A chimeric GB virus B encoding the hepatitis C virus hypervariable region 1 is infectious in vivo

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Two GB virus B (GBV-B) chimeric genomes, GBV-HVR and GBV-HVRh (with a hinge), containing the coding region of the immunodominant hypervariable region 1 (HVR1) of the E2 envelope protein of Hepatitis C virus (HCV) were constructed. Immunoblot analysis confirmed that HVR1 was anchored to the GBV-B E2 protein. To investigate the replication competence and in vivo stability of in vitro-generated chimeric RNA transcripts, two naïve marmosets were inoculated intrahepatically with the transcripts. The GBV-HVR chimeric genome was detectable for 2 weeks post-inoculation (p.i.), whereas GBV-HVRh reverted to wild type 1 week p.i. Sequencing analysis of the HVR1 and flanking regions from GBV-HVR RNA isolated from marmoset serum demonstrated that the HVR1 insert remained unaltered in the GBV-HVR chimera for 2 weeks. Inoculation of a naïve marmoset with serum collected at 1 week p.i. also resulted in viraemia and confirmed that the serum contained infectious particles. All animals cleared the infection by 3 weeks p.i. and remained negative for the remaining weeks. The chimera may prove useful for the in vivo examination of any HCV HVR1-based vaccine candidates.

INTRODUCTION

Hepatitis C virus (HCV) causes acute and chronic hepatitis, which can lead to liver cirrhosis and hepatocellular carcinoma (Alfonso et al., 2004; Alter & Seeff, 2000; Poynard et al., 2003). The infection is resolved in a proportion of acute cases, but persists for life in >80 % of infected patients. Although the current treatment regimen can cure 40–80 % of infected individuals, the number of HCV carriers continues to increase. There is no effective vaccine and HCV infection is considered to be one of the major health problems worldwide (McHutchison, 2004).

HCV is classified within the family Flaviviridae, the members of which are small, icosahedral, enveloped viruses that contain a positive-sense RNA genome (Bartenschlager & Lohmann, 2000; Lindenbach & Rice, 2001). The family Flaviviridae consists of three genera: Flavivirus, Pestivirus and Hepacivirus. It was suggested that three novel proposed members of the Flaviviridae, the GB agents [GB virus (GBV) A, B and C], should be classified in a separate genus within the Flaviviridae or in a subgenus of Hepacivirus (Muerhoff et al., 1995).

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A supplementary table showing oligonucleotide primers designed and used in this study is available in JGV Online.
Although HCV and GBV-B share only 28% amino acid identity in their polyproteins, their genomic organization is identical. The entire genome of both viruses contains a single, long open reading frame that is flanked by 5′ and 3′ untranslated regions; the structural proteins are located towards the N terminus and the non-structural proteins towards the C terminus of the resulting polyprotein.

There is considerable interest in GBV-B/HCV chimeras, which might be used to examine specific functions of HCV genes within a backbone of GBV-B. As proof of principle, the aim of this study was to construct such a chimera that contained the HCV HVR1 region at the N terminus of the GBV-B E2 protein, and to determine whether this chimera was infectious.

**METHODS**

**Cells and plasmids.** COS cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco-BRL) supplemented with 10% fetal bovine serum (PA Biologicals Co.), 100 IU penicillin ml\(^{-1}\) and 100 µg streptomycin ml\(^{-1}\). The pGBB construct (Bukh et al., 1999) containing the full-length GBV-B cDNA was kindly provided by Dr Jens Bukh (NIH, Bethesda, MA, USA). *Escherichia coli* strain JM109 (Promega) was used in all transformation experiments.

**Animals.** Marmosets (*Callithrix jacchus*) were bred and housed at Monash University (Clayton, VIC, Australia). The animals were housed separately in a PC2 facility and maintained under conditions that met all requirements of the Animal Ethics Committee (the project was approved by our local animal committee). All marmosets (except 832) used in this study were female and aged between 3 and 5 years. Blood samples (0.5–1.0 ml) were taken weekly from the femoral vein and separated serum samples were stored at \(-80^\circ\text{C}\).

