GB virus B infection of the common marmoset (Callithrix jacchus) and associated liver pathology

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GB virus B (GBV-B) is a flavivirus that is related closely to hepatitis C virus (HCV) and induces an acute hepatitis when inoculated into several species of New World primates. Common marmosets (Callithrix jacchus) are a widely available, non-endangered primate species that is susceptible to GBV-B infection and develops a characteristic acute hepatitis. Here, animals were found to be susceptible to serially passaged serum and GBV-B transcripts. Hepatic pathology and peripheral viraemia could be quantified biochemically, immunophenotypically and morphologically, and persisted for periods of up to 6 months in some animals. Hepatitis was characterized by a marked influx of CD3+ CD8+ T lymphocytes and CD20+ B cells within the first 2 months of primary infection. The results of this study document the marmoset as another small, non-human primate species in which the pathogenesis of GBV-B can be studied and used as a surrogate model of HCV infection for investigation of pathogenesis and antiviral drug development.

HCV is a member of the virus family Flaviviridae, composed of the flaviviruses, the pestiviruses and the hepatitis C viruses, which have similar genomic organization and replication strategies (Rice, 1996). Bovine viral diarrhoea virus (BVDV) is a pestivirus that has been developed for cell-based screening assays to assess antiviral drug activity (Meyers & Thiel, 1996; Zitzmann et al., 1999). The GB viruses (GBVs), based on their genetic sequences, have been categorized as flaviviruses (Muerhoff et al., 1995). Their historical background has been reviewed (Karayiannis & McGarvey, 1995; Robertson, 2001). Briefly, GBV-B was isolated from New World monkeys (tamarins) after inoculation with serum from a human patient with hepatitis (Deinhardt et al., 1967). Subsequent work has demonstrated and characterized three distinct viral agents termed GBV-A, GBV-B and GBV-C. GBV-C, previously called hepatitis G virus (HGV), has been found in 1–2 % of the human population (Simmons et al., 1995; Linnen et al., 1996). The association of GBV-B with human disease has not been firmly established (Alter et al., 1997). All three flaviviruses are related to HCV, but GBV-B is phylogenetically most closely related to HCV, showing up to 25 % amino acid sequence identity (Muerhoff et al., 1995; Ohba et al., 1996). Although each exhibits different host tropisms and disease sequelae, both HCV and GBV-B have similar cellular mechanisms of processing viral gene products (Reed et al., 1998) and both are associated with persistent infections in their respective hosts (Beames et al., 2001).

The natural host of GBV-B is unknown but several species of New World primates including tamarins (Saguinus sp.)
and owl monkeys (*Aotus* sp.) are susceptible to experimental inoculation (Bukh et al., 2001). Recently, susceptibility of the common marmoset (*Callithrix jacchus*) to GBV-B infection has been demonstrated as an alternative small-animal model (Lanford et al., 2003; Bright et al., 2004). In the report that follows, the successful infection of common marmosets with GBV-B and associated hepatic pathology and inflammatory changes are described. The availability of GBV-B-infected marmosets now allows investigation of both host and viral processes as they relate to the pathogenesis of HCV infection of the liver.

**METHODS**

**Experimental animals.** Animals were housed in an animal biocontainment facility in accordance with the Harvard Medical School’s Standing Committee on Animals and the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996). Prior to inoculation, animals were checked for GBV-A and GBV-B by RT-PCR performed on sera. After GBV-B inoculation, animals were examined prospectively with sequential blood and hepatic biopsies. Blood was obtained for measurement of hepatic enzymes including alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase. The presence of GBV-B viraemia and viral RNA load was detected by RT-PCR.

**Inoculum.** Marmosets were inoculated intravenously with 1·0 ml of a 10⁻³ dilution of GBV-B-infectious serum. This serum was derived from a common marmoset inoculated with 'GB Agent Pool, Mystax 661, 8/93’ kindly provided by Dr Jens Bukh (Hepatitis Viruses Section, NIH, NIAID, USA). Two animals were subject to direct transfection of the liver with recombinant (r)GBV-B RNA, as described previously (Bukh et al., 1999), with the plasmid pGB (kindly provided by Dr Bukh). Subsequently, six animals were inoculated and three were immunosuppressed with oral FK506 (Prograf) at a dose of 0·2 mg kg⁻¹ twice daily for 4 weeks starting 1 week prior to inoculation with GBV-B-infectious serum.

