and inhibition by alpha and beta interferons. J Virol 68:8406–8408
33. Binn LN, Lemon SM, Marchwicki RH, Redfield RR, Gates NL, Bancroft WH (1984) Primary isolation and serial passage of hepatitis A virus strains in primate cell cultures. J Clin Microbiol 20:28–33
34. Bukh J, Miller RH, Purcell RH (1995) Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. Semin Liv Dis 15:41–63
35. Honda M, Brown EA, Lemon SM (1996) Stability of a stem-loop involving the initiator AUG controls the efficiency of internal initiation of translation on hepatitis C virus RNA. RNA 2:955–968
36. Reynolds JE, Kaminski A, Carroll AR, Clarke BE, Rowlands DJ, Jackson RJ (1996) Internal initiation of translation of hepatitis C virus RNA: the ribosome entry site is at the authentic initiation codon. RNA 2:867–878

37. Rijnbrand RCA, Abbink TEM, Haasnoot PCJ, Spaan WJM, Bredenbeek PJ (1996) The influence of AUG codons in the hepatitis C virus 5′ nontranslated region on translation and mapping of the translation initiation window. Virology 226:47–56
38. Kato N, Sekiya H, Ootsuyama Y, Nakazawa T, Hijikata M, Ohkoshi S, Shimotohno K (1993) Humoral immune response to hypervariable region 1 of the putative envelope glycoprotein (gp70) of hepatitis C virus. J Virol 67:3923–3930
39. Shimizu YK, Hijikata M, Iwamoto A, Alter HJ, Purcell RH, Yoshikura H (1994) Neutralizing antibodies against hepatitis C virus and the emergence of neutralization escape mutant viruses. J Virol 68:1494–1500
40. Weiner A, Erickson AL, Kansopon J, Crawford K, Muchmore E, Hughes AL, Houghton M, Walker CM (1995) Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T lymphocyte escape variant. Proc Natl Acad Sci USA 92:2755–2759
41. Lesniewski R, Okasinski G, Carrick R, Van Sant C, Desai S, Johnson R, Scheffel J, Moore B, Mushahwar I (1995) Antibody to hepatitis C virus second envelope (HCV-E2) glycoprotein: a new marker of HCV infection closely associated with viremia. J Med Virol 45:415–422
42. Alberti A, Morsica G, Chemello L, Cavalletto D, Noventa F, Pontisso P, Ruol A (1992) Hepatitis C viraemia and liver disease in symptom-free individuals with anti-HCV. Lancet 340:697–698
43. Lesniewski RR, Boardway KM, Casey JM, Desai SM, Devare SG, Leung TK, Mushahwar IK (1993) Hypervariable 5′-terminus of hepatitis C virus E2/NS1 encodes antigenically distinct variants. J Med Virol 40:150–156
44. Matsumoto M, Hsieh TY, Zhu NL, VanArsdale N, Hwang SB, Jeng KS, Gorbalenya AW, Lo SY, Ou JH, Ware CF, Lai MHC (1997) Hepatitis C virus core protein interacts with the cytoplasmic tail of lymphotoxin-receptor. J Virol 71:1301–1309
45. Chen CM, You LR, Hwang LH, Lee YH (1997) Direct interaction of hepatitis C virus core protein with the cellular lymphotoxin-beta receptor modulates the signal pathway of the lymphotoxin-beta receptor. J Virol 71:9417–9426
46. Ray RB, Meyer K, Steele R, Shrivastava A, Aggarwal BB, Ray R (1998) Inhibition of tumor necrosis factor (TNF-alpha)-mediated apoptosis by hepatitis C virus core protein. J Biol Chem 273:2256–2259
47. Ruggieri A, Harada T, Matsuura Y, Miyamura T (1997) Sensitization to Fas-mediated apoptosis by hepatitis C virus core protein. Virology 229:68–76
48. Lerat H, Berby F, Trabaud MA, Vidalin O, Major M, Trepo C, Inchauspe G (1996) Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. J Clin Invest 97:845–851
49. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C (1996) Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 334:77–81
50. Kurosaki M, Enomoto N, Murakami T, Sakuma I, Asahina Y, Yamamoto C, Ikeda T, Tozuka S, Izumi N, Marumo F, Sato C (1997) Analysis of genotypes and amino acid residues 2209 to 2248 of the NS5A region of hepatitis C virus in relation to the response to interferon-therapy. Hepatology 25:750–753