**Construction of chimeric cDNA.** Following restriction-enzyme digestion of pGBB at the unique NotI and EcoRI sites (Promega), the resulting fragment encoding the GBV-B internal ribosomal entry site (IRES), core, E1 and partial E2 was subcloned into pGEM-11zf (+) (Promega). The resulting recombinant plasmid was named pGEM-NE. Two chimeric GBV-B genomic cDNA molecules were constructed: to construct pGBV-HVR, two GBV-B genome-specific primers (see Supplementary Table S1, available in JGV Online) were designed at the GBV-B E1/E2 junction. The forward primer (FHVR) was anchored at its 5′ end with the nucleotide sequence specific for aa 2–31 of the HCV E2 protein that encompassed aa 2–27 of HCV HVR1 and four additional downstream amino acid residues of the HCV H77 strain [GenBank accession no. AF009606 (Kolykhalov et al., 1997)]. The reverse primer (RHVR) was anchored with the complementary HVR1 sequence. To construct pGBV-HVRh, the primers were designed to incorporate a ‘GGSSG hinge’ sequence at the HVR1/E2 junction (see Supplementary Table S1, available in JGV Online) and to contain a sequence specific for five amino acid residues towards the C terminus of the resulting polyprotein.

**In vitro RNA transcription.** RNA transcripts were synthesized as described previously (Huang et al., 2005). Briefly, plasmids pGBB, pGBV-HVR and pGBV-HVRh were linearized with XhoI, purified by phenol/chloroform extraction and ethanol-precipitated. RNA was synthesized with a MegaScript T7 transcription kit (Ambion). For each transcription, 5 µg linearized plasmid was used in a 50 µl transcription reaction mixture and incubated at 37°C for 2.5 h. An aliquot was examined by non-denaturing 0.7% TAE gel electrophoresis to confirm the quality of the generated RNA fragments. The RNA was stored at \(-80^\circ\text{C}\) before use.

**Intrahepatic inoculation of marmosets with RNA.** All marmosets used in this experiment were initially determined to be GBV-B RNA-negative by RT-PCR examination of serum. The animals were anaesthetized by intramuscular injection of alfaxan followed by isoflurane inhalation (face mask). The RNA inocula were thawed quickly, 3 vols PBS (Invitrogen) was added and 30–50 µl was injected immediately into each of five to seven different sites of the surgically exposed liver. The marmosets were monitored daily for any clinical signs for a total of 24 weeks, then euthanized.

**Real-time RT-PCR.** QiAamp viral RNA Minispin columns (Qiagen) were used to extract viral RNA from serum samples according to the manufacturer’s instructions. Briefly, 25 µl serum was used in each extraction, the RNA was eluted into 60 µl elution buffer and stored at \(-80^\circ\text{C}\). The GBV-B genome was quantified by using a Taqman-based real-time RT-PCR using a primer–probe that hybridized to the GBV-B core gene as described by Beames et al. (2000). The serum viral load was calculated by comparison with a standard curve generated from serial 10-fold dilutions (from \(10^2\) to \(10^5\) copies ml\(^{-1}\)) of in vitro-transcribed full-length RNA.

**Analysis of the HVR1 and flanking regions by RT-PCR.** RNA was extracted from 25–50 µl serum as described above. RT-PCR was performed with a SuperScript One-Step RT-PCR kit (Invitrogen) with 1 µM final concentrations of sense (FG1BVE1) and antisense (R1GBVE2) primers (see Supplementary Table S1, available in JGV Online) in a final volume of 25 µl. An initial RT step was performed at 48°C for 30 min, followed by 94°C for 2 min and 40 cycles of denaturation at 94°C for 20 s and annealing and extension at 60°C for 1 min. Using the same oligonucleotide primers, the resulting product was subsequently reamplified in a PCR including 35 cycles of 94°C for 20 s, 52°C for 20 s and 72°C for 40 s, with a final extension at 72°C for 7 min. The resulting products were analysed in a 3% TAE/agarose gel. The PCR products were then eluted from the gel and subjected to automated cycle sequencing to determine the nucleotide sequence.

