## Chapter 12

# Animal Models for HCV Study

*Linda B. Couto and Alexander A. Kolykhalov*

## ABSTRACT

The study of HCV biology is complicated by the paucity of relevant animal models. The ideal model for studying HCV would be one that adequately represents most aspects of human HCV infection and disease, is affordable, easily available, and reproducible. Currently, the only widely recognized animal model of HCV infection is the chimpanzee, which does not meet all of these desirable attributes. Recently, other models have been used to dissect various aspects of HCV biology and to evaluate novel therapeutics. Each has a unique set of advantages and limitations. Transgenic mouse models have elucidated the pathophysiology of specific viral proteins, but they are limited by their inability to support HCV replication. Xenograft models provide an environment for human hepatocyte engraftment in mice and subsequent infection with HCV. These models are technically challenging, but once optimized they promise to be extremely useful both for the study of HCV biology and for drug development. Alternatively, the GBV-B virus, which efficiently replicates in tamarins and marmosets, represents a surrogate model for the study of HCV. Chimeras between GBV-B and HCV have been created and will be useful in the development of HCV-targeting drugs.

## CHIMPANZEE MODEL

Use of the chimpanzee model to address questions regarding HCV biology is well justified by both the importance of HCV as a major healthcare problem and by the fact that the questions cannot be addressed otherwise. The chimpanzee is the closest genetic relative to human, which explains why many features of hepatitis C disease are so common between humans and chimps. Both humans and chimpanzees have detectable HCV RNA within a few days of infection. Maximum viral titers usually reach $10^5$-$10^7$ RNA genome copies per mL of blood. The rise in viremia is usually followed by an increase in serum liver enzymes, which peak between 2 and 12 weeks. The majority of infected chimpanzees have necroinflammatory changes in liver biopsies; typically the disease is somewhat milder than that observed in humans. Antibodies to HCV antigens usually appear around week 8 or after. This acute phase of infection is followed by transition toward chronic viral persistence. It was initially reported that chimpanzees have lower rates of chronicity compared to humans, ~40% and ~70-85%, respectively. Bassett et al. (1998) reported that a cross-

sectional study in the chimp colony at the Southwest Foundation for Biomedical Research, San Antonio, TX, revealed that out of 46 animals infected with different strains of HCV, only 18 (39%) were viremic based on reverse transcription-PCR analysis. More recent data on infection of naïve animals with HCV suggest that about 60% of all chimps became persistently infected; this rate of persistence is similar for HCV of different genotypes (Bigger et al., 2004; Nam et al., 2004). Chimpanzees with acute resolving infection usually clear virus in plasma during weeks 12 to 24.

As the only animal model for the study of HCV, the chimpanzee was used to provide early characteristics of HCV. Even before HCV had been identified, the chimpanzee was involved in the study of Non-A, Non-B hepatitis (NANBH) virus transmission, in establishment and duration of disease, and in the chronic nature of NANBH infection (Alter et al., 1978; Tabor et al., 1978). The first characteristics of the infectious agent, such as its size (Watanabe et al., 1987), and its inactivation with lipid solvents (i.e. the enveloped nature of the agent) (Bradley et al., 1983; Feinstone et al., 1983) were determined using the chimpanzees. Finally, the HCV genome was isolated and cloned for the first time from chimpanzee plasma with a high infectivity titer of Non-A, Non-B agent (Bradley et al., 1991; Choo et al., 1989).

The major advantages of the chimpanzee model stem from the ability to monitor and analyze the development of the disease from its initiation. Most clinical data on HCV infection in humans is derived from patients who have been infected for a period of time, often decades. Due to the asymptomatic nature of hepatitis C disease, the acute phase of the infection is often not noticeable, and thus, very little data exist regarding the events immediately following infection. In human studies, usually only samples of easily accessible tissues, such as blood, are available. Only a few liver biopsies per year can be performed in infected patients, which preclude efficient analysis of events in the primary tissue of HCV replication. On the contrary, liver biopsy samples from the chimpanzees can be obtained before the exposure and at planned intervals post-inoculation. These well controlled samples allowed the analysis of events starting immediately after HCV infection, such as changes in gene regulation and cellular immune responses to viral antigens. Furthermore, the possibility to rechallenge animals that cleared a previous infection, allowed memory immune responses to be analyzed (Bassett et al., 2001; Bigger et al., 2004; Farci et al., 1992; Ilan et al., 2002b; Prince et al., 1992; Weiner et al., 2001), as well as an analysis of HCV vaccine development (Forns et al., 2000; Houghton, 2000). Finally, the chimpanzee model was instrumental in the establishment of HCV infectious molecular clones (Kolykhalov et al., 1997; Yanagi et al., 1997). The virus recovered from an *in vitro* synthesized RNA resulted in the development in chimpanzees of classical signs of hepatitis disease, such as viremia, elevated serum

levels of hepatic enzymes, histologic changes in the liver, and the development of HCV specific antibodies, thus formally proving that HCV is the causative agent of the disease.

## REVERSE GENETICS/FUNCTIONAL ANALYSIS OF HCV

The ability to test the infectivity of molecular clones in chimpanzees allowed for the first time a reverse genetics analysis. This established the critical importance of all genome coded enzymatic activities, as well as some *cis*-acting elements in the HCV genome, for virus replication (Kolykhalov et al., 2000; Bukh et al., 1999). Though the HCV replicon tissue culture model is extremely useful for genetic analysis, it is restricted by the fact that replication of HCV RNA is not dependent on structural proteins (see Chapter 11). Thus, the chimpanzee model was required to demonstrate that the hypervariable region 1 (HVR1) of the envelope protein E2 is not critical for virus replication *in vivo*, and can be removed altogether (Forns et al., 2000). This result was somewhat unexpected since the HVR1 was considered among the primary regions of HCV to interact with the host immune system (Farci et al., 1996; Kato et al., 1993). In another experiment, it was demonstrated that p7 is absolutely essential for infectivity of HCV, and that the amino- and/or carboxyl-terminal intraluminal tails of p7 contain sequences with genotype-specific function (Sakai et al., 2003).

## MONOCLONAL INFECTIONS

Many experiments using patient-derived virus were complicated by the fact that HCV exists as a set of quasispecies. Replication of the viral genome depends on the genome-encoded RNA dependent RNA polymerase, which lacks proofreading activity (see Chapter 10). As a consequence, viral replication results in the accumulation of numerous genetic variants, called quasispecies. The quasispecies nature of an inocula was thought to explain the initial evolution of the virus *in vivo*, as well as the escape of the virus from the immune response (Hijikata et al., 1995; Kojima et al., 1994; Okamoto et al., 1992). Recovery of virus from *in vitro* synthesized infectious RNA allowed the creation of "monoclonal" virus pools, derived from a single cDNA molecule. Chimpanzee serum, collected during the first weeks following intrahepatic inoculation of infectious RNA, exhibited no genetic variability (Major et al., 1999), therefore, providing a unique starting material for the study of viral evolution and of virus-host interactions. Infection of chimpanzees with such virus simplifies studies of HCV, since the interpretation of results is not complicated by the quasispecies nature of the inocula. Thus, Major at al. (2004) published detailed results of the analysis of ten chimps all inoculated with the same monoclonal virus, representing the dominant variant in the most studied patient isolate H77. Six out of ten infected animals became chronically infected, which implied that the presence of quasispecies in the inocula is not a requirement for establishing chronicity. The acute phase of infection was similar in all animals, whether they resolved the infection or became chronically infected. The