**Virus quantification.** Quantification of GBV-B was performed using previously described protocols (Beames et al., 2000). One-step RT-PCR amplification utilized the following primer pair and probe: forward, 5'-AAGGAGCAAAAGCGCAAGT-3'; reverse, 5'-CATC-ATGGTTACACGAATTTCG-3'; and probe, 5'-6Fam-AGCG-GATGCTCGGGCCTGTA-Tamra-3' (6Fam, 6-carboxyfluorescein; Tamra, 6-carboxytetramethylrhodamine). The primer pairs and fluorescence reporter probe for the sequence analysis were synthesized commercially (Applied Biosystems). RT-PCR was employed for quantifying GBV-B serum titres (TaqMan system, Applied Biosystems, Sequence Detection System). A reference standard of GBV-positive serum was calculated to contain 1×10⁷ GBV genome equivalents (g.e.) ml⁻¹ in a head-to-head comparison with an rGBV RNA transcript standard (Bukh et al., 1999).

**Histology.** In initial experiments, eight animals were inoculated intravenously with serially passaged GBV-B serum. Two of these animals were euthanized and necropsies were performed at 4 weeks after infection. One animal was euthanized at 7 weeks and the remainder were followed to 22 weeks. Hepatic biopsies were obtained under general anaesthesia with ultrasonic guidance and samples divided for histological evaluation, RNA isolation and determination of lymphocyte subsets. Sections of liver were stained with haematoxylin and eosin (H&E) and examined for morphological evidence of hepatic inflammation. To examine the immunophenotype of cells within the liver, tissues were also stained for CD3, CD8, CD20 and HLA-DR (Dako). Tissue sections (5 μm) were immunostained using an avidin–biotin–horseradish peroxidase complex (ABC) technique with diaminobenzene chromogen as described previously (Mansfield et al., 1995; Wykrzykowska et al., 1996). Lymphocytes were also isolated by mechanical disruption and Ficoll separation from hepatic biopsies and stained for CD3, CD8, CD4, CD20 and HLA-DR (Dako). Lymphocyte subsets were determined by fluorescence-activated cell sorting (FACS) analysis (Genain & Hauser, 1996).

**Peripheral blood mononuclear cell (PBMC) analysis.** Whole blood was collected at weekly intervals from two marmosets inoculated with infectious GBV-B serum as described above. PBMCs were isolated from whole blood by density-gradient centrifugation. Briefly, after centrifugation of 2 ml whole blood in EDTA anticoagulant at 4500 r.p.m. using a Sorvall SH3000 rotor, the plasma was removed and the cell pellet (1 ml) suspended in 3 ml RPMI and layered onto 3 ml lymphocyte separation medium, followed by centrifugation at 4500 r.p.m. The PBMC interface (0·7 ml) was collected with a needle and syringe, diluted twofold in PBS and centrifuged at 4500 r.p.m. The cell pellets (50 μl) were diluted into 1·5 ml PBS, followed by centrifugation at 4500 r.p.m. In one case, PBMCs were stained for CD3⁺ and isolated by FACS analysis as described above. The final cell pellets were suspended in 100 μl PBS and stored at −80°C. Total cell RNA was isolated and titres of GBV-B RNA were quantified in both the plasma and PBMC extracts by the procedures described above. At 7 weeks post-inoculation (p.i.), animals were euthanized for the recovery of primary hepatocytes used for *in vitro* assays.

**RESULTS**

**Susceptibility of the common marmoset to GBV-B infection.**

The natural host of GBV-B is unknown; however, several New World primates develop a characteristic hepatopathy following experimental inoculation. Following inoculation with infectious serum in a time-course pathogenesis study, 8/8 (100%) marmosets became infected, with peripheral viraemia detected as early as 2 weeks p.i. (Table 1). Direct transfection of rGBV-B into the liver of two animals resulted in detection of peripheral viraemia beginning 2 weeks p.i. (Table 2).

