

HEPATOLOGY HIGHLIGHTS

Harvey J. Alter, *Viral Hepatitis Editor*

The Inner Life of an HBV Surface Mutant

The expanded use of hepatitis B vaccines and the immune pressure associated with hepatitis B immunoglobulin administered in the transplantation setting has led to an increasing number of HBV surface antigen mutations. The most common of these mutations is a glycine-to-arginine substitution at amino acid position 145 (G145R). Kalinina et al. studied the biologic properties of the G145R mutant compared with the wild-type (wt) virus by constructing infectious plasmids under a cytomegalovirus promoter and then transfecting human hepatoma cells. The key finding is that the mutation did not affect production of HBV DNA, messenger RNA, or surface proteins. The noteworthy biologic effect of the mutation was reduction of virion secretion by 80% compared with wt; the secretion of nonvirion (empty-particle) small S protein was not affected. In addition, mutant virion was significantly more sensitive to detergent treatment, implying diminished stability of its envelope. In noteworthy cotransfection studies, Kalinina and colleagues demonstrated that increasing amounts of mutant virus decreased wt virus secretion by up to 70% in a dose-dependent manner. This finding suggests that mutant S protein may hamper core envelopment by wt surface protein. In contrast, the secretory capacity of the mutant virus was rescued (*i.e.*, enhanced) when mutant cells were cotransfected with wt virus.

Thus, the G145R mutant preferentially reduces secretion of infectious virions, probably because the presence of the basic Arg145 residue disrupts interactions between acidic residues on the outer surface of the core protein and basic residues on the inner surface of the envelope protein. Impaired envelopment of the core then would result in decreased virion secretion but normal small surface antigen secretion; this has clinical significance, as it should reduce the infectivity of persons with homogeneous mutant populations and perhaps even those with mixed populations. Overall, because of its low infectivity and surface instability, the mutant agent does not have a selective advantage over wt except when there is persisting immune pressure, as during the administration of hepatitis B immunoglobulin. In transplant recipients, when hepatitis B immunoglobulin is withdrawn, the wt virus returns rapidly. In addition, it has been shown in chimpanzees that animals infected with the mutant virus rapidly revert to wt if there is no exogenous immune pressure. Thus, although there is theoretic concern that this surface antigen mutant could become dominant, its low virion production and surface instability diminish its clinical import and suggest that such mutations will not be a major threat to the ex-

panded use of hepatitis B vaccines. (See HEPATOLOGY 2003;38: 1273–1281.)

There's No Ignorin' Cyclosporin: An Unexpected Effect on HCV Replication

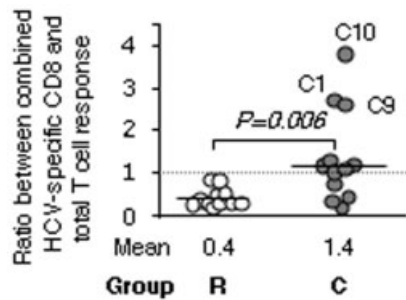
The availability of an HCV subgenomic replicon system has allowed the relatively easy screening of drugs for their antiviral effects. In the process of exploring various types of compounds, Watashi et al. found that cyclosporin A had a profound inhibitory effect on HCV protein expression in both replicon cells and cultured human hepatocytes. Treatment with either 1 $\mu\text{g}/\text{mL}$ cyclosporin A or 100 units/mL interferon (IFN) alfa (positive control) for 7 days reduced NS5A and NS5B protein production to undetectable levels, with a sequential decline over the 7-day study interval. In contrast, treatment with FK506, another immunosuppressive agent that similarly inhibits calcinurin, had no effect on HCV protein production. Indirect immunofluorescence showed that NS5A levels were reduced in all cells, whereas a control nonviral cell marker was unaffected by cyclosporin A. The inhibitory effects on nonstructural proteins were validated by demonstrating similar inhibition of replicon RNA levels after treatment with cyclosporin A and IFN but not after treatment with FK506; quantitative studies found that the HCV replicon RNA titer was reduced to 1/500 of its original value and that the replicon level of cells treated with both cyclosporin A and IFN was approximately 1/5 that of cells treated with IFN alone.