maximal viral titers were 0.5-1 log higher in animals with chronic infection, usually between $10^6$ and $10^7$ RNA copies/ml. The viral load increased quickly during the first 1-2 weeks after infection (mean doubling time = 0.5 days) until reaching $10^3$ to $10^5$ copies/ml. This was followed by a significant delay in virus accumulation (mean doubling time = 7.5 days) over the next several weeks, during which titers increased by only 2 to 3 $\log_{10}$. Viral titers began to decrease in all animals as alanine amino transferase (ALT) responses increased, with peak RNA titers preceding ALT peaks by 2 to 3 weeks. ALT elevations in the serum are believed to be markers of hepatocyte death and could be due to killing of infected or bystander liver cells by the host immune response. Both the height and the time of the ALT peaks were similar between the groups. Following the ALT peak the viral titers decreased, and all infections resulted in 1 of 2 outcomes: persistence, with virus titers reaching a steady state at approximately $10^4$ to $10^5$ RNA copies/mL; or clearance, with titers continuing to decrease below levels of quantitation (<200 copies/mL in the study). After the decrease in viral titers, ALT levels returned to baseline in all animals despite significant levels of virus in those animals with persistent infection. The animals that became persistently infected were followed for 82-216 weeks after infection. Very low levels of the virus were observed in some animals after the clearance from time to time up to 1 year. This was attributed to the use of a highly sensitive RT-PCR method for virus detection (40 RNA copies/mL) and to very long follow-up of the cleared animals.

An HCV-specific antibody response in the Major at al. (2004) study was mounted during weeks 7-14 (usually on weeks 9-10) in the chronic group and during weeks 6-9 (majority on weeks 8-9) in the animals that resolved the infection. On the contrary, antibodies to the HVR1 were detectable only in the chronic group (in 5 out of 6 animals), but not in the resolved group. In general, anti-HVR1 antibodies correlate with anti-E1/E2 antibodies (Bartosch et al., 2003; Major et al., 1999). In an overlapping study by Logvinov et al. (2004) no neutralizing antibody (nAb) responses were detected in three animals that cleared the virus, whereas strain-specific nAbs were detected in six of the seven chronically infected animals after approximately 50 weeks of infection. These data suggest that nAbs do not play a role in the control of virus infection. This data correlates between human and chimpanzee (Prince et al., 1999).

## CELLULAR IMMUNE RESPONSE

The role of the cellular immune response was also addressed in the chimpanzee model. The early events in a human infection are difficult to analyze, since liver tissue samples are not available during the acute phase of infection, nor are control liver samples taken from before the infection. Analysis of T-cell responses showed that animals who terminated the infection mounted a strong cytotoxic T lymphocyte (CTL) response (Cooper et al., 1999; Nascimbeni et al., 2003), and that CD8+ CTLs

are better correlated with protection against HCV infection than antibodies. The appearance of these cells in the liver several weeks after infection was temporary associated with increases in liver enzymes in the plasma and with a temporary decrease in viral load in plasma . Thimme at al. (2002) showed that initial HCV spread outpaces the T cell response by demonstrating that HCV rapidly induces, but is not controlled by IFN-alpha and IFN-beta. Viral clearance follows the appearance and accumulation of HCV-specific IFN-γ-producing T cells in the liver. The importance of memory CD8+ T cells in the control of HCV infection was confirmed by antibody-mediated depletion of this lymphocyte subset in a chimpanzee (who had recovered from two previous infections) just before a third infection with the same dose and strain of HCV. Virus replication was significantly prolonged despite the presence of memory CD4+ T helper cells primed by the two prior infections, and it was not terminated until HCV-specific CD8+ T cells recovered in the liver (Shoukry et al., 2003). This was in sharp contrast to the second infection, when the effector function was not delayed, and the viremia was terminated within 14 days, i.e. 28 days earlier than during the third infection. Control of this second infection was kinetically linked to the rapid acquisition of virus-specific cytolytic activity by liver resident CD8+ T cells and expansion of memory CD4+ and CD8+ T cells in the blood. Though memory CD4+ T cells were intact in the CD8+ T cell-depleted animals, they did not facilitate rapid clearance of the virus. It does not, of course, rule out a critical supporting role for the helper T cells in protective immunity. This was addressed in other two chimpanzees that had cleared the infection. These animals were treated with an anti-CD4 monoclonal antibody before reinfection with HCV (Grakoui et al., 2003). The treatment resulted in significant reduction of circulating CD4+ T cells for over one year, that, in turn, resulted in persistent, low-level viremia despite functional intra-hepatic memory CD8+ T cell responses. Incomplete control of HCV replication by memory CD8+ T cells in the absence of adequate CD4+ T cell help was associated with emergence of viral escape mutations in class I major histocompatibility complex (MHC)-restricted epitopes and in the failure to resolve HCV infection.

Most studies of T-cell mediated immunity to HCV suggest that this response is essential for resolution of infection. However some animals with high numbers of intrahepatic CD4+ and CD8+ T cells did not clear the infection completely. In these animals, a decrease of viral titers during the acute phase was followed by viral persistence. One explanation of this fact was provided by Erickson et al.(Erickson et al., 2001), who showed that HCV in three persistently infected chimpanzees acquired mutations in multiple epitopes that impaired class I MHC binding and/or CTL recognition. Most escape mutations appeared during acute infection and remained fixed in the viral population for years without further diversification. A statistically significant increase in the amino acid replacement rate was observed in epitopes versus adjacent regions of HCV proteins. In contrast, most epitopes

were intact in animals that resolved hepatitis C spontaneously. Other explanations for the inefficiency of the HCV-specific CD8+ T cells include the possibility that at least a portion of the cells is anergic or arrested at an early stage of differentiation (Gruener et al., 2001; Ulsenheimer et al., 2003; Wedemeyer et al., 2002), that infected hepatocytes are resistant to immune recognition, and/or that HCV-specific CD8+ T repressor cells are present that produce anti-inflammatory cytokines, such as IL-10 (Accapezzato et al., 2004).

Prediction of the outcome of an HCV infection is complicated by the fact that despite the well documented correlation between the spontaneous resolution of infection and the presence of a vigorous cellular response, this correlation is not absolute. In the study by Thompson et al. (2003), it was shown that neither the chimpanzees that remained chronically infected, nor the animals that resolved the infection mounted a significant cellular response. Only weak and transient T helper responses were detected during the acute phase in all animals. This study suggests that chimpanzees may recover from HCV infection by mechanisms other than the induction of readily detectable HCV-specific T-cell responses.

## LIVER GENE EXPRESSION

Changes in liver gene expression in response to HCV infection were measured in acute phase samples of chimpanzees who either became chronically infected, temporarily controlled the infection, or cleared the infection (Bigger et al., 2001; Ilan et al., 2002b). In another study, chronic phase samples were compared for changes in gene expression in the livers of 10 chimpanzees (Bigger et al., 2004). Hundreds of genes were shown to be up or down regulated in response to HCV infection. Some changes in the expression profile are expected, such as changes due to the response to viral double-stranded (ds) RNA, which includes type 1 interferons (IFNs) and the IFN response genes. Changes due to the innate and adaptive immune response to the infection are also predicted, including activation and infiltration of NK cells, macrophages, and lymphocytes. In addition, changes due to the hepatocyte response to the cytokines expressed by the immune cells are expected. To determine the set of genes whose expression most likely reflects the initial host response to HCV in the liver, Su et al. (2002) attempted to identify genes with expression patterns that strongly correlated with the amount of HCV RNA in the serum of all of the chimps over the entire time course profiled, and found 27 such transcripts. Many of these genes were known to be stimulated by IFN-$\alpha$, including STAT1, 2'-5' oligoadenylate synthase (OAS), and Mx1, which are well known to exert antiviral activity through inhibition of translation, activation and repression of transcriptional activity, and mRNA degradation (de Veer et al., 2001). Similarly, Bigger et al. (2001) found that during the first phase of infection the most notable changes in gene expression occurred in numerous IFN response genes that increased dramatically, some as early as day 2 post-infection. Moreover, Su et al. (2002) showed that although