**Anti-core and anti-HVR1 ELISAs.** Truncated GBV-B core protein was expressed in *E. coli* (D. Li & E. J. Gowans, unpublished data). The recombinant His-tagged protein was purified by nickel-resin chromatography and used in ELISA as described previously (Trowbridge et al., 1996). Marmoset serum samples were diluted 1:100 in PBS, then added to the plates and bound antibodies were detected with sheep anti-human IgG horseradish peroxidase conjugate (Chemicon) and tetramethylbenzidine (TMB) substrate. The reactions were stopped by the addition of 1 M sulphuric acid and absorbance values were read at 450 nm. The anti-HVR1 ELISA was performed as described above, using plates coated with the homologous synthetic HVR1 peptide.

**Alanine transaminase (ALT) assay.** ALT levels in the serum samples were assayed with an ALT assay kit (Sigma) according to the manufacturer’s protocol. In this assay, 20 µl serum (stored at 4°C for <7 days as recommended by the kit supplier) was used.
RESULTS

Natural history of infection resulting from wild-type, in vitro-generated RNA transcripts

Initially, the natural history of wild-type GBV-B infection in the marmosets was examined after intrahepatic injection of RNA transcribed in vitro from pGBB. The Taqman RT-PCR was able to detect a minimum of 100 copies of the full-length synthetic GBV-B and was linear over a 4-log range from $10^2$ to $10^5$ copies ml$^{-1}$ (data not shown). One week post-inoculation (p.i.), both inoculated animals, 839 and 848, were shown to be viraemic, with titres of $10^4$ and $8.9 \times 10^6$ genomic copies ml$^{-1}$, respectively (Fig. 1). The animals reached the maximum viral titre of $10^7$–$10^8$ genomic copies ml$^{-1}$ at 3 weeks p.i. The viral titre dropped sharply at 4 weeks p.i., although both animals remained viraemic up to week 7 p.i. A transient viraemia was detected in both animals at 10 weeks p.i. We were unable to detect any increase in ALT during the course of infection, in agreement with published data (Bright et al., 2004).

As shown in Fig. 1, both animals developed antibody to GBV-B core protein at 4 weeks p.i. The antibody was transient in marmoset 839, but remained detectable in marmoset 848 throughout the course of the infection.

Construction of chimeric genomes

Two strategies were employed to generate the chimeric genomes encoding the HCV HVR1 epitope ligated to the 5' end of the GBV-B E2 protein. One construct fused the HVR1 directly to the E2 protein, whereas in the other, the flexible GGSSG hinge was inserted between HVR1 and E2 to prevent any possible effects of the insert on the correct folding of the GBV-B E2 protein. After construction of the chimeric cDNA, the encoded GBV-B E1/E2 junctions were confirmed by nucleotide sequencing (Fig. 2) and by analysis of the PCR products, as standards for additional studies described below. For this assay (HVR1-RT-PCR), the primers (see Supplementary Table S1, available in JGV Online) were designed in the GBV-B E1 and E2 coding regions flanking the E1/E2 junction (GenBank accession no. NC_001655). Using these primers, PCR products of 204, 297 and 312 bp were generated, as predicted, from pGBB, pGBV-HVR and pGBV-HVRh, respectively (data not shown).

HCV HVR1 is anchored to the N terminus of GBV-B E2

Correct processing of the polyprotein of members of the Flaviviridae is a critical step in the production of infectious virus. It was shown recently that the wild-type GBV-B core, E1 and E2 precursor protein was processed efficiently in mammalian cells (Ghibaudo et al., 2004). To determine whether the mutated polyprotein was cleaved correctly, the entire coding region of E1 and E2 in the wild-type and the chimeric GBV-B genomes was amplified by PCR from pGBV-HVRh, pGBV-HVR and pGBB using primers