Among animals inoculated with infectious serum, a rapid rise in viraemia 3–4 weeks p.i., attaining titres of > 10⁹ GBV-B g.e. ml⁻¹, could be demonstrated and was associated with an increase in hepatic enzymes. Two characteristic patterns of peripheral viraemia were observed and correlated with hepatic viral load (Fig. 1). In some animals, peak viraemia was achieved in 4–6 weeks followed by rapid clearance from both plasma and the hepatic tissue. In other animals, peak viral load was delayed and such animals remained viraemic for periods of up to 6 months. Intrahepatic GBV-B was detected only during periods of peripheral viraemia.

**Liver histology during GBV-B infection of marmosets.**

Biopsy or necropsy samples of liver were obtained prior to and following experimental infection and evaluated histologically (Fig. 2). Hepatitis was evident as early as 4 weeks
after inoculation and recognized initially as multifocal random non-suppurative inflammation within the hepatic parenchyma. Subsequently, marked lymphocytic infiltrates developed within portal tracts (Fig. 2a). Portal lymphocytic hepatitis was associated with erosion of the limiting plate and areas of piecemeal necrosis (Fig. 2b). Immunophenotypically, the hepatitis was characterized by the infiltration of large numbers of CD3\(^+\) and CD8\(^+\) lymphocytes within both the hepatic parenchyma and portal areas (Fig. 2c). CD20\(^+\) lymphocytes were observed primarily within portal tracts (Fig. 2d) and progressed to form well-defined lymphoid nodules in some animals. Alterations in liver enzymes correlated directly with the morphological changes observed histologically. The increase in alkaline phosphatase coincided with a prominent portal inflammatory cell infiltration, suggesting ongoing damage to the biliary epithelium. Increased numbers of CD3\(^+\) lymphocytes were observed within septal duct epithelium (Fig. 2e) and were associated with increased expression of HLA-DR, an MHC II antigen, on biliary epithelium (Fig. 2f).

**Immunophenotype of infiltrating lymphocytes**

Intrahepatic lymphocytes were isolated from liver tissues collected 1 and 2 months p.i. and the immunophenotype of the cells was determined (Fig. 3). Compared with pre-inoculation samples, there was a statistically significant increase in CD3\(^+\) CD8\(^+\) T lymphocytes (0 vs 4 weeks, \(P = 0.006\), and 0 vs 8 weeks, \(P = 0.016\); Mann–Whitney rank sum test) that correlated with changes noted histologically.

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**Table 1.** Experimental inoculation of common marmosets with GBV-B

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<th>Animal</th>
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<tr>
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**Table 2.** Susceptibility of the common marmoset to GBV-B-infectious RNA transcripts and tissue culture supernatants obtained from transfected hepatocytes

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<thead>
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<th>Animal</th>
<th>Time p.i. (weeks)</th>
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**Fig. 1.** Patterns of GBV-B viraemia following experimental inoculation of common marmosets. Two patterns in peripheral viral load were noted. Peak viral loads of >10\(^8\) RNA copies (ml plasma)\(^{-1}\) were reached between 4 and 20 weeks p.i. Peripheral viraemia (filled circles) persisted past 24 weeks in some animals (a), but was cleared in 12–15 weeks in others (b). GBV-B genomic copies in hepatic biopsies (shaded bars) and elevations of serum alkaline phosphatase levels (solid lines) correlated with measures of peripheral viraemia.
This population of cells comprised primarily cytotoxic T lymphocytes, an MHC class I-restricted immune cell that most likely plays a critical role in controlling viral infection. Concurrent with these alterations, a statistically significant increase in CD20\(^+\) B cells was also observed (0 vs 4 weeks, \(P = 0.014\), and 0 vs 8 weeks, \(P = 0.009\); Mann–Whitney rank sum test). An increase in the number of CD3\(^+\) CD4\(^+\) helper T lymphocytes, an MHC class II-restricted immune cell, was not evident. A slight increase in lymphocytes expressing the MHC class II antigen HLA-DR was observed on lymphocytes, but the overall population of naïve T cells, CD45RA\(^+\) cells, did not reflect this trend.