HCV-infected cells successfully induce the transcription of many antiviral IFN-α-stimulated genes, this response has little or no effect on viral titer or outcome. Analysis of gene expression in 10 chronically infected chimpanzees confirmed that many IFN-stimulated genes were transcriptionally elevated, suggesting an ongoing response to IFN and/or dsRNA (Bigger et al., 2004). On the contrary, transient and sustained viral clearance was uniquely associated with the induction of IFN-γ-induced genes and other genes involved in antigen processing and presentation and the adaptive immune response (Su et al., 2002). IFN-γ-induced genes are known to be expressed as a result of the homing and activation of immune cells to the liver (Boehm et al., 1997). Surprisingly, induction of IFN-γ itself was not detected in Su et al. (2002) microarray analysis, probably due to its low expression levels. The increases in IFN-γ mRNA levels were detected by RT-PCR in all tested animals in the Major at al. (2004) study, and these increases coincided with ALT elevations and decreases in viral titers in the plasma. In the cohort of animals infected with the same monoclonal virus, the induction of IFN-γ was observed both in those animals with self-limiting infections and in chronically infected animals. Additionally, the level of induction did not correlate with spontaneous clearance of the virus; IFN- γ induction was at least as high in the group of animals that progressed to chronic infections as for those that eventually cleared HCV. Also, the levels of IFN- γ mRNA remained elevated into the post-acute phase to a similar degree in all animals. Out of four genes reported in this study for which data were collected, two genes had profiles of the expression that correlated with the outcome; induction of the CD3e and MIP-1a were observed only in animals that cleared the virus. The initial peaks of CD3 also coincided with the control of virus replication.

A single genotype 3-infected animal was available for analysis in study performed by Bigger et al. (2004), and this animal exhibited increased expression of a number of genes potentially involved in steatosis compared to the levels of expression in animals with genotype 1 infections.

Unlike human patients, for whom the timing of infection, route of infection, the source and nature of the virus are often not known, the chimpanzee provides a well controlled clinically relevant model for the study of HCV. However, it is extremely limited in its availability, as well as by its expense. As a consequence, many results have been generated in experiments with low numbers of animals. In order to generate more statistically credible data, alternative animal models that are readily available and less expensive are needed.

## TRANSGENIC MOUSE MODELS

A number of transgenic mouse models have been developed to examine the potential pathogenic effects of the HCV core protein and/or the envelope glycoproteins on hepatocytes. Conflicting results have been reported, and therefore it has been

hard to make any conclusive statements regarding the pathogenicity of the HCV structural proteins. In one study, the core protein of HCV (genotype 1a) was shown to be produced in mouse liver at levels similar to that seen in chronically infected HCV individuals, and pathogenesis was not observed over the course of 18 months (Pasquinelli et al., 1997). However, in another similar transgenic line expressing the core protein from HCV (genotype 1b), vacuolations in the liver were observed which led to steatosis in animals at 3-12 months of age (Moriya et al., 1997). Steatosis is the abnormal accumulation of fat within hepatocytes, and it appears to be a factor affecting chronic hepatitis C progression in humans (Rubbia-Brandt et al., 2004). In follow-up studies, Moriya et al. (1998) reported the formation of gross hepatic nodules in animals at 16 months of age, and the development of hepatocellular carcinoma (HCC) in some animals, suggesting that the core protein plays an important role in HCC. In addition, an age dependent increase in oxidative stress within the liver, as measured by an increase in lipid peroxidation and a decrease in glutathione levels, was observed in the transgenic mice that developed HCC (Moriya et al., 2001). As endogenous oxidants are an important class of naturally occurring carcinogens that act by producing genetic alterations, this may contribute to the etiology of HCC. In a transgenic line that was created to express not only core, but also E1 and E2 of HCV (genotype 1b), hepatitis was observed at 10-15 months, but no neoplastic nodules or carcinomas were observed during 4 years of cumulative observation in animals that ranged in age from 4-20 months.(Honda et al., 1999). Another transgenic line expressing these same three transgenes showed no evidence of liver pathology during the six months these animals were evaluated (Kawamura et al., 1997). In addition, no histological abnormalities associated with the expression of the envelope proteins alone were observed in transgenic mice up to 18 months of age (Koike et al., 1995; Pasquinelli et al., 1997). The conflicting results regarding the pathology associated with the HCV structural proteins could be due to differences in the mouse strains used to generate the transgenic animals. Alternatively, the discrepancies could be attributed to the different promoters that were used to express the transgenes, which resulted in different levels of protein.

One of the drawbacks of using transgenic models to study the potential pathogenesis of HCV proteins is the fact that the animals are tolerant to the transgenic protein, and thus, the role of the immune response to HCV proteins cannot be evaluated. To overcome this limitation, Wakita et al. (1998) constructed a transgenic model that allows conditional expression of the core, E1,E2, and NS2 proteins of HCV (genotype 1b) using the Cre/*loxP* recombination system. An adenoviral vector expressing Cre DNA recombinase was used to induce the expression of the HCV proteins. Upon infection of these mice with the adenoviral vector, acute hepatitis was observed and a humoral response to core protein was detected, indicating that the transgenic animals were immunocompetent for HCV proteins. To determine the role of the cellular immune response in the development of hepatitis, CD4+ and CD8+

T cells were depleted by administration of anti-CD4 and anti-CD8 monoclonal antibodies. In the absence of T cells, no histopathological differences were observed between Ad-infected and uninfected transgenic mice, suggesting that HCV structural proteins are not directly cytopathic to hepatocytes, but rather a cellular immune response to these proteins is responsible for the hepatitis observed. However, a caveat to this interpretation is that adenoviral vectors alone cause hepatitis in a T cell-dependent manner, so it is not clear if the hepatitis observed was caused by the HCV structural proteins or the adenovirus (Yang et al., 1996). In a follow-up study (Wakita et al., 2000), these authors reported that injection of the Ad-Cre vector increased the CD8+ lymphocyte infiltration in the livers of transgenic mice more than that in non-transgenic mice, and ALT levels were higher in the former. In addition, CTLs isolated from the livers of transgenic mice were HCV specific, suggesting that HCV structural proteins are indirectly responsible for liver injury, and that the host immune response plays a role in the pathogenesis of HCV. The Cre/*loxP* system is a useful model to evaluate host/viral protein interactions, but it could be improved by expressing the Cre protein from a non-inflammatory viral vector, such as an adeno-associated viral vector.

Although still controversial, HCV infection has been reported to be associated with several extrahepatic manifestations, including hypertrophic and dilated cardiomyopathy. Using transgenic mice expressing the HCV core protein, Omura et al. (2005) recently demonstrated the development of histological changes consistent with cardiomyopathy after 12 months of age. However, the pathogenicity of these cardiac complications is not well understood.

