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Short communication

Investigation into the ability of GB virus B to replicate in various immortalized cell lines

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Abstract

GB virus B (GBV-B) is the most closely related virus to the hepatitis C virus (HCV) and is an attractive surrogate model system for HCV drug development efforts. Unfortunately, GBV-B can only be grown in the primary hepatocytes of certain non-human primates. We grew GBV-B in tamarins and marmosets and then used this virus in the absence and presence of lipofection reagents to try to infect 20 different cell lines including human primary hepatocytes and marmoset primary hepatocytes. GBV-B only replicated in marmoset primary hepatocytes. We isolated primary hepatocytes from GBV-B-positive and negative tamarins and marmosets and tried to immortalize the cells using SV40 large T-antigen or cell fusion. GBV-B stable cell lines were constructed in Huh7 and HepG2 cell lines, but there was no evidence for viral replication or a response to antiviral agents in these lines. Infectious full-length GBV-B RNA could be transfected into Vero, Huh7 and HepG2 at high efficiency, however there was no evidence for GBV-B replication or a response to antiviral agents. None of these approaches were successful and an in vitro model of GBV-B replication using immortalized cell lines was not produced. We hypothesize that these immortalized cell lines lack liver-specific factors that are required for GBV-B replication.
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Keywords: GB virus B; Surrogate model system; Immortalization

The development of new antiviral agents to combat the hepatitis C virus (HCV) has been hampered by our inability to grow the virus in vitro. As such, related viruses are often used as surrogate models of HCV replication in HCV drug development efforts. The virus GB virus B (GBV-B) appears to be the closest phylogenetic relative of HCV (Muerhoff et al., 1995). Like HCV, the GBV-B virus is also hepatotrophic and GBV-B infection causes an acute self-resolving hepatitis in several non-human primates (NHPs, Beames et al., 2001).

The utility of the GBV-B primary hepatocyte model for antiviral screening purposes was demonstrated recently using ribavirin (RBV), human interferon alpha (IFN) and an HCV protease inhibitor (Beames et al., 2000; Lanford et al., 2001; Bright et al., 2004). The downside of the model is that to date, only tamarins or marmoset primary hepatocytes are known support their replication in vitro (Beames et al., 2000; Lanford et al., 2001, 2003; Bright et al., 2004; Jacob et al., 2004). This makes the use of this model system cost-prohibitive for many researchers and not amenable to high-throughput applications. As such, we sought to identify and/or create immortalized cell lines capable of supporting GBV-B replication.

At the time that these studies were initiated, the tamarins *Saguinus mystax* and *S. oedipus* were known to be susceptible to GBV-B infection, while it was not certain if the common marmoset (*Callithrix jocchus*) would be. Since these tamarin species are protected and endangered respectively, the use of other NHP animal hosts was highly desirable. Fig. 1 shows the infection profile observed using both a tamarin (*S. mystax*) and a marmoset. As observed recently by others, the titer of GBV-B was higher in the tamarin than in the marmoset (Bright et al., 2004; Lanford et al., 2003). At week 5 the animals were exsanguinated and their plasma was prepared and frozen to −80 °C for use as the source of GBV-B virus for our experiments. The tamarin plasma had a GBV-B titer of 4.2×10^5 genomic equivalents per ml (ge/ml), while the marmoset plasma contained 3.0×10^4 ge/ml GBV-

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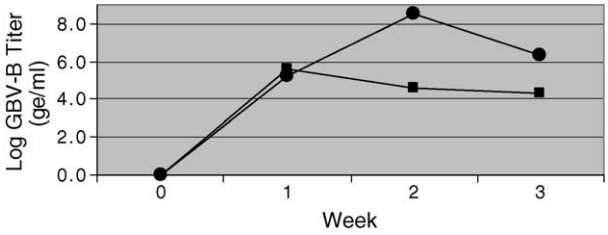

Fig. 1. Course of GBV-B infection in tamarins and marmosets. A moustached tamarin (*S. mystax*) and a common marmoset (*C. jocchus*) were inoculated intravenously with 100 μl GBV-B virions (ATCC VR-806). Viral RNA was extracted from plasma at the indicated timepoints using QIAamp viral RNA minikits (QIAGEN). GBV-B titers in genomic equivalents per ml (ge/ml) were determined by quantitative TaqMan RT-PCR (Beames et al., 2000; Lanford et al., 2001). A GBV-B capsid protein-encoding plasmid (pZGBcoreF) that contains the amplified region of interest (kindly provided by Dr. Robert Lanford, Southwest Foundation for Biomedical Research, San Antonio, TX) was used to transcribe the internal quantification control RNA for the GBV-B TaqMan RT-PCR in vitro using the MEGAscript SP6 transcription kit (Ambion). Both animals were found to be negative for GBV-B RNA in the TaqMan RT-PCR assay before the infection was initiated.