**Treatment of animals with FK506**

Immunosuppressive therapy is known to alter the pathogenicity of viral infection. All marmosets (3/3) treated with FK506 1 week prior to inoculation showed viraemia 2 weeks p.i., similar to untreated controls (3/3) (Table 3). In two animals, GBV-B viraemia persisted for 24 weeks p.i., with or without FK506 treatment. However, the viral load in the animal treated with FK506 was 0.5–1.5 logs greater than that measured in the untreated control up to 9 weeks p.i. (5.04 × 10\(^7\) compared with 8.41 × 10\(^5\), respectively).

**Fig. 2.** GBV-B hepatitis in the common marmoset. Hepatic necropsy and biopsy material was obtained from animals during the course of GBV-B infection and evaluated by H&E staining and immunocytochemistry. (a) Lymphocytic infiltrates expand and disrupt normal hepatic cord architecture (H&E staining, original magnification ×200). (b) Piecemeal necrosis and erosion of the limiting plate (H&E staining, original magnification ×1000). (c) Multifocal random non-suppurative hepatitis is composed primarily of CD3\(^+\) lymphocytes within hepatic parenchyma. Original magnification ×400. Inset: CD3\(^+\) lymphocytes within portal tracts. (d) CD20\(^+\) B cells within portal tracts (ABC immunostaining, original magnification ×400). (e) CD3\(^+\) lymphocytes infiltrating biliary epithelium of the septal duct (ABC immunostaining, magnification ×1000). (f) Increased expression of HLA-DR within biliary epithelium of the septal duct (ABC immunostaining, original magnification ×1000).

**Fig. 3.** Alterations in hepatic lymphocyte subsets. Sequential hepatic biopsies were obtained from animals prior to inoculation and at 4 and 8 weeks p.i. and analysed by FACS to determine the relative proportion of lymphocyte subsets. Statistically significant increases in CD3\(^+\) CD8\(^+\) (○) and CD20\(^+\) (▼) lymphocytes were observed, but there was no increase in CD3\(^+\) CD4\(^+\) lymphocytes (●).
Table 3. Experimental inoculation of common marmosets with GBV-B with and without treatment with FK506

Peripheral viraemia and hepatic infection were detected by RT-PCR as early as 2 weeks p.i. Animals were euthanized at predetermined time points to examine morphological alterations in hepatic tissue.

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<th>Animal</th>
<th>Treatment</th>
<th>Time p.i. (weeks)</th>
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<td>13</td>
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*These animals were euthanized.

Fig. 4. Treatment of animals with the immunosuppressive agent FK506 results in enhanced hepatic pathology. Hepatic biopsy material was obtained at 15 weeks p.i. from control (a, b) and FK506-treated (c, d) animals. Tissues were evaluated by H&E staining (a, c) and infiltrating lymphocytes were identified by staining for CD3\(^+\) antigen (b, d). Peripheral viraemia was monitored on plasma extracted from serum at the specified time points. Original magnification × 100.
Necropsy or biopsy samples of liver were obtained from both FK506-treated and control animals at 2, 4, 8 and 15 weeks p.i. No changes in histological features were noted between the treated and control groups at 2, 4 and 8 weeks. At 15 weeks p.i., the liver from the untreated control animal appeared normal, with minimal lymphocytic infiltration (Fig. 4a). A normal distribution of lymphocytes in the liver parenchyma was observed upon staining for CD3+ cells (Fig. 4b). These features were in contrast to the liver of the FK506-treated animal, in which a multifocal hepatitis was apparent (Fig. 4c), with a significant increase in the number of T lymphocytes infiltrating the liver (Fig. 4d).

Detection of GBV-B in PBMCs

RNA extracted from pre-inoculation sera and PBMCs from two additional marmosets (animals cj500-00 and cj96-01) were found to be negative for GBV-B RNA. Animal cj500-00 showed a rapid increase in GBV-B serum titres beginning at 1 week p.i., fluctuating between $1 \times 10^5$ and $3 \times 10^5$ g.e. ml$^{-1}$ and then increasing steadily to $6 \times 10^6$ g.e. ml$^{-1}$ at 7 weeks p.i. (Fig. 5a). Animal cj96-01 exhibited a delayed onset of viraemia, beginning at 3 weeks p.i. and gradually reaching $4 \times 10^5$ g.e. ml$^{-1}$ at 7 weeks p.i.