![Fig. 1. Natural history of GBV-B infection in marmosets following intrahepatic inoculation with RNA transcripts derived from the pGBB plasmid (wild-type cDNA). Marmosets (Mar.) 839 and 848 were injected with RNA transcripts and blood samples were tested for viral RNA and ALT (see Methods). Selected serum samples were also examined for anti-GBV-B core antibody. The limit of detection of the real-time RT-PCR is shown as a dotted line.](http://vir.sgmjournals.org)
FSP-coreE1 and R-E2-V5 (see Supplementary Table S1, available in JGV Online). The products were cloned in frame with an encoded V5 epitope at their 3′ ends and the resultant plasmids were named pGH1, pGH2 and pGH3, respectively. COS cells were transfected with each expression vector by using FuGene 6 (Roche) and analysed 48 h later by SDS-PAGE. To deglycosylate the E2 glycoproteins, the total protein was treated with 200 U peptide N-glycosidase F (New England Biolabs) according to the manufacturer’s instructions, prior to immunoblot analysis (Haqshenas et al., 2002). A chemiluminescence kit (Amersham Biosciences) was used to detect the immunocomplexes. An anti-V5 monoclonal antibody (Invitrogen) detected proteins in the untreated samples that ranged from 45 to 50 kDa in the wild-type and chimeric samples, whereas in the deglycosylated samples, the antibody detected bands of approximately 36 kDa in the chimeric samples and approximately 32 kDa in the wild-type samples (Fig. 3a). Most importantly, a polyclonal rabbit anti-HVR1 serum (Sakai et al., 2003), a gift from Dr Robert Purcell (NIH, Bethesda, MA, USA), only detected bands in the chimeric samples, but not the wild-type sample (Fig. 3b). These bands were 45–50 and 36 kDa in the untreated and deglycosylated samples, respectively, consistent with the anti-V5 data (Fig. 3a), and were not detected either in untransfected cells or in cells transfected with the same plasmid encoding β-galactosidase (data not shown). These experiments demonstrated that the HVR1 remained fused to the N terminus of the GBV-B E2 protein.

Intrahepatic injection of in vitro-transcribed chimeric GBV-B/HCV HVR1 RNA results in infection

The marmosets were injected by the intrahepatic route with in vitro-transcribed RNA as described in Methods. One week after injection of the chimeric RNA, the marmosets were shown to be viraemic by Taqman RT-PCR (data not shown). The viral load was $4 \times 10^4$ and $2.7 \times 10^3$ genome copies ml$^{-1}$ in marmosets 888 and 857 that received RNA derived from pGBB-HVR and pGBB-HVRh, respectively (Fig. 4). Both animals remained viraemic 2 weeks after injection, although the viral load was reduced by 1 log in week 2 (Fig. 4). Thereafter, the viral load was undetectable, indicating that the animals had cleared the infection. In contrast, marmosets 839 and 848, which were injected with wild-type GBV-B RNA, showed a viral load of approximately $4 \times 10^6$ genomes ml$^{-1}$ and remained viraemic for 7 weeks (Fig. 1). Neither marmoset injected with the chimeric RNA seroconverted to either the GBV-B core protein or the HVR1 domain, as shown by ELISA (data not shown). We presume that the brief period of viraemia was insufficient to elicit a detectable immune response. These results indicate that the replication of the chimeric genomes was impaired severely. Nine weeks after clearance of the chimeric viruses, marmosets 888 and 857 were infected with wild-type GBV-B RNA, showed a viral load of approximately $4 \times 10^6$ genomes ml$^{-1}$ and remained viraemic for 7 weeks (Fig. 1). Neither marmoset injected with the chimeric RNA seroconverted to either the GBV-B core protein or the HVR1 domain, as shown by ELISA (data not shown). We presume that the brief period of viraemia was insufficient to elicit a detectable immune response. These results indicate that the replication of the chimeric genomes was impaired severely. Nine weeks after clearance of the chimeric viruses, marmosets 888 and 857 were infected with wild-type GBV-B RNA, showed a viral load of approximately $4 \times 10^6$ genomes ml$^{-1}$ and remained viraemic for 7 weeks (Fig. 1). 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Fig. 3. Immunoblot analysis of wild-type and chimeric proteins. COS cells were transfected with pGH1, pGH2 and pGH3 encoding HVR1 plus hinge chimera, HVR1 chimera and wild type, respectively. Anti-V5 monoclonal antibody (a) and anti-HVR rabbit polyclonal antibody (b) were used to detect chimeric proteins in an immunoblot analysis.

Fig. 4. Infection profile following intrahepatic injection of marmosets with the chimeric RNA. Marmosets 888 (●) and 857 (■) were injected with RNA transcripts from GBV-HVR and GBV-HVRh, respectively. Serum samples taken at weekly intervals were examined by real-time RT-PCR and virus titre is plotted against time after injection. The positive cut-off of the assay is shown as a dotted line.
resulted a delayed transient viraemia (X. Dong, G. Haqshenas & E. J. Gowans, unpublished data).