Thus, the authors make a very strong case that cyclosporin A inhibits HCV growth *in vitro*. They then demonstrated that the mechanism of action of cyclosporin A was independent of IFN action by showing that cyclosporin A did not induce the expression of key IFN- α -downstream genes, such as 2',5'-oligoadenylate synthetase. Finally, they demonstrated that the antiviral effect of cyclosporin A was independent of its immunosuppressive activity in that cyclosporin A did not inhibit the calcinurin/NF-AT pathway in cultured hepatocytes. This pathway is essential to cyclosporin A immunosuppression.

These data suggest that cyclosporin A may be a useful addition to antiviral therapy; however, optimism is tempered by the finding that patients who have undergone transplants and receive cyclosporin A generally replicate HCV at high levels. A possible explanation for this disparity is that *in vivo*, the direct antiviral effect of cyclosporin A may be overwhelmed by its opposing immunosuppressive effect, which might foster viral replication by mechanisms that currently are undefined. Thus, these promising *in vitro* findings may not translate directly to the clinic. Nonetheless, proof of principle has been established, and there is some evidence that the cyclosporin A analogue

NIM811, currently under study, manifests antiviral activity in the absence of immunosuppressive activity. One easily could envision a cocktail of IFN, ribavirin, and a nonimmunosuppressive cyclosporin A analogue. Furthermore, elucidation of the mechanism of the antiviral activity of cyclosporin A may reveal new critical steps in HCV replication and novel targets for other antiviral therapies. (See HEPATOLOGY 2003;38:1282–1288.)

It's the CD4 Cell, Stupid!



I must begin with the disclaimer that I am highlighting a study in which I am a coauthor. The disclaimer is that despite my participation, the study of Sugimoto et al. is excellent and makes many noteworthy and synthesizing observations.

There has been unanimity in multiple publications regarding the critical role that CD4 cells play in recovery from HCV infection. Sugimoto et al. expand these observations by using overlapping peptides across most of the HCV genome (envelope excluded) as assay targets rather than preselecting established human leukocyte antigen–restricted T-cell epitopes and by using *ex vivo* techniques that do not require T-cell expansion before study. The investigators created 36 pools, each containing 10 or 11 overlapping peptides, and demonstrated that reactivity to even a single peptide within the pool was readily detected in the pooled assay. Using the enzyme-linked immunosorbent spot assay for IFN- γ , they demonstrated that: 1) consistent with other studies, recovered patients had far more vigorous and broadly reactive CD4 responses than did chronically infected patients; 2) although the CD4 response differed significantly between recovered and chronically infected patients, there was no difference in CD8 responses; 3) depletion of CD4 cells in recovered patients resulted in diminished *ex vivo* response, whereas it resulted in enhanced reactivity in chronically infected patients, suggesting that in chronic infection, there is a population of CD4 cells that suppress CD8 reactivity (this was the most unique finding); 4) the suppression of T cells in chronically infected patients was related to cells that were CD4⁺ and CD25⁺ (Tregs); this effect was dose dependent and HCV antigen specific; the frequency of Tregs was significantly greater in chronically infected patients than in recovered patients ($P = .002$); and 5) the weak, circulating HCV-specific type 1 T-cell response in chronically infected patients was inversely correlated with viral titer, consistent with the observation of higher viral titers in immunosuppressed patients. Overall, these findings suggest that HCV may promote its own survival by up-regulating Tregs that suppress HCV-specific T cells. The mechanism of this interaction has not yet been elucidated.

Like the study of Sugimoto et al., most studies of cell-mediated immunity in HCV infection compare recovered patients with chronically infected patients and surmise what may have occurred during acute infection. The chimpanzee model allows one to assess acute-phase cellular samples that rarely are avail-

able in humans. Wollard et al. characterized CD4 responses during acute HCV infection in the chimpanzee. Notably, they found that CD4⁺ T cells targeting HCV nonstructural proteins could be detected with proliferation assays by Week 6 postinfection but that such cells did not produce IFN- γ until Week 8, coincident with a 10-fold drop in viral titer. In addition, IFN- γ -producing cytotoxic CD8 cells with broad HCV specificity, including E-1, were found in the liver and peripheral blood mononuclear cells. Recovery was not correlated with antibody responses to core, NS-3, or NS-5, and there was no detectable antibody to envelope proteins. Thus, the findings of this chimpanzee study support the critical role played by CD4 cells and the combined interactions of CD4 and effector CD8 cells in the resolution of acute HCV infection.

