Comparison of tamarins and marmosets as hosts for GBV-B infections and the effect of immunosuppression on duration of viremia

Robert E. Lanford, a, * Deborah Chavez, a Lena Notvall, a and Kathleen M. Brasky b

a Department of Virology and Immunology, Southwest National Primate Research Center, and Southwest Foundation for Biomedical Research, San Antonio, TX 78227, USA
b Department of Laboratory Animal Medicine, Southwest National Primate Research Center, and Southwest Foundation for Biomedical Research, San Antonio, TX 78227, USA

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Abstract

GBV-B virus is a close relative to hepatitis C virus (HCV) that causes hepatitis in tamarins, and thus, is an attractive surrogate model for HCV. In this study, we demonstrate that the host range of GBV-B extends to the common marmoset with an infection profile similar to that observed for tamarins. Marmoset hepatocytes were susceptible to in vitro infection with GBV-B. Virus was efficiently secreted into the medium, and approximately 25% of hepatocytes were positive for NS3 staining. In an attempt to induce persistent infections, tamarins were immunosuppressed with FK506 and inoculated with GBV-B. Although no chronic infections were induced, the duration of viremia was increased in most animals. In one animal, the duration of viremia was extended to 46 weeks, but viral clearance occurred 18 weeks after stopping FK506 therapy. The greater availability of marmosets in comparison to tamarins will greatly facilitate future research efforts with this model.

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Introduction

GBV-B is the virus most closely related to hepatitis C virus (HCV) based on phylogenetic analysis (Muerhoff et al., 1995; Robertson et al., 1998; Ohba et al., 1996) and as such GBV-B represents an important surrogate model for HCV infections. The history of the GB agent is complex and originates with the inoculation of tamarins with serum obtained from a surgeon with the initials G.B. who contracted hepatitis (for review, see Beames et al., 2001). In the 1960s, Deinhardt inoculated tamarins with this serum and some of the animals appeared to have contracted hepatitis from the inoculation (Deinhardt et al., 1967). Although extensive research on the agent was performed following these initial studies, the technological limitations at the time prevented the isolation and characterization of the agent. Nearly three decades later, the nucleic acids from two viruses were cloned from serum representing a serial passage of the original tamarin serum (Simons et al., 1995b). The viruses were designated GBV-A and GBV-B due to their relationship to the original GB studies. A third related virus was subsequently cloned from human serum and designated GBV-C (Simons et al., 1995a) or hepatitis G virus (Linnen et al., 1996). All three viruses are closely related to HCV with GBV-B being the only hepatotropic member of the group and the most closely related to HCV (Muerhoff et al., 1995; Ohba et al., 1996).

Subsequent studies have suggested that GBV-B is of New World primate origin and that it was isolated by chance during the inoculation of human serum into tamarins already infected with GBV-B. This assumption is based on the fact that GBV-B has not been recovered from humans and the fact that GBV-B has a very narrow host range for
tamarins and other closely related New World monkeys (this study and Bukh et al., 2001). The fact that it has not been recovered a second time from tamarins may be due to the rapid resolution of the acute infection in tamarins, the low propensity of the virus to induce persistent infections, and the limited number of wild-caught tamarins that have been examined immediately upon introduction into captivity. In contrast, GBV-A is closely related to the human GBV-C virus, and members of this family frequently cause persistent infections and have been isolated from a number of primates (Bukh and Apgar, 1997; Leary et al., 1996; Birkenmeyer et al., 1998).

The GBV-B/tamarin model overcomes a number of limitations encountered working with HCV (Beames et al., 2001; Lanford and Bigger, 2002). GBV-B replicates to levels several logs higher than what is observed in HCV-infected humans and chimpanzees, thus making the detection of viral RNA and antigens more feasible. The tamarin represents a less expensive, more readily available, and smaller animal model than the chimpanzee. Although limited replication of HCV in vitro has been reported for a number of systems, the tamarin primary hepatocyte system is more suitable for virological studies (Beames et al., 2000). This system has been used to examine the antiviral activity of poly (IC) for GBV-B and to demonstrate the induction of error-prone replication by ribavirin (Lanford et al., 2000). Although the recently developed (Lohmann et al., 1999) and improved (Lohmann et al., 2001; Blight et al., 2000) HCV replicon system will greatly advance many types of studies with HCV, it cannot replace the need for a virus-based culture system and a small animal model.