**In vitro-synthesized GBV-HVR chimeric genomes generated infectious particles in vivo**

In order to demonstrate that the serum samples containing the chimeric GBV-HVR particles were infectious, a naïve marmoset (832) was inoculated by the intravenous route with 20 μl serum collected at week 1 p.i. from marmoset 888 that was infected successfully after intrahepatic injection of pGBV-HVR-derived chimeric RNA. Marmoset 832 became viraemic 1 week later and remained viraemic for a further 1 week (data not shown). The viral titre was $10^3$ genomic copies ml$^{-1}$. We did not detect any viral RNA in the pre-bleed serum sample that was checked on at least two occasions. Thus, although the viral load was low, it is clear that the chimeric virus was able to infect a naïve marmoset.

**Genomic stability of the GBV-B chimeras**

To confirm that the genomic RNA that was detected in the serum of the marmosets was derived from the input RNA and to determine whether any mutations appeared in the chimeric genomes during virus replication that might explain the transient nature of the observed viraemia, we analysed the HVR1 and flanking regions. At weeks 1 and 2 p.i., amplification of the HVR1 region from the chimeric viral genome present in the serum obtained from marmoset 888 injected with GBV-HVR transcripts resulted in a product of 297 bp (Fig. 5a), corresponding to the size of the chimeric fragment derived from the cDNA standard described above. In contrast, analysis of the serum samples collected from marmoset 857 at week 1 after injection with GBV-HVRh chimera demonstrated that the serum contained a mixed population of chimeric (312 bp product) and wild-type (204 bp product) viruses (Fig. 5a). We were unable to amplify any virus-specific products from the week 2 sample from this animal (857), although the animal was still viraemic as determined by real-time RT-PCR. In the week 2 sample from this animal (marmoset 857), the only observed product (Fig. 5b) was non-specific, as determined by nucleotide sequencing. Sequence analysis of the chimeric PCR products revealed that the chimeric genomes of both GBV-HVR (weeks 1 and 2 p.i.) and GBV-HVRh (week 1 p.i.) remained unaltered. Interestingly, the nucleotide sequence of the revertant showed that the nucleotide and deduced amino acid sequences matched the wild-type sequence exactly. Thus, the chimeric viruses and the wild-type revertant were cleared rapidly from the circulation. We also examined the stability of the chimeric genome in marmoset 832 and showed that the chimeric genome was detectable 1 week p.i., although another band that was larger than wild-type, but smaller than the chimeric product, was also detected (Fig. 5c). Unfortunately, the serum of this animal is no longer available to determine the nucleotide sequence of this PCR product. This animal remained negative for GBV-B RNA over the following 13 weeks (data not shown).

**DISCUSSION**

In this study, we generated and tested GBV-B/HCV chimeric RNA genomes for their infectivity and stability. Chimeric genomes of GBV-B containing the HCV HVR1 coding sequence were replication-competent and were packaged into infectious particles, as determined by the successful infection of a naïve animal with the week 1 p.i. serum. This is the first demonstration of a GBV-B/HCV infectious chimera that contains a chimeric protein, although an IRES chimera was reported previously (Rijnbrand et al., 2005).