## XENOGRAFT MODELS

Two xenograft models for studying HCV have been developed and are now being used to evaluate HCV biology and anti-HCV therapies. Both models rely on transplantation of human hepatocytes into mice and subsequent repopulation of the mouse liver. One model utilizes Alb-uPA transgenic mice, which carry a tandem array of four murine urokinase-type plasminogen activator genes under the control of a liver-specific albumin promoter (Heckel et al., 1990). Over-expression of the transgene is cytotoxic to hepatocytes and results in a hypofibrinogenemic state and fatal neonatal bleeding. Spontaneous inactivation of the transgene occurs in a portion of the cells, giving them a growth advantage over transgene-containing cells, and the uPA-negative cells eventually repopulate up to 90% the liver (Sandgren et al., 1991). Mercer et al. (2001) combined the properties of these mice with those of immunodeficient SCID mice to develop a model system that allows human hepatocyte engraftment in mouse liver, and used this as a small animal model to study HCV infection. In this model transgenic uPA mice are transplanted with a million human hepatocytes, and engraftment occurs over the course of 4-6 weeks. Following engraftment the animals are inoculated with HCV-infected serum from

human donors. Evidence for replication of HCV in these animals was confirmed by demonstrating viral titers of $1x10^4$-$1x10^6$ copies/ml for periods up to 35 weeks. In addition**,** the authors claimed to detect negative-strand RNA, supporting the contention that *bona fide* HCV viral replication occurs in the animals. Viral replication was further validated by serially passaging the virus through three generations of mice, during which viral RNA levels increased 37,500 times. Considering dilution of the virus during passaging, this could not be attributed to the original human inoculum. These results clearly demonstrate that both replication of the HCV genome and production of fully infectious viral particles is possible in this animal model.

Recently, this chimeric mouse/human model was used to evaluate novel anti-HCV therapies. In one report (Hsu et al., 2003), the mice were used in a gene therapy approach to treat HCV by delivering a modified form of the BH3-interacting death agonist (BID). BID is a member of the Bcl-2 family of pro-apoptotic proteins and is crucial for death receptor-mediated apoptosis (Esposti, 2002). It is activated upon cleavage by caspase 8, and induces an increase in the permeability of the outer mitochondrial membrane, leading to release of apoptogenic proteins, such as cytochrome c. In this study, the endogenous cleavage site of BID was engineered to contain a specific cleavage site recognized by the NS3/NS4A protease of HCV. An adenoviral vector encoding the modified BID was delivered to the livers of SCID/Alb-uPA mice that had been previously infected with HCV. Animals were evaluated for serum HCV RNA titers, as well as, clinical and liver pathology. HCV-infected animals had initial HCV titers that ranged from $1x10^4$-$5x10^7$ genome equivalents/ml. The animals with lower titers were able to completely clear the infection following Ad-BID vector administration, while a 2-3 log decrease in HCV viral titers was observed in animals with higher initial titers. Histological examination of the livers of animals inoculated with both HCV and Ad-BID showed extensive cell death, and a TUNEL assay confirmed apoptosis in their livers.

A company called KMT Hepatech (Edmonton, Alberta) has now been founded on the basis of this chimeric human/mouse technology, that provides "KMT mice" to collaborators interested in evaluating potential HCV therapies. Although not yet published in a peer-reviewed journal, a scientific poster on the company's website details the use of this model to evaluate two anti-HCV therapeutics that have been used successfully to treat HCV in humans (P-187 $10^{th}$ HCV Meeting Dec2-6,2003 Kyoto, Japan; KMT Hepatech website). The two drugs tested were IFN-α-2B and the novel protease inhibitor, BILN2061, which has been shown in human clinical trials to reduce viral RNA levels by 2-3 logs (Lamarre et al., 2003). Homozygous SCID/Alb-uPA mice transplanted with human hepatocytes were treated with either IFN-α-2B or BILN2061, and statistically significant reductions in HCV viral loads were observed with both agents. Thus, two therapies that have been shown

to be effective against HCV in humans, and one novel gene therapy, are able to reduce viral titers in HCV-infected "KMT mice", thereby validating the model for evaluation of novel anti-HCV therapies.

Another mouse model that has been developed to study HCV replication is a modification of the "Trimera mouse". Trimera mice are created by total body irradiation of normal mice, followed by reconstitution with bone marrow from SCID mice. These animals are subsequently engrafted with human hematopoietic cells or solid tissues, such as liver. Since the resulting animal is comprised of three genetically distinct sources of tissue, the name Trimera mouse was coined. These mice were originally created to study the development of human B and T cells, but they are now also used to study HCV. When human liver fragments are transplanted, engraftment rates of up to 85% have been reported one month post-implantation (Ilan et al., 2002a). When healthy human liver tissue fragments were infected *ex vivo* with HCV positive serum, viremia was detected in 50% of transplanted animals. Mean viral loads of up to $1x10^5$ copies /ml peaked on day 18, and subsequently declined by day 25. Direct transplantation of infected human livers also resulted in viremia in mice for approximately one month. The decline in viremia is the result of fibrosis and necrosis of the human liver tissue, which limits the evaluation of potential anti-HCV therapeutics. In addition to the presence of viral RNA in the serum of mice, HCV RNA was also detected in the implanted human liver tissue. Whereas positive-strand RNA was observed on day 0, negative-strand RNA was not observed until day 9, suggesting that HCV replication occurs. The trimeric model has been shown to support replication of HCV 1a, 1b, 2a, and 3a.

Having established that *bona fide* HCV replication occurs in the Trimera mouse model, it was used to evaluate the efficacy of two novel anti-HCV agents. A small molecule HCV IRES inhibitor and an anti-HCV monoclonal antibody showed modest dose-dependent reductions in the viral load of HCV during the treatment period, which returned to pretreatment levels following cessation of drug treatment (Ilan et al., 2002a). Viability of the hepatocytes was assessed by measuring the levels of human serum albumin (HSA) mRNA in grafts from control and treatment groups. Similar levels of HSA mRNA were observed, demonstrating that the reduced viral load was not due to a hepatotoxic effect of the drug. In these studies the maximal initial viral load was approximately $7x10^4$ copies/ml. This level of viremia will need to improve before potent anti-viral agents, that have the ability to knock down HCV titers by several logs can be tested. Another issue with the model is that viremia persists for only one month, limiting the timeframe available to test the efficacy of drugs. At present, protocols to increase this window using antifibrotic agents are being attempted.

Maeda et al. (2004) recently proposed an alternative to the Trimera model, which involves engrafting human liver tissue into non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Although very good engraftment (approximately 90%) was observed in these mice, inoculation of these animals with either HCV-infected human serum or culture media containing an infectious HCV molecular clone resulted in HCV viral titers that were near the limit of detection of the PCR-based assay. HCV sequences were detected in the engrafted liver tissue by *in situ* PCR, but at present this does not represent a robust animal model for studying HCV replication or for evaluating anti-HCV therapies.

All xenograft models that have been described rely on RT-PCR for quantification of HCV RNA. To reliably detect changes in titer, the viral load should be at least 10,000 copies/ml, in order to see efficacy in the range of a one log decrease in titer. The main advantage of these models is that they represent a potentially less expensive *in vivo* model for studying HCV relative to the chimpanzee. Another advantage of the xenograft models is that HCV infection and replication occurs in human hepatocytes as opposed to chimpanzee or other non-human primate hepatocytes. Compared to the tissue culture replicon systems, adaptive mutations in the HCV genome do not seem to be required for replication in the xenograft models. However, none of these models has yet to achieve widespread utility due to the technical difficulty in breeding and creating the mice, the limited availability of human hepatocytes, variable human cell engraftment and inconsistent viral titers. If these difficulties can be overcome, these models will greatly aid the study of HCV biology and pathogenesis, as well as facilitate the development of new therapies for HCV. In order to expand the application of these mouse models for studying the role of the immune system in the pathogenesis of HCV, it will be necessary to reconstitute the mice with components of the human immune system.