B by TaqMan RT-PCR (Beames et al., 2000; Lanford et al., 2001).

Primary hepatocytes were isolated from the liver of the GBV-B infected tamarin and marmoset as well as from marmosets which were not infected with GBV-B following the procedures that were used previously to culture these cells (Lanford and Estlack, 1998; Beames et al., 2000; Lanford et al., 2001). The marmoset liver taken from the GBV-B-infected animal showed severe diffuse fatty changes that were apparent both in the gross pathology and microscopically (data not shown). The primary hepatocytes isolated in all the experiments performed were then transformed using two methods: SV40 T-antigen and cell fusion.

A recombinant retrovirus expressing a temperature-sensitive (ts) form of the SV40 large T-antigen (SV40 tsA58 T antigen) and resistance to neomycin (kindly provided by Dr. Byung-Ho Kim, Kyung Hee University College of Medicine, Korea) was used as the transforming agent, following their procedures (Kim et al., 2000). The use of the temperature-sensitive SV40 T antigen allows us to examine GBV-B replication in a conditionally immortalized cell line. The cells should grow indefinitely at the permissive temperature and show a differentiation of liver-specific gene expression and a loss of the ability of the cells to proliferate when placed at the non-permissive temperature. A maximum of 12 colonies of immortalized cells per transformation were isolated with cloning cylinders and expanded via standard methodology (Mortensen et al., 1997). None of the clones analyzed was found to express GBV-B RNA by TaqMan RT-PCR (Table 1).

The primary hepatocytes were also immortalized by fusion with a hypoxanthine guanine phosphoribosyl transferase deficient ($HPRT^-$) hepatoma cell line to create hybrid hepatocytoma cell lines. Rat Fao hepatoma cells (kindly provided by Dr. Ernst Petzinger, Justus-Liebig University, Giesen, Germany) lack the enzymes HPRT and thymidine kinase and do not survive in hypoxanthine–aminopterin–thymidine (HAT)

Table 1
Experiments to immortalize primary hepatocytes

Hepatocytes	GBV-B status	Methodology	Results[a]
Tamarin	+	SV40 tsTAg	0/1
Marmoset	+	$HPRT^-$ fusion	0/1
Marmoset	–	SV40 tsTAg	0/3
Marmoset	–	$HPRT^-$ fusion	0/2

[a] Immortalization events observed per independently performed experiment.

media due to purine nucleotide starvation. The protocol used previously was followed (Petzinger et al., 1994). Surviving hybrid clones were isolated and expanded as above. None of the clones was found to be express GBV-B RNA via TaqMan RT-PCR (Table 1).

We attempted to infect various immortalized cell lines by exposing cultures at ∼75% confluence to GBV-B virions (MOI ∼0.3 and ∼1) for 6 h. The cultures were then rinsed twice with media and grown for a week. Supernatant samples were taken from the cultures at days 2–3 and 6–7 and analyzed for GBV-B RNA content using TaqMan RT-PCR. We also took the same virion samples and combined them with lipofectAMINE2000 according to the manufacturer's recommendations (Invitrogen) before adding them to cells. This strategy was implemented to identify cell lines that were not susceptible to direct infection with this virus, but could support GBV-B replication once virions had entered the cell. This approach has been used successfully in other model systems (Sharma et al., 1997). The results of this study are shown in Table 2. None of the cell lines examined other than marmoset primary hepatocytes was able to support the replication of GBV-B. The finding that human primary hepatocytes were not permissive for GBV-B replication supports the contention that GBV-B is a monkey virus and not a human virus (Bukh et al., 2001).