The RNA extracted from PBMCs isolated at weekly intervals was analysed for GBV-B content (Fig. 5b). Measurable levels of GBV-B were not detected at any time point of infection in animal cj96-01. GBV-B genomes were quantified in the PBMC pellets from animal cj500-00 that were proportionate to viraemia. The titre of GBV-B in PBMCs appeared to correlate with serum titres, although the quantities in PBMCs were 1–2 logs greater than would have been expected if serum virus co-purified with the PBMCs during the isolation procedures. The titre of GBV-B in PBMCs reached a plateau at $2 \times 10^4$ g.e. (µg RNA)$^{-1}$ by 5–7 weeks p.i. as serum titres increased progressively. PBMCs collected from a marmoset at 14 weeks p.i. were stained for CD3+ cells, isolated by FACS and analysed for GBV-B content. Titres of GBV-B were enhanced fivefold in the non-CD3-staining lymphocyte population.

DISCUSSION

The results of this study document the use of the common marmoset as a small non-human primate in which the pathogenesis of GBV-B can be studied as a surrogate model of human HCV infection. Marmosets are a widely available, non-endangered primate species. They are susceptible to GBV-B infection and develop a characteristic hepatitis. Here, hepatic pathology and peripheral viraemia were found to persist for periods of at least 6 months and could be quantified biochemically, immunophenotypically and morphologically. All animals showed signs of infection 2–4 weeks p.i. followed by a period of viraemia of up to 24 weeks. The infection of naive marmosets via transfection of rGBV-B into the liver resulted in patterns of viraemia similar to that following inoculation of infectious serum.

This facet of the study also demonstrated the capacity to examine molecular clones of GBV-B to identify virulence factors of the virus. Recently, a chronic infection was documented in one of two tamarins inoculated with rGBV-B (Martin et al., 2003). Whether this is characteristic for the clone or due to individual responses to infection will require further investigation. However, similar variations in the duration of viraemia were observed in our study of marmosets (duration of 8–12 weeks vs >24 weeks). Larger cohorts of experimentally infected animals and head-to-head comparison of infectious clones would be valuable in clarifying the factors that determine virus persistence.

In comparison with the tamarin model, the marmoset is equally susceptible to GBV-B infection. In a study presented by Bright et al. (2004), GBV-B-infectious serum from tamarins led to increased infectivity upon serial passage in the marmoset model, from a rate of infection with tamarin serum of 65% (13/20 marmosets) to a rate of 100% (9/9 marmosets) with marmoset-derived material.
The onset of viraemia peaked at 3 weeks p.i. and attained serum titres of approximately $10^9$–$10^{10}$ g.e. ml$^{-1}$ before clearing virus by 8 weeks p.i. The onset of viraemia in the study reported here occurred as early as 1–2 weeks p.i. followed by increasing titres to 7 weeks p.i. averaging $10^8$ g.e. ml$^{-1}$. In a report by Lanford et al. (2001), viraemia was detected in tamarins as early as 1–2 weeks p.i., with sustained viral titres of $10^7$ g.e. ml$^{-1}$ for 12 weeks until clearance of virus from the serum. In a study comparing the viraemia in GBV-B-infected marmosets and tamarins, the kinetics of infection were similar (Lanford et al., 2003). In this report, persistent GBV-B infection of immunologically normal marmosets was documented for periods of up to 24 weeks p.i.

Immunosuppressive treatment of marmosets prior to inoculation of GBV-B resulted in higher viral load and more severe liver pathology. These data suggest that the virus may have established a more productive infection in the liver during immunosuppressive treatment, leading to a more vigorous immune response once the suppression diminished. Although both an FK506-treated and control animal showed evidence of chronic infection for 24 weeks, a larger cohort of animals, treated in a similar fashion, must be monitored for a period of >6 months to determine the full outcome of immunosuppressive therapy. Immunosuppressive therapy on GBV-B-infected tamarins resulted in some persistent infections (Lanford et al., 2003). These results coupled with those presented here imply that immune modulation of either New World primate model will allow us to investigate factors that contribute to the establishment of the persistent carrier state.