HCV and GBV-B polyproteins possess approximately 25–30% homology at the amino acid level (Muerhoff et al., 1995), while the 5’ and 3’ untranslated regions are more distinct (Muerhoff et al., 1995; Bukh et al., 1999; Rijnbrand et al., 2000). This high level of homology has lead to the anticipation that antiviral compounds developed for HCV will be active against GBV-B. This concept is supported by the observation that the GBV-B NS3 protease correctly processes the HCV polyprotein (Scarselli et al., 1997) and that HCV/GBV-B chimeric NS3 proteins are enzymatically active (Butkiewicz et al., 2000). Infectious cDNA clones of GBV-B have been produced that induced hepatitis upon intrahepatic inoculation of tamarins with in vitro transcribed RNA (Bukh et al., 1999; A. Martin et al., in preparation).

The tamarin has been the preferred model for infections with GBV-B and several species of *Saguinus* have been shown to be susceptible to GBV-B, including *oedipus, mystax, labiatus,* and *nigricolis* (Beames et al., 2000; Lanford et al., 2001; Schlauder et al., 1995; Bukh et al., 1999; Deinhardt et al., 1967). Owl monkeys are also susceptible to GBV-B infection, but the levels of viremia are several logs lower than those observed in tamarins, making this animal model less amenable for most studies (Bukh et al., 2001). Although in the past many species of tamarins were bred for biomedical research, few colonies remain today. Thus, although tamarins are currently the preferred model for GBV-B studies, they are limited in availability. In contrast, common marmosets (*Callithrix jaccus*) are easier to manage as breeding colonies and are currently bred for biomedical research at a number of facilities. Although some confusion exists in the literature, because some species of tamarins were at one time referred to as marmosets, no previous studies appear to have directly assessed the susceptibility of the common marmoset for GBV-B infections, nor have they been evaluated since the development of quantitative RT-PCR for GBV-B RNA and immunological assays for antibody responses.

In this study, we demonstrate that marmosets are susceptible to infection with GBV-B, and marmoset hepatocytes are equivalent to tamarin hepatocytes as a culture system for GBV-B. In addition, although immunosuppression extended the duration of viremia in some animals, no chronic infections were observed in four immunosuppressed tamarins.

**Results and discussion**

**Comparison of infection profiles for GBV-B in tamarins, marmosets, and spider monkeys**

To examine the susceptibility of the common marmoset for GBV-B infections, two animals were inoculated with GBV-B at $5.6 \times 10^7$ genome equivalents (GE) and were monitored for viral RNA by quantitative RT-PCR assay (TaqMan), for antibody response to the NS3 protein by enzyme-linked immunosorbent assay (ELISA), and for liver pathology by elevations in the liver enzyme alanine transaminase (ALT) (Fig. 1). Both animals exhibited high levels of viremia within 2 weeks postinoculation, the first time point examined. The level of viral RNA in the serum of marmoset 17067 was $1.1 \times 10^8$ GE/ml of serum by week 2. Viremia peaked on week 6 at $3.4 \times 10^8$ GE/ml of serum and began to decline by week 10. Viremia clearance was observed on week 16. An antibody response to NS3 was first observed at week 8 and increased to peak values by week 10, the time at which viremia began to decline. No significant increase in ALT, AST, or GGT was observed at any time. Marmoset 17089 had a lower level of viremia that peaked on week 4 at $2.0 \times 10^7$ GE/ml and began to decline by week 8. Viremia was undetectable first at week 16, but was again positive on week 18, and then negative thereafter. Similar to 17067, no increase in liver enzymes was detected, but in contrast to 17067, no antibody response to NS3 was detected by ELISA (Fig. 1). Although some differences were observed in the infection profiles for the two marmosets, they were both within the range we have observed in tamarin infections.

For comparison purposes, two tamarin profiles (*oedipus*, 12024 and 12026) are presented in Fig. 2. Both animals exhibited increases in viremia to greater than $4 \times 10^8$ GE/ml of serum by week 2 postinoculation. Clearance of viremia occurred by 16–18 weeks. Although increases in ALT were observed in both animals by week 2 postinocu-
peak ALT levels were not observed until week 10–12. The early rise in ALT could be due either to the innate immune response to infected cells or possibly to some cytopathic effect due to the high levels of GBV-B replication. The decline in viremia tended to occur after peak ALT. This was especially evident in tamarin 12024. Both the lack of an ALT rise in marmosets and the differences in the kinetics of ALT rise and viremia decline in the tamarins suggest that destruction of infected hepatocytes is not the primary mechanism of viral clearance and that noncytolytic, cytokine-mediated clearance of viral RNA is likely involved. Although the limited data from these animals suggest that some differences may exist between GBV-B infections in marmosets and tamarins, we have observed tamarins that do not have significant rises in ALT and lack anti-NS3 responses, as well. The assays for antibodies to the other viral antigens have not been developed. No response to NS5B has been observed in any of the animals studied thus far. All of the anti-GBV-B ELISA assays should be considered preliminary at this point, and greater immunological responses may become apparent with improved assays.