Stable cell lines expressing GBV-B were created by subcloning the infectious full-length GBV-B cDNA from pGBB (Bukh et al., 1999; kindly provided by Dr. Jens Bukh, NIH) using Not I and Xho I sites into pCMV-Script (Stratagene), a vector which contains a strong CMV promoter/enhancer and a gene conferring resistance to neomycin allowing for selection of colonies using G418. Both ends of the cloned insert were sequenced and found to be identical with the pGBB sequence. This plasmid pGBV-B-neo was transfected into Huh7 and HepG2 human hepatoma cell lines using LipofectAMINE Plus lipofection reagent (Invitrogen). Clones were selected using G418 and the cloning and manipulation of the cells was performed as above. A total of 12 clones were expanded from the HepG2 transfection and five from the Huh7 cells. The supernatants from two of the HepG2 clones produced $1.5–1.6 \times 10^6$ ge/ml GBV-B RNA while one Huh7 clone produced 3.4×10^3 ge/ml. All the other clones were negative for GBV-B RNA. These GBV-B RNA-positive cells, unlike NHP primary hepatocytes, did not show a reduction in viral RNA in response to IFN and RBV and other antiviral agents tested (data not shown). As such, we suspect that

Table 2
Examination of cell lines for ability to support GBV-B replication

Cell type	Cell line examined	GBV-B replication	
		Virions	Virions and lipofection reagent
Bovine kidney	MDBK	–	–
Cynmolgus monkey primary hepatocytes	Hepatocytes	–	NT[a]
Human cervical epithelium	HeLa	–	–
Human hepatoma	HepG2	–	–
	Hep2–287	–	–
	Huh7[b]	–	–
	H5[c]	–	NT
	H8[c]	–	NT
	H16[c]	–	NT
Human hepatoma/HCV RNA replicon	Huh7 ET[d]	–	–
Human hepatoma/HBV stable cell line	HepG2 2.2.15	–	–
Human primary hepatocytes	Hepatocytes	–	–
Human PBMCs	PBMCs	–	NT
Human stellate	LX2[e]	–	NT
Human T-cell	CEM-SS	–	–
Marmoset primary hepatocytes	Hepatocytes	+	NT
Monkey kidney	Vero	–	–
Rat hepatocytoma	E13[f]	–	–
Rhesus monkey primary hepatocytes	Hepatocytes	–	NT
Tamarin EBV-transformed B-cell	B95-8	–	–

[a] NT, not tested.
[b] Both our in-house Huh7 cell line and that kindly provided by Dr. Ralf Bartenschlager, University of Heidelberg, Germany.
[c] Kindly provided by Dr. Avrind Patel, MRC, UK.
[d] Kindly provided by Dr. Ralf Bartenschlager, University of Heidelberg, Germany.
[e] Kindly provided by Dr. Scott Friedman, Mount Sinai School of Medicine, NY.
[f] Kindly provided by Dr. Ernst Petzinger, Justus-Liebig University, Giesen, Germany.

the GBV-B RNA that was observed in the clones was entirely derived from transcription from the vector and not a result of intracellular replication of GBV-B RNA. In order to address this, we next transcribed infectious GBV-B full-length RNA in vitro from pGBB (Bukh et al., 1999), mixed the RNA with lipofectAMINE2000 (Invitrogen) and added this to Vero, Huh7 and HepG2 cell cultures. Cellular RNA was extracted from the cells over the course of a week using RNeasy 96-kits (QIAGEN) and GBV-B levels were analyzed using TaqMan RT-PCR. We observed a dose-dependent increase in GBV-B RNA levels with the amount of GBV-B RNA transfected, but the levels of GBV-B RNA fell rapidly over time indicating that there was no significant intracellular replication of the GBV-B RNA in these cell lines (data not shown). Since GBV-B RNA replicons can replicate efficiently in Huh7 cells (De Tomassi et al., 2002) it seems unlikely that an inability to translate the GBV-B RNA was responsible for this effect. Also, as with the GBV-B RNA-positive stable cell lines, this GBV-B RNA transfection system also did not respond to IFN or RBV (data not shown).

In summary, of the 20 different cell lines tested only marmoset primary hepatocytes were unable to support the replication of GBV-B. We were unsuccessful in the immortalization of primary hepatocytes from tamarins and marmosets using either SV40 large T-antigen or through cell fusion experiments. Stable cell lines expressing GBV-B RNA were created using Huh7 and HepG2 cells, however there was no indication that these cell lines were replicating GBV-B. This finding seems unexpected since GBV-B RNA replicons do replicate in Huh7 cells (De Tomassi et al., 2002), however the identical situation occurs with HCV, which replicates poorly, if at all, in a variety of immortalized cell lines, while HCV RNA replicons replicate to high levels in Huh7 cells (Bartenschlager and Lohmann, 2000). Since GBV-B can replicate in the primary hepatocytes of only certain NHP species it is possible that these cells contain liver-specific factors that are required for GBV-B replication that are not present in the cell lines or in the differentiated hepatocytes which we utilized in this study.

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