51. Gale MJ Jr, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, Polyak SJ, Gretch DR, Katze MG (1997) Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology 230:217–227
52. Choo Q-L, Kuo G, Ralston R, Weiner A, Chien D, Van Nest G, Han J, Berger K, Thudium K, Kuo C, Kansopon J, McFarland J, Tabrizi A, Ching K, Moss B, Cummins LB, Houghton M, Muchmore E (1994) Vaccination of chimpanzees against infection by the hepatitis C virus. Proc Natl Acad Sci USA 91:1294–1298
53. Klavinskis LS, Whitton JL, Oldstone MBA (1989) Molecularly engineered vaccine which expresses an immunodominant T-cell epitope induces cytotoxic T lymphocytes that confer protection from lethal virus infection. J Virol 63:4311–4316
54. González A, Esteban JI, Madoz P, Viladomiu L, Genesca J, Muñoz E, Enríquez J, Torras X, Hernández JM, Quer J, Vidal X, Alter HJ, Shih JW, Esteban R, Guardia J (1995) Efficacy of screening donors for antibodies to the hepatitis C virus to prevent transfusion-associated hepatitis: Final report of a prospective trial. Hepatology 22:439–445
55. Nishioka K (1996) Transfusion-transmitted HBV and HCV. Vox Sang 70:4–8
56. Davis GL, Balart LA, Schiff ER, Lindsay K, Bodenheimer HC. Jr, Perillo RP, Carey W, Jacobson IM, Payne J, Dienstag JL, VanThiel DH, Tamburro C, Lefkowitch J, Albrecht J, Meschievitz C, Ortego TJ, Gibas A, Hepatitis Interventional Therapy Group (1989) Treatment of chronic hepatitis C with recombinant interferon alfa: A multicenter randomized, controlled trial. N Engl J Med 321:1501–1506
57. Di Bisceglie AM, Martin P, Kassianides C, Lisker-Melman M, Murray L, Waggoner J, Goodman Z, Banks SM, Hoofnagle JH (1989) Recombinant interferon alfa therapy for chronic hepatitis C: a randomized, double-blind, placebo-controlled trial. N Engl J Med 321:1506–1510
58. Sánchez-Royuela F, Porres JC, Moreno A, Castillo I, Martinez G, Galiana F, Carreño V (1991) High doses of recombinant-interferon or gamma-interferon for chronic hepatitis C: A randomized, controlled trial. Hepatology 13:327–331
59. Nishiguchi S, Kuroki T, Nakatani S, Morimoto H, Takeda T, Nakajima S, Shiomi S, Seki S, Kobayashi K, Otani S (1995) Randomised trial of effects of interferon-α on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. Lancet 346:1051–1055
60. Kiyosawa K, Furuta S (1994) Hepatitis C virus and hepatocellular carcinoma. In Reesink HW (Ed) Hepatitis C Virus. Karger, Basel, pp 98–120
61. Mazzella G, Accogli E, Sottili S, Festi D, Orsini M, Salzetta A, Novelli V, Cipolla A, Fabbri C, Pezzoli A, Roda E (1996) Alpha interferon treatment may prevent hepatocellular carcinoma in HCV-related liver cirrhosis. J Hepatol 24:141–147
62. McCormick JB, King IJ, Webb PA, Scribner CL, Craven RB, Johnson KM, Elliott LH, Belmont-Williams R (1986) Lassa fever. Effective therapy with ribavirin. N Engl J Med 314:20–26
63. Smith DW, Frankel LR, Mathers LH, Tang AT, Ariagno RL, Prober CG (1991) A controlled trial of aerosolized ribavirin in infants receiving mechanical ventilation for severe respiratory syncytial virus infection. N Engl J Med 325:24–29

64. Toltzis P, Huang AS (1986) Effect of ribavirin on macromolecular synthesis in vesicular stomatitis virus-infected cells. Antimicrob. Agents Chemother 29:1010–1016
65. Rankin JT Jr, Eppes SB, Antczak JB, Joklik WK (1989) Studies on the mechanism of the antiviral activity of ribavirin against reovirus. Virology 168:147–158
66. Bodenheimer HC Jr, Lindsay KL, Davis GL, Lewis GH, Thung SN, Seeff LB (1997) Tolerance and efficacy of oral ribavirin treatment of chronic hepatitis C: a multicenter trial. Hepatology 26:473–477
67. Di Bisceglie AM, Conjeevaram HS, Fried MW, Sallie R, Park Y, Yurdaydin C, Swain M, Kleiner DE, Mahaney K, Hoofnagle JH, Wright D (1995) Ribavirin as therapy for chronic hepatitis C—a randomized, double-blind, placebo-controlled trial. Ann Intern Med 123:897–903