The common marmoset is an accepted model to study inflammatory diseases such as demyelination associated with multiple sclerosis in humans (Genain & Hauser, 1996; Villoslada et al., 2001; Genain et al., 1996). The origins for utilizing this New World primate as an alternative in vitro model for hepatitis research have been described as well (Stephensen et al., 1991). Based on the susceptibility of the marmoset to infection with GBV-B, we believe it should be useful as a small primate model to investigate the mechanism of hepatic damage and inflammatory changes during the course of viral infection. The presence of lymphoid nodules was particularly interesting, as this is often cited as a defining change in chronic HCV infection in man. The increase in alkaline phosphatase coincided with a prominent portal inflammatory cell infiltration. This suggests ongoing damage to the biliary epithelium, which is a feature of chronic HBV or HCV infections. The finding of increased numbers of MHC class I-restricted CD8$^+$ lymphocytes, indicative of cytotoxic T cells, is in accordance with previous studies showing CD8$^+$ cells predominant in the liver during either chronic HBV or HCV infections (Fiore et al., 1997). The contribution of class I-restricted CD8$^+$ T cells to liver injury during virus clearance has been demonstrated in other animal models of viral hepatitis (Fiore et al., 1997). However, HCV infections persist despite an immune response, and the role of CD8$^+$ cells and whether HCV is able to escape immune elimination are issues of pathogenesis that need to be studied further (Cerny & Chisari, 1999; Ferrari et al., 1999). In this study, MHC class I expression in association with the observed increase in CD8$^+$ cells was not examined. An increase in the CD4$^+$ T lymphocytes was not detected; whether this reflects a dysfunction of the immune response requires further experimentation.

Extrahepatic sites of replication are characteristic for some members of the Flaviviridae, specifically BVDV (Liebler-Tenorio et al., 1997). BVDV can replicate in PBMCs and antigen expression can be localized to several organs including brain tissues (Bielefeldt-Ohmann et al., 1987; Gruber et al., 1993). Extrahepatic manifestations of disease have not been well characterized in animal models for HCV (Gruber et al., 1993). Several papers suggest that HCV and HGV (GBV-C) may be lymphotropic (Fogeda et al., 1999; Afonso et al., 1999; Rodriguez-Inigo et al., 2000) and a recent paper describes the identification of HCV genomic material in brain tissue from humans (Radkowski et al., 2002).

In this study, the data suggest that lymphocytes may be an extrahepatic site of GBV-B replication in the common marmoset. Although there was variation in detecting GBV-B in PBMCs from infected animals, the quantified levels of GBV-B in RNA extracted from PBMCs were 1–2 logs greater than could be accounted for by the dilution factor of contaminating serum carried through the PMBC isolation procedure (1:7200). It is possible that GBV-B adhered to the erythrocytes or to the platelets in the PMBC preparation. However, if this were the case, we would expect detectable levels of GBV-B in the PMBCs of cj96-01, which were prepared in parallel with cj500-00 samples. GBV-B was not detected in the PBMCs of cj96-01 and in a control experiment we did not detect GBV-B in normal PBMCs seeded with infectious virus. Furthermore, a recent report (Ducoulombier et al., 2004) describes the absence of HCV compartmentalized to the CD4$^+$ and CD8$^+$ fractions of PMBCs isolated from chronic carriers. This attests to our finding of increased GBV-B titres in the non-CD3$^+$ fraction of PMBCs isolated from infected marmosets.

As discussed previously, the availability of the marmoset for research purposes provides an affordable, assessable animal resource allowing experimental studies utilizing the surrogate GBV-B system. Our data show characteristic immunophenotypic and morphological features following GBV-B inoculation of marmosets that have been observed during chronic HCV infections in man. This work and that reported by others further supports the use of New World primates infected with GBV-B as a surrogate model for the study of HCV pathogenesis.

**ACKNOWLEDGEMENTS**

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