## SMALL NON-HUMAN PRIMATE MODELS

The chimpamzee is the only truly validated animal model for studying HCV, but because they are an endangered species, expensive to work with, and the subject of ethical debates, other non-human primates were evaluated for infection by HCV. Early studies concluded that NANBH virus could infect marmosets (Feinstone et al., 1981) and tamarins (Karayiannis et al., 1983), both of which are New World monkeys. However, these studies were undertaken before the NANBH virus group had been subdivided into identifiable agents and before specific diagnostic tests for HCV were available. Once these were obtained, it was concluded that chimpanzees, but no other non-human primates, were susceptible to HCV infection (Garson et al., 1997).

Despite this, the pursuit of a small-animal model to study HCV has not abated, and the GBV-B virus, which efficiently replicates in tamarins and marmosets, is an

example of a surrogate model of HCV that has been gaining credibility. Originally identified as a "GB hepatitis agent", it was transmitted to tamarins from the blood sample of a surgeon (whose initials are GB) that was suffering from acute hepatitis (Deinhardt et al., 1967). All infected tamarins developed an acute hepatitis, and subsequently, the GB hepatitis agent was identified as containing two distinct RNA viruses: GBV-A and GBV-B (Simons et al., 1995). It was later shown that GBV-A does not replicate in the tamarin liver, whereas GBV-B causes hepatitis. Sequence analysis of the GBV-A and GBV-B genomes suggested that they belong to the Flaviviridae family. The GBV-B virus contains a positive-sense, single-stranded RNA genome of 9399 nucleotides, and it was shown to be most closely related to HCV. Additional experimental infection of tamarins using either the original serum sample, or serially passaged infected tamarin serum, confirmed that GBV-B causes acute hepatitis (Beames et al., 2000; Bright et al., 2004; Bukh et al., 1999). In three different species of tamarins, viremia with peak viral titers in excess of $1x10^8$ genomic equivalents/ml was observed between 2 and 14 weeks post-injection, which subsequently cleared by 16 weeks post-injection. Viremia was accompanied by an increase in liver enzymes and inflammation in the liver. Because tamarins, like chimpanzees, are in limited supply, another New World monkey, the common marmoset, has been assessed for susceptibility to GBV-B infection. Marmosets are easier to manage as breeding colonies and are currently bred for biomedical research in a number of facilities. Several reports have confirmed the susceptibility of marmosets to GBV-B infection (Bright et al., 2004; Jacob et al., 2004; Lanford et al., 2003). Viral titers of over $1x10^8$ genome equivalents/ml peaked around 6 week post-injection and viral clearance was complete by week 16. Increases in some liver enzymes were observed, which correlated with inflammation in the liver, as the result of infiltration of $CD8^+$ lymphocytes. This GBV-B marmoset model was recently tested as a small-animal model for HCV by evaluating several anti-HCV therapeutics. A small-molecule inhibitor of the HCV NS3 protease was also shown to inhibit GBV-B replication *in vivo* (Bright et al., 2004). This inhibitor reduced GBV-B viral replication by more than three logs. This was the first time an anti-HCV therapeutic was demonstrated to be effective in an animal model other than the chimpanzee, and provides validation of the GBV-B/marmoset model as a surrogate for studying HCV. Not only is this model valuable for evaluating therapies directed against the NS3 protease, it may soon be shown to be useful for testing therapies targeting other regions of the HCV genome.

One of the major differences between the course of infection of GBV-B in New World monkeys to that of HCV in humans is the tendency for the former to result in an acute infection and the propensity of the latter to lead to chronic hepatitis. However, in one recent study viremia was observed for >2 yrs following infection of one tamarin with GBV-B (Martin et al., 2003). In this case, the animal was infected by direct intrahepatic inoculation of synthetic RNA. It is not clear if this

was related to the outcome, but in any case, it enhances the value of the GBV-B tamarin or marmoset model.

The cloning and sequencing of GBV-B revealed some differences and similarities between GBV-B and HCV (Muerhoff et al., 1995) and allowed the construction of chimeric viruses to be made between the two. The genomic organization and structure of GBV-B and HCV are similar; each containing a single long ORF flanked by 5' and 3' nontranslated regions (NTR). The 5' portion of the ORF was predicted to encode structural proteins, while the 3' portion of the ORF encodes nonstructural proteins (Muerhoff et al., 1995)**.** Even though the homology of the predicted polyproteins between GBV-B and HCV is low (25-30%), the hydropathy plots of the polyproteins are very similar. The early work that led to the realization of chimeric viruses was the demonstration that the GBV-B and HCV NS3 protease share substrate specificity (Scarselli et al., 1997)**.** This guided the construction of a functional chimeric GBV-B/HCV protease that consisted of an N-terminal HCV protease domain and a C terminal GBV-B RNA helicase domain. The chimeric NS3 retained protease activity capable of processing both GBV-B and HCV substrates, and retained helicase activity similar to that of the native GBV-B NS3 (Butkiewicz et al., 2000)**.** The ability to construct a chimeric NS3 polypeptide with HCV protease and GBV-B helicase activities suggested that it may be possible to create viable chimeric GBV-B/HCV viruses that could be used to test protease inhibitors in the tamarin and/or marmoset model. For this to be realized, the development of an infectious clone of GBV-B was required. Although the GBV-B genome was initially cloned and sequenced in 1995, RNA transcribed from this clone was not infectious. It was not until 1999, when it was shown that the 3'NTR extends an additional 259 nucleotides, that an infectious clone was generated (Bukh et al., 1999)**.** This set the stage for the construction and evaluation of chimeric viruses. This area is currently in its infancy and chimeric viruses may eventually serve as models for testing HCV protease, helicase, or polymerase inhibitors, as well as, therapeutic agents that target the viral RNA.

A recent publication (Rijnbrand et al., 2005) describes a functional chimeric virus derived from GBV-B, in which a functionally important HCV regulatory sequence was substituted for the analogous sequence in the 5' NTR of GBV-B genome. Domain III of the GBV-B NTR, which binds directly to the 40S ribosome subunit, was replaced with the corresponding domain from HCV. Inoculation of tamarins with RNA transcripts derived from this chimeric clone led to recovery of viable virus, which resulted in acute hepatitis. This result demonstrates that domain III of HCV can substitute for the similar domain in GBV-B and can support both translation and viral replication *in vivo*. However, the kinetics of viremia were noticeably different, and the unusual infection profile was shown to be due to the accumulation of compensatory mutations that arose to support efficient viral

replication. This is an exciting new model for the evaluation of HCV replication and for use in drug screening, as this chimeric virus will allow the investigation of potential anti-HCV therapies targeted to domain III of the HCV IRES.

Similar chimeras may soon be available in the 3'NTR region of GBV-B, following the functional analysis of mutant 3'NTR sequences. The GBV-B 3'NTR consists of a short sequence of 27 nucleotides, followed by a poly(U) tract of 23 nucleotides, and a 3' terminal sequence that consists of 309 nucleotides. By deleting specific regions of the 3'NTR and testing the mutants by intrahepatic transfection of tamarins with the transcribed RNAs, functionally important areas of the 3'NTR were identified. Deletion of both the poly (U) tract and the short proximal sequence killed the virus; however, deletion of just one of these elements resulted in viable viruses (Nam et al., 2004). The authors also showed that insertion of a long heterologous sequence in the proximal sequence resulted in the recovery of a virus that had deleted the majority of the insert, but retained a short fragment of the heterologous sequence. This suggests that it should be possible to insert short DNA sequences into the GBV-B 3'NTR.

Studies using chimeric replicating viruses in the marmoset model, while providing a new modality for testing anti-HCV therapies, will certainly have limitations. These viruses may not completely mimic the HCV viral life cycle and the insertion of HCV elements into the GBV-B sequence may not accurately reflect the natural accessibility of these elements in the HCV genome. However, it represents a new small-animal model alternative to the chimpanzee and will be useful in evaluating at least some potential drug candidates.