To better appreciate the variation observed in viremia in both marmosets and tamarins, the profiles of two marmosets (dashed lines) and four tamarins (solid lines) were superimposed in Fig. 3. At this time, profiles from a sufficient number of both marmosets and tamarins are not available to conclude whether subtle differences exist in GBV-B infections between the two animal models. However, the data demonstrate that GBV-B infections in marmosets are sim-
ilar to those observed in tamarins and that the marmoset will be a useful animal model for future GBV-B studies.

**In vitro infection of marmoset hepatocytes with GBV-B**

One of the major advantages of the use of the GBV-B model as a surrogate for HCV studies is the availability of a primary hepatocyte culture system for in vitro replication and antiviral studies. To evaluate the use of marmoset hepatocytes for in vitro studies, an in vitro growth curve was conducted using marmoset hepatocytes. Cultures were infected 3 days after plating. The cultures were washed two times following infection to remove residual virus, and cultures were harvested at various times over 72 h to examine GBV-B RNA levels in cell RNA and culture medium (Fig. 4). Time 0 was immediately after inoculation and represents the level of virus attached or internalized by the cells or released from the cultures into the medium after the washes. By 48 h postinoculation, peak levels of viral RNA were observed in the cells ($1.3 \times 10^7$ GE per culture) and culture medium ($2.6 \times 10^5$ GE per culture), and a 590-fold increase in secreted viral RNA occurred from 8 to 48 h.

To determine the percentage of cells infected, immunohistochemical staining for GBV-B NS3 was performed on marmoset hepatocytes 3 days postinfection. The staining procedure utilized the Fast Red substrate for alkaline phosphatase, and the cells were counterstained with hematoxylin. A vivid red staining was apparent in greater than 25% of the cells (Fig. 5A). The Fast Red substrate can also be detected by fluorescence microscopy (Fig. 5B). The detection of infected cells using this method represents a significant improvement over our previously described immunofluorescence staining for NS3 (Beames et al., 2000).

These studies indicated that the marmoset cultures were suitable for GBV-B in vitro replication studies. The culture medium used in our studies was developed over 10 years ago to support a variety of differentiated hepatocyte functions over a long period of culture. The system has been used successfully with human, chimpanzee, baboon, rhesus, marmoset, and tamarin hepatocytes, in studies that involved lipoprotein metabolism, metabolic functions, HBV, HDV, HCV, and GBV-B (Beames et al., 2000, 2001; Jacob et al., 1989; Lanford et al., 1989, 1994; Lanford and Estlack, 1998; Rainwater and Lanford, 1989; Stephensen et al., 1991; Sureau et al., 1991; Tam et al., 1997; White et al., 1993, 1994). One limitation of the system, besides the dependence on the use of primary cultures, was the complexity of the original serum-free medium supplemented with hormones, growth factors, and nutrients. We examined a simplified version of this medium in these studies (see Materials and methods), but prior to adoption of the simplified medium, we examined replication and secretion of GBV-B in marmoset cultures maintained in the original (medium A) and simplified (medium B) media. Cultures were inoculated in triplicate with either tamarin or marmoset serum containing GBV-B and harvested 3 days postinoculation. The results indicate that both media support GBV-B infection, replication, and secretion and that similar infections were obtained using either tamarin and marmoset serum (Fig. 6). Other serum-free media formulations may be suitable for cultivation of marmoset hepatocytes for GBV-B replication studies, especially since these studies are short term in nature. We have continued the use of a complex formulation (the simplified formula B shown above), because this medium supports numerous hepatocyte
functions over long culture times in a number of different species of primate hepatocyte cultures.

Effect of immunosuppression on GBV-B infections

Previous studies have suggested that experimental infections with GBV-B do not induce persistent infections. This is supported by the failure to observe persistent infections in wild-caught tamarins and marmosets. However, recent data indicate that persistent infections are possible. A single animal receiving an intrahepatic inoculation with infectious GBV-B RNA developed a persistent infection (A. Martin et al., in preparation). To some degree, this limits the utilization of this model, since one would like to have persistently infected animals for a variety of studies including studies on pathogenesis and evaluation of antiviral strategies. Two approaches for the induction of persistent infections include induction of transient immunosuppression during infection and infection of animals at birth prior to the maturation of the immune system.