A cohesive picture is emerging in which the CD4 cell is the critical determinant of an effective cell-mediated immune response, which, in concert with CD8 effector cells, can resolve HCV infection in 20%–30% of cases. Nonetheless, in the majority of infected patients, there is specific impairment of CD4 proliferation and IFN- γ production that results in viral persistence. The CD4 cell appears to be rendered anergic to HCV, and the presumption is that tolerance is the result of specific, but as yet incompletely defined, interactions with viral proteins. In addition, it now appears that HCV can stimulate CD4⁺/CD25⁺ T regulatory cells that further suppress CD8 effector cells. This virus-induced immune paralysis, together with the enormous variability of the HCV quasispecies, makes this agent ideally suited to evade host immune responses and establish lifelong infections. In short, this is the virus that came to dinner. (See HEPATOLOGY 2003;38:1437–1448.)

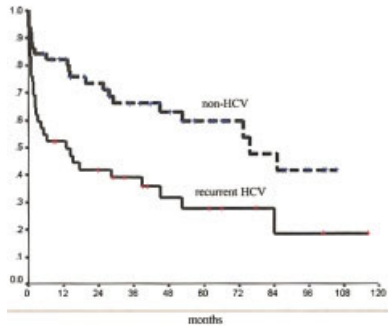
Are Reactive Oxygen Species Helpful or Harmful in HCV Infection?

Generally, I am unreactive to articles on reactive oxygen species, but the study of Choi et al. caught my attention, due to the quality of the work and its implications for HCV pathogenesis and (possibly) treatment. Choi et al. used bicistronic, subgenomic, and hybrid genomic replicons to infect HuH7 hepatoma cells and then measured the effects of physiologic amounts of reactive oxygen species; H₂O₂ caused a dose-dependent decrease of up to 60% in both positive- and negative-stranded HCV RNA and similar changes in NS3/NS5 protein levels. These negative effects could be reversed by the addition of an antioxidant compound. Labeling studies and replication assays on cytoplasmic lysates demonstrated that the decline in RNA levels was not due to rapid degradation, but rather to diminished replication, particularly in the Golgi fractions. The presumption is that reactive oxygen species interfere with HCV replication complexes within the Golgi apparatus, probably by affecting signal pathways.

This raises a dilemma. It previously has been postulated that production of reactive oxygen species plays a role in HCV-related liver destruction and thus has a deleterious effect. The study of Choi et al. suggests that reactive oxygen species impair viral replication and thus have a beneficial effect. The net effect is unknown. The use of antioxidants as adjunctive therapy to counteract the presumed deleterious effects of reactive oxygen species in liver dis-

ease is under study. If used against HCV infection, could antioxidants increase viral replication by neutralizing the antireplicative effects of reactive oxygen species? Now that's something to be re-active to. (See HEPATOLOGY 2004;39:81–89.)

The Second Time Around: Retransplantation for Recurrent Hepatitis C



Roayaie et al. present a sobering view of the outcome of retransplantation for recurrent HCV. Of 116 patients listed more than 90 days after initial transplantation, 32% died while waiting, 24% still are waiting, and 44% (51

patients) received a second transplant. Forty-two of these 51 lost their first transplant because of HCV-induced cirrhosis and are the focus of the study. Of the 42, only 13 (31%) are alive, at a median of 41 months follow-up; 20 patients (48%) died after < 6 months, 65% of these 20 due to sepsis; 9 (21%) died > 6 months after transplantation, 66% of these 9 due to recurrent HCV. Overall, 70% of patients receiving second transplants died after < 3 years due to early sepsis or late complications of HCV. The median survival time was 12.9 ± 6.7 months. Of the 13 living patients, only 4 are faring well. Seven patients underwent a third transplantation; five of these seven died. Patients who underwent retransplantation for HCV fared significantly worse ($P = .002$) than did those who received transplants for other conditions. The most significant predictors of poor outcome in a multivariate analysis were PT (Prothrombin Time) > 16 and donor age > 60 years. The pretransplantation MELD score was not correlated with survival after the second transplantation.