Although the JFH1 strain of HCV (Wakita et al., 2005; Zhong et al., 2005) and inter- and intra-genotypic chimeric viruses of this strain (Lindenbach et al., 2005; Pietschmann et al., 2006) introduced a remarkable opportunity for in vitro study of HCV, the lack of a suitable animal model for in vivo study of HCV has prompted researchers to evaluate alternative models. Other hepatitis viruses are equally difficult to study, and animal models of **Hepatitis B virus** (Mason et al., 1980; Schultz et al., 2004) and **Hepatitis E virus**...
GBV-B is closely related genetically to HCV and causes hepatitis in tamarins and marmosets (Bukh et al., 2001; Ghibaudo et al., 2004; Jacob et al., 2004; Lanford et al., 2003). However, a major dissimilarity between GBV-B infection and HCV infection is that GBV-B does not cause persistent infection, as the infection only lasts for 8–12 weeks. Nevertheless, in some exceptional circumstances, GBV-B results in persistent infection (Martin et al., 2003; Nam et al., 2004). Our laboratory showed recently that binding of vitamin B12 to domain IV of the HCV and GBV-B IRESs inhibited translation specifically from these two IRES elements (Li et al., 2004), providing further evidence of the close relationship of the two viruses. Bright et al. (2004) also demonstrated that GBV-B can be used as a surrogate model for HCV to study the effects of protease inhibitors on virus replication. In a recent attempt to generate GBV-B chimeric viruses, Nam et al. (2004) constructed a chimeric RNA molecule comprising GBV-B with a green fluorescent protein (GFP) gene controlled by the encephalomyocarditis virus IRES. However, during the first replication cycles, almost the entire GFP was deleted and, consequently, it was not possible to generate particles that packaged the chimeric genome. More recently, a GBV-B chimera that contained stem–loop III from the HCV IRES element was shown to be replication-competent after intrahepatic inoculation of in vitro-transcribed RNA (Rijnbrak et al., 2005). The initial viraemia was low and limited to 2 weeks, similar to the results with our HVR1 chimeras and those described in a previous study in which marmosets were infected with a low dose of virus (Bright et al., 2004). The IRES chimera generated adaptive mutations that resulted in a rebound of viraemia. In contrast, we did not observe rebound viraemia, even in marmosets that were followed for up to 8 weeks. We were able to reinfect our marmosets with wild-type GBV-B to demonstrate that the animals were susceptible. However, sequence analysis of the PCR products derived from the sera of the chimera-infected animals failed to identify any mutations, including stop codons, frame shifts or deletions, that might explain the short duration of the viraemia. The PCR product with a wild-type size (Fig. 5, lane 2) was not the result of contamination, as this band was not detected in any other sample from this animal or others. Moreover, by using PCR, we ruled out the possibility of cross-contamination of the recombinant plasmid preparations that were used in the in vitro RNA synthesis with the plasmid encoding the wild-type genome (data not shown). Surprisingly, the revertant virus that appeared at week 1 post-transfection with the transcripts generated from pGBV-HVRh did not survive in vivo. The low titre of this virus is likely to be responsible for the short duration of the revertant. However, we cannot exclude the possibility of introduction of a lethal mutation in other regions of the genome. Unfortunately, due to insufficient serum from that time point, coupled with the low titre, we were unable to determine the nucleotide sequence of the entire genome of the revertant.

The crystal structure of the GBV-B E2 protein is not yet available. However, the HVR1 epitope at the N terminus of HCV E2 protein is exposed on the surface of virions, as antibody to this region is neutralizing (Farci et al., 1996). Thus, it is likely that the N terminus of the GBV-B E2 is also exposed to the surface of the virus and we chose this location to insert the HCV HVR1 region. Following intrahepatic injection of the RNA, chimeric RNA was detected in the circulation, but the duration of viraemia was short compared with that of wild-type GBV-B. Although we did not show formally that the chimeric RNA was contained in a viral particle, we have shown previously that the half-life of in vitro-transcribed RNA in serum containing 10% fetal calf serum is 4 min (K.L. Jones & E.J. Gowans, unpublished data) and, consequently, the appearance of chimeric RNA in the serum is indicative of de novo synthesis and secretion in virus particles. To demonstrate this point, we infected a naïve marmoset successfully by using serum from an animal that was injected with the chimeric RNA. However, as the viral titre obtained from intrahepatic RNA inoculation was low, we were unable to show the exposure of the HVR1 epitope on the surface by immune-capture RT-PCR (data not shown), and studies to examine this in more detail are currently ongoing.

In conclusion, we generated and tested GBV-B/HCV chimeric RNA genomes for their infectivity and stability. Currently, we are vaccinating marmosets with a homologous HVR1 peptide and testing the infectivity of the chimeric virus in the vaccinated animals. Because a potential vaccine, designed to elicit anti-HVR1, can be expected to prevent the chimeric virus from reaching the liver, we believe that the chimeric virus, despite the short duration of viraemia, is a valuable tool to examine any potential vaccines encompassing the HVR1 region.

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GBV-B/HCV HVR1 chimeras