We have focused on the former approach. Partial success was attained in inducing a viremia of longer duration with GBV-B by transient immunosuppression with FK506. A total of four tamarins (S. mystax) were infected with GBV-B.

![Fig. 5. Immunohistochemical staining for GBV-B NS3. Marmoset hepatocytes were grown on glass coverslips and were harvested 3 days after inoculation with GBV-B. Cells were fixed in acetone for 10 min, stained for NS3 using a rabbit anti-NS3/GST serum, developed using an alkaline phosphatase conjugate and Fast Red substrate, and counterstained with hematoxylin. Fast Red can be viewed using both light and fluorescence microscopy. The top panel (A) depicts infected cells viewed under light microscopy, while the bottom panel (B) depicts cells from a different field of the same coverslip viewed under fluorescence microscopy using a Texas Red filter.](image1)

![Fig. 6. Comparisons of in vitro infection of marmoset hepatocytes maintained in different serum-free media. Cultures were inoculated in triplicate with either tamarin or marmoset serum containing GBV-B and were harvested 3 days postinoculation. The levels of viral RNA in the cells and culture medium were similar whether the hepatocytes were cultured in Medium A (top) or Medium B (bottom), and whether cultures were inoculated with marmoset serum (left side) or tamarin serum (right side). Medium A and Medium B represent our previously published serum-free medium and the simplified version listed under Materials and methods, respectively.](image2)
during the course of these studies (Fig. 7). Immunosuppression was initiated 3 weeks prior to inoculation and was maintained until week 30 (12033) or at least 4 weeks after viral clearance. Immunosuppression was induced by twice daily, oral administration of FK506 at 100 μg/kg. The most successful attempt at inducing persistent infection was with 12033. The animal was maintained on FK506 for 30 weeks without any apparent adverse effects. At the time that treatment was discontinued, the animal still exhibited a viremia of greater than 10^6 GE/ml. The animal seroconverted for antibody to NS3 on week 32 and had a minor ALT flare on week 38 that resolved. The animal continued to have a stable high-level viremia; however, RNA levels began to decline on week 46. The animal spontaneously cleared on week 48, 18 weeks after discontinuation of FK506. The anti-NS3 antibody began to decline immediately upon viral clearance. As in previous animals, the animal cleared viremia in the absence of a rise in ALT, suggesting a noncytopathic, cytokine-mediated viral clearance. The success of immunosuppression was less apparent in the other three tamarins. Two cleared viremia in 24 weeks (12034 and 16461) and one in 16 weeks (16460). The dose of FK506 was doubled at week 18 for both 16460 and 16461, but this may have been too late to have an effect on the outcome of the infection. Animal 16461 had an antibody response to NS3 at week 20, prior to a decline in viremia, and while still on FK506 therapy. No anti-NS3 response was observed in 12034 or 16460. Although no rise in ALT was noted in 16461, both 12034 and 16460 had elevations in ALT (73 and 112 IU/ml, respectively), and the peak in ALT occurred prior to a decline in viremia. Although initially disappointing that persistent infections were not obtained with transient immunosuppression, the data (especially with 12033) are encouraging that altered immunosuppressive regimes may be more effective at least in providing animals with stable, extended viremia. Similar to cyclosporin, FK506 is a calcineurin inhibitor and is commonly used to prevent rejection of liver transplants and may be used in combination with other immunosuppressive and antiproliferative drugs (Moser, 2002). Combination therapy decreases organ rejection at lower drug levels, and thus decreases adverse effects, and may be beneficial in the induction of persistent infection in GBV-B-infected tamarins and marmosets. Alternatively, animals can be inoculated at birth in an attempt to obtain persistently infected animals. The value of having a colony of persistently infected animals for future studies indicates that additional efforts are warranted.

Materials and methods

**Animals**

Moustached tamarins (*S. mystax*), cotton top tamarins (*S. oedipus*), common marmosets (*C. jaccus*), and black-handed spider monkeys (*Ateles paniscus*) were housed at the Southwest National Primate Research Center at the Southwest Foundation for Biomedical Research. Animals were cared for by members of the Department of Laboratory Animal Medicine in accordance with the Guide for the Care and Use of Laboratory Animals and all protocols were approved by the Institutional Animal Care and Use Committee. FK506 (tacrolimus or Prograf) was purchased from Fujisawa Pharmaceutical Co. The animals were provided oral FK506 twice daily at 100 μg/kg. The blood trough levels were measured several times to ensure that therapeutic levels were being obtained.