68. Di Bisceglie AM, Shindo M, Fong T-L, Fried MW, Swain MG, Bergasa NV, Axiotis CA, Waggoner JG, Park Y, Hoofnagle JH (1992) A pilot study of ribavirin therapy for chronic hepatitis C. Hepatology 16:649–654
69. Dusheiko G, Main J, Thomas H, Reichard O, Lee C, Dhillon A, Rassam S, Fryden A, Reesink H, Bassendine M, Norkrans G, Cuypers T, Lelie N, Telfer P, Watson J, Weegink C, Sillikens P, Weiland O (1996) Ribavirin treatment for patients with chronic hepatitis C: results of a placebo-controlled study. J Hepatol 25:591–598
70. Lai MY, Kao JH, Yang PM, Wang JT, Chen PJ, Chan KW, Chu JS, Chen DS (1996) Long-term efficacy of ribavirin plus interferon alfa in the treatment of chronic hepatitis C. Gastroenterology 111:1307–1312
71. Reichard O, Norkrans G, Fryden A, Braconier JH, Sonnerborg A, Weiland O (1998) Randomised, double-blind, placebo-controlled trial of interferon alpha-2b with and without ribavirin for chronic hepatitis C. The Swedish Study Group (see comments). Lancet 351:83–87
72. Bellobuono A, Mondazzi L, Tempini S, Silini E, Vicari F, Ideo G (1997) Ribavirin and interferon-alpha combination therapy vs interferon-alpha alone in the retreatment of chronic hepatitis C: a randomized clinical trial. J Viral Hepatal 4:185–191
73. Di Bisceglie AM, Bacon BR, Kleiner DE, Hoofnagle JH (1994) Increase in hepatic iron stores following prolonged therapy with ribavirin in patients with chronic hepatitis C. J Hepatol 21:1109–1112
74. Takahashi T, Hosoya M, Kimura K, Ohno K, Mori S, Takahashi K, Shigeta S (1998) The cooperative effect of interferon-alpha and ribavirin on subacute sclerosing panencephalitis (SSPE) virus infections, in vitro and in vivo (In Process Citation). Antiviral Res 37:29–35
75. Ning Q, Brown D, Parodo J, Cattral M, Gorczynski R, Cole E, Fung L, Ding JW, Liu MF, Rotstein O, Phillips MJ, Levy G (1998) Ribavirin inhibits viral-induced macrophage production of TNF, IL-1, the procoagulant fgl2 prothrombinase and preserves Th1 cytokine production but inhibits Th2 cytokine response [In Process Citation]. J Immunol 160:3487–3493
76. Jiang Z, Kunimoto M, Patel JA (1998) Autocrine regulation and experimental modulation of interleukin-6 expression by human pulmonary epithelial cells infected with respiratory syncytial virus (In Process Citation). J Virol 72:2496–2499

77. Yamada Y, Natsumeda Y, Weber G (1988) Action of the active metabolites of tiazofurin and ribavirin on purified IMP dehydrogenase. Biochemistry 27:2193–2196
78. Grakoui A, McCourt DW, Wychowski C, Feinstone SM, Rice CM (1993) Characterization of the hepatitis C virus-encoded serine proteinase: Determination of proteinase-dependent polyprotein cleavage sites. J Virol 67:2832–2843
79. Tanji Y, Hijikata M, Hirowatari Y, Shimotohno K (1994) Hepatitis C virus polyprotein processing: kinetics and mutagenic analysis of serine proteinase-dependent cleavage. J Virol 68:8418–8422
80. Love RA, Parge HE, Wickersham JA, Hostomsky Z, Habuka N, Moomraw EW, Adachi T, Hostomska Z (1996) The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. Cell 87:331–342
81. Tai CL, Chi WK, Chen DS, Hwang LH (1996) The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3). J Virol 70:8477–8484
82. Kim JL, Morgenstern KA, Griffith JP, Dwyer MD, Thomson JA, Murcko MA, Lin C, Caron PR (1998) Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. Structure 6:89–100
83. Behrens SE, Tomei L, De Francesco R (1996) Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. EMBO J 15:12–22
84. Lohmann V, Korner F, Herian U, Bartenschlager R (1997) Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. J Virol 71:8416–8428
85. Pestova TV, Shatsky IN, Fletcher SP, Jackson RJ, Hellen CU (1998) A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. Genes Dev 12:67–83