## SUMMARY AND CONCLUSIONS

The chimpanzee remains the best animal model for studying the biology of HCV, as it is the only animal that is susceptible to HCV infection and replication, and because the liver disease observed in chimpanzees mimics the pathology seen in humans. Nevertheless, this model is difficult to use due to its expense, inaccessibility, and ethical considerations, and thus, efforts to develop other smaller animal models have continued. A major advantage of a small-animal model is that large numbers of animals can be employed, thus, providing statistically significant data. A few mouse models have been in the development now for about one decade. Transgenic mice expressing HCV structural proteins, for example, can be used to detect potential pathophysiological features of specific viral proteins. However, these animals cannot be used to probe questions about the viral life cycle. In addition, in all but one transgenic model, the mice are tolerant to the HCV proteins preventing the immune response against these proteins from being evaluated. In one model, conditional expression of HCV proteins is possible. Since the host immune response to HCV is believed to play an important role in disease, this latter model may be very

useful. Several mouse/human xenograft models are also being used to study HCV biology and to evaluate potential anti-HCV therapeutics. These models, although very promising, still suffer from a lack of reproducibility and require skilled and experienced individuals to create them. Another drawback, is that the animals are immunocompromised, and thus virus/host interactions cannot be assessed fully. Finally, the use of GBV-B/HCV chimeric viruses that infect and replicate in New World monkeys offer the advantages of direct virus infection of a small animal, without the complication and irreproducibility of hepatocyte engraftment. Although these viruses may not perfectly recapitulate HCV biology, drugs that target specific regions of these chimeric viruses can be evaluated using this model. All of the animal models for studying HCV have their limitations, but careful selection of a model will allow investigators to ask specific questions regarding HCV infection, replication, pathogenesis, and/or drug sensitivity, and important information can be gleaned.

## REFERENCES

Accapezzato, D., Francavilla, V., Paroli, M., Casciaro, M., Chircu, L. V., Cividini, A., Abrignani, S., Mondelli, M. U., and Barnaba, V. (2004). Hepatic expansion of a virus-specific regulatory CD8(+) T cell population in chronic hepatitis C virus infection. J Clin Invest *113*, 963-972.

Alter, H. J., Purcell, R. H., Holland, P. V., and Popper, H. (1978). Transmissible agent in non-A, non-B hepatitis. Lancet *1*, 459-463.

Bartosch, B., Bukh, J., Meunier, J. C., Granier, C., Engle, R. E., Blackwelder, W. C., Emerson, S. U., Cosset, F. L., and Purcell, R. H. (2003). *In vitro* assay for neutralizing antibody to hepatitis C virus: evidence for broadly conserved neutralization epitopes. Proc Natl Acad Sci U S A *100*, 14199-14204.

Bassett, S. E., Brasky, K. M., and Lanford, R. E. (1998). Analysis of hepatitis C virus-inoculated chimpanzees reveals unexpected clinical profiles. J Virol *72*, 2589-2599.

Bassett, S. E., Guerra, B., Brasky, K., Miskovsky, E., Houghton, M., Klimpel, G. R., and Lanford, R. E. (2001). Protective immune response to hepatitis C virus in chimpanzees rechallenged following clearance of primary infection. Hepatology *33*, 1479-1487.

Beames, B., Chavez, D., Guerra, B., Notvall, L., Brasky, K. M., and Lanford, R. E. (2000). Development of a primary tamarin hepatocyte culture system for GB virus-B: a surrogate model for hepatitis C virus. J Virol *74*, 11764-11772.

Bigger, C. B., Brasky, K. M., and Lanford, R. E. (2001). DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection. J Virol *75*, 7059-7066.

Bigger, C. B., Guerra, B., Brasky, K. M., Hubbard, G., Beard, M. R., Luxon, B. A., Lemon, S. M., and Lanford, R. E. (2004). Intrahepatic gene expression during chronic hepatitis C virus infection in chimpanzees. J Virol *78*, 13779-13792.

Blight, K. J., Kolykhalov, A. A., and Rice, C. M. (2000). Efficient initiation of HCV RNA replication in cell culture. Science *290*, 1972-1974.

Boehm, U., Klamp, T., Groot, M., and Howard, J. C. (1997). Cellular responses to interferon-gamma. Annu Rev Immunol *15*, 749-795.

Bradley, D. W., Krawczynski, K., Beach, M. J., and Purdy, M. A. (1991). Non-A, non-B hepatitis: toward the discovery of hepatitis C and E viruses. Semin Liver Dis *11*, 128-146.

Bradley, D. W., Maynard, J. E., Popper, H., Cook, E. H., Ebert, J. W., McCaustland, K. A., Schable, C. A., and Fields, H. A. (1983). Posttransfusion non-A, non-B hepatitis: physicochemical properties of two distinct agents. J Infect Dis *148*, 254-265.

Bright, H., Carroll, A. R., Watts, P. A., and Fenton, R. J. (2004). Development of a GB virus B marmoset model and its validation with a novel series of hepatitis C virus NS3 protease inhibitors. J Virol *78*, 2062-2071.

Bukh, J., Apgar, C. L., and Yanagi, M. (1999). Toward a surrogate model for hepatitis C virus: An infectious molecular clone of the GB virus-B hepatitis agent. Virology *262*, 470-478.

Butkiewicz, N., Yao, N., Zhong, W., Wright-Minogue, J., Ingravallo, P., Zhang, R., Durkin, J., Standring, D. N., Baroudy, B. M., Sangar, D. V., et al. (2000). Virus-specific cofactor requirement and chimeric hepatitis C virus/GB virus B nonstructural protein 3. J Virol *74*, 4291-4301.

Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science *244*, 359-362.

Cooper, S., Erickson, A. L., Adams, E. J., Kansopon, J., Weiner, A. J., Chien, D. Y., Houghton, M., Parham, P., and Walker, C. M. (1999). Analysis of a successful immune response against hepatitis C virus. Immunity *10*, 439-449.

de Veer, M. J., Holko, M., Frevel, M., Walker, E., Der, S., Paranjape, J. M., Silverman, R. H., and Williams, B. R. (2001). Functional classification of interferon-stimulated genes identified using microarrays. J Leukoc Biol *69*, 912-920.

Deinhardt, F., Holmes, A. W., Capps, R. B., and Popper, H. (1967). Studies on the transmission of human viral hepatitis to marmoset monkeys. I. Transmission of disease, serial passages, and description of liver lesions. J Exp Med *125*, 673-688.

Erickson, A. L., Kimura, Y., Igarashi, S., Eichelberger, J., Houghton, M., Sidney, J., McKinney, D., Sette, A., Hughes, A. L., and Walker, C. M. (2001). The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. Immunity *15*, 883-895.

Esposti, M. D. (2002). The roles of Bid. Apoptosis *7*, 433-440.

Farci, P., Alter, H. J., Govindarajan, S., Wong, D. C., Engle, R., Lesniewski, R. R., Mushahwar, I. K., Desai, S. M., Miller, R. H., Ogata, N., and et al. (1992).

Lack of protective immunity against reinfection with hepatitis C virus. Science *258*, 135-140.

Farci, P., Shimoda, A., Wong, D., Cabezon, T., De Gioannis, D., Strazzera, A., Shimizu, Y., Shapiro, M., Alter, H. J., and Purcell, R. H. (1996). Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. Proc Natl Acad Sci U S A *93*, 15394-15399.