Thus, if hepatitis C is severe the first time around, it will be more severe the second time around, and still more severe the third time around. This raises the eternal dilemma of the seed versus the soil. Is this process selecting bad hosts or virulent agents? Are there more-virulent strains of HCV that initially result in cirrhosis and then cause increasingly rapid cirrhosis as they reinfect each new liver, or are there hosts who, by virtue of genetic make-up and/or impaired immunity, constitute the 20%–30% who develop cirrhosis from the primary infection and then again each time they receive an HCV-naïve liver? This issue cannot be resolved at present, but it is fundamental to our understanding of the pathogenesis of hepatitis C.

This study also raises difficult ethical issues. Given these poor outcomes in a subset of patients, are we justified in using the limited number of available livers for retransplantation rather than primary transplantation in patients who might fare better? Are we under obligation to provide a second liver, if necessary, once committed to a patient for the primary transplantation? These will remain incredibly difficult questions as long as the supply of organs remains limited. Overall, we need better seed, better soil, and more livers (or liver equivalents). (See HEPATOLOGY 2003;38:1428–1436.)

Out of the Livers of Babes

A significant proportion of HCV-infected adults have no identified route of exposure to the virus. On the premise that these cases might be derived from neonatal 'minitransfusions', Casiraghi et al. reviewed the hospital records of children born in 1968 in a single hospital in Italy. One hundred sixty-six infants were found to have received a minitransfusion, in which blood from a single donor was transfused in small aliquots to multiple recipients. Thirty-six such donors were traced, and 1 was found to be positive for anti-HCV after he donated 4 units of blood that were divided and transfused to 43 infants. Thirty-one of these 43 presumably exposed recipients were traced 35 years after their minitransfusions; 18 of the 31 (58%) had anti-HCV, and 16 of these 18 were positive for HCV RNA. All 16 viremic recipients and the donor shared a common genotype and were related by phylogenetic analysis. Seventeen of 18 infected recipients had no other risk factors for HCV acquisition. Eleven of the 16 viremic patients underwent liver biopsy; 5 with normal alanine aminotransferase levels refused biopsy. Thirty-five years after infection with HCV, the mean Ishak inflammatory score was 4.6 ± 0.8 , and the mean fibrosis score was 1.4 ± 1.1 . No patient had more than mild inflammatory activity, and 9 of 11 (82%) had no fibrosis or only 1+ fibrosis. Two patients (18%) had Stage 3 or 4 bridging fibrosis; none had cirrhosis. Liver biopsy was repeated 5 years later in 5 untreated patients and showed no substantial change.

This is a noteworthy study from two perspectives. First, it demonstrates that careful epidemiologic study can uncover parenteral routes of HCV infection in many cases in which the infectious origin initially is not apparent. The use of minitransfusions is not unique to Italy and has been practiced widely, including in the United States, where the distribution of single-donor blood to multiple infant recipients was common as recently as 1985. Such recipients would not know or remember that they had received transfusions when questioned decades later about HCV exposure. This is only one means of occult parenteral transmission. It is probable that until awareness of acquired immunodeficiency syndrome resulted in more cautionary medical practices, many patients were infected through the use of multidose vials for vaccines, topical anesthetics, or other medications, and perhaps through the use of inadequately sterilized multiuse instruments in medical and dental practices. Such iatrogenic transmission has been well documented in the hepatitis B epidemic, due to yellow fever vaccine; in the HCV epidemic in Egypt, due to schistosomal treatments; and in cases of HCV infection traced to folk medicine practices in Japan. I suspect that such occult medical exposures account for most hepatitis C infections that are not caused by standard blood transfusion or intravenous drug use.

Second, the study by Casiraghi et al. further supports an increasing number of studies that have demonstrated the more benign outcome of HCV infections that are acquired in infancy, childhood, or young adulthood. Thus, even after follow-up periods of 20–50 years, cirrhosis and hepatocellular carcinoma rarely have been encountered in persons infected before age 40, and particularly before age 20, unless there have been major comorbidities, such as human immunodeficiency virus infection or alcoholism. (See HEPATOLOGY 2004;39:90–96.)