**Hepatocyte cultures**

Primary marmoset hepatocytes were isolated by collagenase perfusion as previously described (Beames et al., 2000; Lanford and Estlack, 1998). Cells were frozen in liquid nitrogen at the time of isolation and were revived and plated on collagen-coated culture dishes as needed. Cultures were maintained in a modification of our originally described, hormonally defined, serum-free medium (Lanford and Estlack, 1998). The simplified, serum-free medium was composed of Williams medium E supplemented with 10 mM HEPES, pH 7.4, 2 mM glutamine, 500 μg/ml bovine serum albumin loaded with 5 μg/ml linoleic acid, 100 ng/ml epidermal growth factor, 10 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 1 μM dexamethasone, 1 μM ethanalamine, and 50 μg/ml gentamicin sulfate. Cells were grown in six-well dishes for 3 days prior to infection. Inoculations were performed with 5 μl of GBV-B containing serum (approximately 2 × 10^5 to 4 × 10^6 GE) in 1 ml of serum-free medium for 6 h at 37°C followed by two washes to remove residual inoculum. Culture medium was changed three times per week. Hepatocytes grown on glass coverslips were harvested at 3 days postinfection and used for immunohistochemical staining. Coverslips were fixed for 10 min in acetone and were stained with rabbit antiserum directed against bacterial-expressed GST-NS3 protein. Immunohistochemistry was performed using the DAKO Envision System for alkaline phosphatase with Fast Red substrate as described by the manufacturer.

**TaqMan quantification of GBV-B RNA**

GBV-B RNA was isolated from cells or medium by extraction with RNAzol (Biotecx Laboratories, Leedo, TX), and total cell RNA was quantified by optical density. GBV-B RNA was quantified by a real time, 5′ exonuclease RT-PCR (TaqMan) assay using a primer/probe combination that recognized a portion of the GBV-B capsid gene as previously described (Beames et al., 2000). The primers (5′-AACGAGCAAAAGCCCAGATC and 626R: 5′-CATCATGGATACCACTTGT) and probe (579P: 5′-FAM-AGCGCGATGCTGCCCTC-TAMRA) were selected using the Primer Express software designed for this purpose and were purchased from PE Biosystems.
Fig. 7. Effect of immunosuppression on duration of viremia in GBV-B-infected tamarins. Tamarins were immunosuppressed by twice daily oral administration of 100 μg/kg FK506. Animals were inoculated with GBV-B 3 weeks after initiation of FK506 therapy, and FK506 was discontinued 30 weeks postinoculation (12033) or after two consecutive RT-PCR-negative time points (4 weeks). The blood trough levels of FK506 were measured at multiple times and found to be within the recommended therapeutic range for liver transplantation. The dose of FK506 was doubled at week 18 for 16460 and 16461. Quantitative real-time RT-PCR (TaqMan) assays were used to determine the levels of GBV-B RNA in the serum, and values were expressed in genome equivalents (GE)/ml of serum (shaded bars). Serum samples that were tested but were RT-PCR negative are indicated by shaded triangles. Liver damage, as measured by serum ALT levels (open circles), and seroconversion for antibody to the NS3 protein (Anti-NS3 ELISA absorbance, triangles) are indicated by line graphs.
H₂O₂ was incubated at room temperature until color development. Antibodies to NS3 in GBV-B-infected animals were monitored with ELISA using purified NS3 (Beames et al., 2000). Purified NS3 protein (10 ng per well) was bound to Immunlon 2, 96-well plates (Dynatech Laboratories, VA) in borate-buffered saline (145 mM NaCl, 6 mM NaOH, 48 mM H₂BO₃, 50 mM KCl pH to 8.2) overnight at 4°C. All ELISA incubations were for 1 h at 37°C, and wells were washed four times with PBS-0.05% Tween 20 between incubation steps. Unoccupied protein-binding sites were blocked with 5% bovine serum albumin (BSA) in PBS. Serial tamarin serum samples were diluted 1:40 in antibody diluent, 0.5% BSA–PBS–0.05% Tween 20. Bound antibody was detected with goat anti-human IgG horseradish peroxidase conjugate diluted 1:1000 in antibody diluent. The substrate (100 μl of 1 mg/ml ABTS (Sigma) in 0.03% H₂O₂) was incubated at room temperature until color development was stopped by the addition of 50 μl of 1% SDS. Plates were read at 405 nm.

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