Feinstone, S. M., Alter, H. J., Dienes, H. P., Shimizu, Y., Popper, H., Blackmore, D., Sly, D., London, W. T., and Purcell, R. H. (1981). Non-A, non-B hepatitis in chimpanzees and marmosets. J Infect Dis *144*, 588-598.

Feinstone, S. M., Mihalik, K. B., Kamimura, T., Alter, H. J., London, W. T., and Purcell, R. H. (1983). Inactivation of hepatitis B virus and non-A, non-B hepatitis by chloroform. Infect Immun *41*, 816-821.

Forns, X., Payette, P. J., Ma, X., Satterfield, W., Eder, G., Mushahwar, I. K., Govindarajan, S., Davis, H. L., Emerson, S. U., Purcell, R. H., and Bukh, J. (2000). Vaccination of chimpanzees with plasmid DNA encoding the hepatitis C virus (HCV) envelope E2 protein modified the infection after challenge with homologous monoclonal HCV. Hepatology *32*, 618-625.

Garson, J. A., Whitby, K., Watkins, P., and Morgan, A. J. (1997). Lack of susceptibility of the cottontop tamarin to hepatitis C infection. J Med Virol *52*, 286-288.

Grakoui, A., Shoukry, N. H., Woollard, D. J., Han, J. H., Hanson, H. L., Ghrayeb, J., Murthy, K. K., Rice, C. M., and Walker, C. M. (2003). HCV persistence and immune evasion in the absence of memory T cell help. Science *302*, 659-662.

Gruener, N. H., Lechner, F., Jung, M. C., Diepolder, H., Gerlach, T., Lauer, G., Walker, B., Sullivan, J., Phillips, R., Pape, G. R., and Klenerman, P. (2001). Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. J Virol *75*, 5550-5558.

Heckel, J. L., Sandgren, E. P., Degen, J. L., Palmiter, R. D., and Brinster, R. L. (1990). Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. Cell *62*, 447-456.

Hijikata, M., Mizuno, K., Rikihisa, T., Shimizu, Y. K., Iwamoto, A., Nakajima, N., and Yoshikura, H. (1995). Selective transmission of hepatitis C virus *in vivo* and *in vitro*. Arch Virol *140*, 1623-1628.

Honda, A., Arai, Y., Hirota, N., Sato, T., Ikegaki, J., Koizumi, T., Hatano, M., Kohara, M., Moriyama, T., Imawari, M., et al. (1999). Hepatitis C virus structural proteins induce liver cell injury in transgenic mice. J Med Virol *59*, 281-289.

Houghton, M. (2000). Strategies and prospects for vaccination against the hepatitis C viruses. Curr Top Microbiol Immunol *242*, 327-339.

Hsu, E. C., Hsi, B., Hirota-Tsuchihara, M., Ruland, J., Iorio, C., Sarangi, F., Diao, J., Migliaccio, G., Tyrrell, D. L., Kneteman, N., and Richardson, C. D. (2003). Modified apoptotic molecule (BID) reduces hepatitis C virus infection in mice with chimeric human livers. Nat Biotechnol *21*, 519-525.

Ilan, E., Arazi, J., Nussbaum, O., Zauberman, A., Eren, R., Lubin, I., Neville, L., Ben-Moshe, O., Kischitzky, A., Litchi, A., et al. (2002a). The hepatitis C virus (HCV)-Trimera mouse: a model for evaluation of agents against HCV. J Infect Dis *185*, 153-161.

Ilan, E., Eren, R., Lubin, I., Nussbaum, O., Zauberman, A., Dagan, S., Arazi, J., Neville, L., Ben-Moshe, O., Kischitzky, A., et al. (2002b). The Trimera mouse: a system for generating human monoclonal antibodies and modeling human diseases. Curr Opin Mol Ther *4*, 102-109.

Jacob, J. R., Lin, K. C., Tennant, B. C., and Mansfield, K. G. (2004). GB virus B infection of the common marmoset (Callithrix jacchus) and associated liver pathology. J Gen Virol *85*, 2525-2533.

Karayiannis, P., Scheuer, P. J., Bamber, M., Cohn, D., Hurn, B. A., and Thomas, H. C. (1983). Experimental infection of Tamarins with human non-A, non-B hepatitis virus. J Med Virol *11*, 251-256.

Kato, N., Sekiya, H., Ootsuyama, Y., Nakazawa, T., Hijikata, M., Ohkoshi, S., and 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.

Kawamura, T., Furusaka, A., Koziel, M. J., Chung, R. T., Wang, T. C., Schmidt, E. V., and Liang, T. J. (1997). Transgenic expression of hepatitis C virus structural proteins in the mouse. Hepatology *25*, 1014-1021.

Koike, K., Moriya, K., Ishibashi, K., Matsuura, Y., Suzuki, T., Saito, I., Iino, S., Kurokawa, K., and Miyamura, T. (1995). Expression of hepatitis C virus envelope proteins in transgenic mice. J Gen Virol *76 ( Pt 12)*, 3031-3038.

Kojima, M., Osuga, T., Tsuda, F., Tanaka, T., and Okamoto, H. (1994). Influence of antibodies to the hypervariable region of E2/NS1 glycoprotein on the selective replication of hepatitis C virus in chimpanzees. Virology *204*, 665-672.

Kolykhalov, A. A., Agapov, E. V., Blight, K. J., Mihalik, K., Feinstone, S. M., and Rice, C. M. (1997). Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. Science *277*, 570-574.

Kolykhalov, A. A., Mihalik, K., Feinstone, S. M., and Rice, C. M. (2000). Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3' nontranslated region are essential for virus replication *in vivo*. J Virol *74*, 2046-2051.

Lamarre, D., Anderson, P. C., Bailey, M., Beaulieu, P., Bolger, G., Bonneau, P., Bos, M., Cameron, D. R., Cartier, M., Cordingley, M. G., et al. (2003). An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. Nature *426*, 186-189.

Lanford, R. E., Chavez, D., Notvall, L., and Brasky, K. M. (2003). Comparison of tamarins and marmosets as hosts for GBV-B infections and the effect of immunosuppression on duration of viremia. Virology *311*, 72-80.

Logvinoff, C., Major, M. E., Oldach, D., Heyward, S., Talal, A., Balfe, P., Feinstone, S. M., Alter, H., Rice, C. M., and McKeating, J. A. (2004). Neutralizing antibody response during acute and chronic hepatitis C virus infection. Proc Natl Acad Sci U S A *101*, 10149-10154.

Major, M. E., Mihalik, K., Fernandez, J., Seidman, J., Kleiner, D., Kolykhalov, A. A., Rice, C. M., and Feinstone, S. M. (1999). Long-term follow-up of chimpanzees inoculated with the first infectious clone for hepatitis C virus. J Virol *73*, 3317-3325.

Major, M. E., Dahari, H., Mihalik, K., Puig, M., Rice, C. M., Neumann, A. U., and Feinstone, S. M. (2004). Hepatitis C virus kinetics and host responses associated with disease and outcome of infection in chimpanzees. Hepatology *39*, 1709-1720.

Martin, A., Bodola, F., Sangar, D. V., Goettge, K., Popov, V., Rijnbrand, R., Lanford, R. E., and Lemon, S. M. (2003). Chronic hepatitis associated with GB virus B persistence in a tamarin after intrahepatic inoculation of synthetic viral RNA. Proc Natl Acad Sci U S A *100*, 9962-9967.

Moriya, K., Nakagawa, K., Santa, T., Shintani, Y., Fujie, H., Miyoshi, H., Tsutsumi, T., Miyazawa, T., Ishibashi, K., Horie, T., et al. (2001). Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. Cancer Res *61*, 4365-4370.

Moriya, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Ishibashi, K., Matsuura, Y., Miyamura, T., and Koike, K. (1997). Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol *78 ( Pt 7)*, 1527-1531.

Muerhoff, A. S., Leary, T. P., Simons, J. N., Pilot-Matias, T. J., Dawson, G. J., Erker, J. C., Chalmers, M. L., Schlauder, G. G., Desai, S. M., and Mushahwar, I. K. (1995). Genomic organization of GB viruses A and B: two new members of the Flaviviridae associated with GB agent hepatitis. J Virol *69*, 5621-5630.

Nam, J. H., Faulk, K., Engle, R. E., Govindarajan, S., St Claire, M., and Bukh, J. (2004). *In vivo* analysis of the 3' untranslated region of GB virus B after *in vitro* mutagenesis of an infectious cDNA clone: persistent infection in a transfected tamarin. J Virol *78*, 9389-9399.

Nascimbeni, M., Mizukoshi, E., Bosmann, M., Major, M. E., Mihalik, K., Rice, C. M., Feinstone, S. M., and Rehermann, B. (2003). Kinetics of CD4+ and CD8+ memory T-cell responses during hepatitis C virus rechallenge of previously recovered chimpanzees. J Virol *77*, 4781-4793.

Okamoto, H., Kojima, M., Okada, S., Yoshizawa, H., Iizuka, H., Tanaka, T., Muchmore, E. E., Peterson, D. A., Ito, Y., and Mishiro, S. (1992). Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: variability and stability. Virology *190*, 894-899.

Pasquinelli, C., Shoenberger, J. M., Chung, J., Chang, K. M., Guidotti, L. G., Selby, M., Berger, K., Lesniewski, R., Houghton, M., and Chisari, F. V. (1997). Hepatitis C virus core and E2 protein expression in transgenic mice. Hepatology *25*, 719-727.

Prince, A. M., Brotman, B., Huima, T., Pascual, D., Jaffery, M., and Inchauspe, G. (1992). Immunity in hepatitis C infection. J Infect Dis *165*, 438-443.

Prince, A. M., Brotman, B., Lee, D. H., Ren, L., Moore, B. S., and Scheffel, J. W. (1999). Significance of the anti-E2 response in self-limited and chronic hepatitis C virus infections in chimpanzees and in humans. J Infect Dis *180*, 987-991.

Rijnbrand, R., Yang, Y., Beales, L., Bodola, F., Goettge, K., Cohen, L., Lanford, R. E., Lemon, S. M., and Martin, A. (2005). A chimeric GB virus B with 5' nontranslated RNA sequence from hepatitis C virus causes hepatitis in tamarins. Hepatology *41*, 986-994.

Rubbia-Brandt, L., Fabris, P., Paganin, S., Leandro, G., Male, P. J., Giostra, E., Carlotto, A., Bozzola, L., Smedile, A., and Negro, F. (2004). Steatosis affects chronic hepatitis C progression in a genotype specific way. Gut *53*, 406-412.

Sakai, A., Claire, M. S., Faulk, K., Govindarajan, S., Emerson, S. U., Purcell, R. H., and Bukh, J. (2003). The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. Proc Natl Acad Sci U S A *100*, 11646-11651.

Sandgren, E. P., Palmiter, R. D., Heckel, J. L., Daugherty, C. C., Brinster, R. L., and Degen, J. L. (1991). Complete hepatic regeneration after somatic deletion of an albumin-plasminogen activator transgene. Cell *66*, 245-256.

Scarselli, E., Urbani, A., Sbardellati, A., Tomei, L., De Francesco, R., and Traboni, C. (1997). GB virus B and hepatitis C virus NS3 serine proteases share substrate specificity. J Virol *71*, 4985-4989.

Shoukry, N. H., Grakoui, A., Houghton, M., Chien, D. Y., Ghrayeb, J., Reimann, K. A., and Walker, C. M. (2003). Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection. J Exp Med *197*, 1645-1655.

Simons, J. N., Pilot-Matias, T. J., Leary, T. P., Dawson, G. J., Desai, S. M., Schlauder, G. G., Muerhoff, A. S., Erker, J. C., Buijk, S. L., Chalmers, M. L., and et al. (1995). Identification of two flavivirus-like genomes in the GB hepatitis agent. Proc Natl Acad Sci U S A *92*, 3401-3405.

Su, A. I., Pezacki, J. P., Wodicka, L., Brideau, A. D., Supekova, L., Thimme, R., Wieland, S., Bukh, J., Purcell, R. H., Schultz, P. G., and Chisari, F. V. (2002). Genomic analysis of the host response to hepatitis C virus infection. Proc Natl Acad Sci U S A *99*, 15669-15674.

Tabor, E., Gerety, R. J., Drucker, J. A., Seeff, L. B., Hoofnagle, J. H., Jackson, D. R., April, M., Barker, L. F., and Pineda-Tamondong, G. (1978). Transmission of non-A, non-B hepatitis from man to chimpanzee. Lancet *1*, 463-466.

Thimme, R., Bukh, J., Spangenberg, H. C., Wieland, S., Pemberton, J., Steiger, C., Govindarajan, S., Purcell, R. H., and Chisari, F. V. (2002). Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. Proc Natl Acad Sci U S A *99*, 15661-15668.

Ulsenheimer, A., Gerlach, J. T., Gruener, N. H., Jung, M. C., Schirren, C. A., Schraut, W., Zachoval, R., Pape, G. R., and Diepolder, H. M. (2003). Detection

of functionally altered hepatitis C virus-specific CD4 T cells in acute and chronic hepatitis C. Hepatology *37*, 1189-1198.

Wakita, T., Katsume, A., Kato, J., Taya, C., Yonekawa, H., Kanegae, Y., Saito, I., Hayashi, Y., Koike, M., Miyamoto, M., et al. (2000). Possible role of cytotoxic T cells in acute liver injury in hepatitis C virus cDNA transgenic mice mediated by Cre/loxP system. J Med Virol *62*, 308-317.

Wakita, T., Taya, C., Katsume, A., Kato, J., Yonekawa, H., Kanegae, Y., Saito, I., Hayashi, Y., Koike, M., and Kohara, M. (1998). Efficient conditional transgene expression in hepatitis C virus cDNA transgenic mice mediated by the Cre/loxP system. J Biol Chem *273*, 9001-9006.

Watanabe, T., Katagiri, J., Kojima, H., Kamimura, T., Ichida, F., Ashida, M., Hamada, C., and Shibayama, T. (1987). Studies on transmission of human non-A, non-B hepatitis to marmosets. J Med Virol *22*, 143-156.

Wedemeyer, H., He, X. S., Nascimbeni, M., Davis, A. R., Greenberg, H. B., Hoofnagle, J. H., Liang, T. J., Alter, H., and Rehermann, B. (2002). Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. J Immunol *169*, 3447-3458.

Weiner, A. J., Paliard, X., Selby, M. J., Medina-Selby, A., Coit, D., Nguyen, S., Kansopon, J., Arian, C. L., Ng, P., Tucker, J., et al. (2001). Intrahepatic genetic inoculation of hepatitis C virus RNA confers cross-protective immunity. J Virol *75*, 7142-7148

Yang, Y., Jooss, K. U., Su, Q., Ertl, H. C., and Wilson, J. M. (1996). Immune responses to viral antigens versus transgene product in the elimination of recombinant adenovirus-infected hepatocytes *in vivo*. Gene Ther *3*, 137-144.

Yanagi, M., Purcell, R. H., Emerson, S. U., and Bukh, J. (1997). Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. Proc Natl Acad Sci U S A *94*, 8738-8743.