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Antiviral activity and host gene induction by tamarin and marmoset interferon- α and interferon- γ in the GBV-B primary hepatocyte culture model

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Abstract

GBV-B induces hepatitis in tamarins and marmosets and is a surrogate model for HCV infections. Here, we cloned and characterized the antiviral activity of tamarin and marmoset interferon (IFN) α and IFN γ . Potent antiviral activity was observed for tamarin and marmoset IFN α in primary hepatocyte cultures infected with GBV-B. The antiviral activity was greater in cultures exposed to IFN α prior to GBV-B infection, suggesting that either GBV-B was capable of inhibition of the antiviral activity of exogenous IFN α or that the preexisting endogenous IFN response to the virus reduced efficacy to exogenous IFN α . IFN γ also exhibited antiviral activity in GBV-B infected hepatocytes. The transcriptional response to IFN α in marmoset hepatocytes was characterized using human genome microarrays. Since the GBV-B hepatocyte culture model possesses a functional innate immune response, it will provide opportunities to explore the nature of the antiviral response to a virus closely related to HCV.

Keywords

HCV; hepatitis C virus; primate; microarray; innate immunity; ISG; liver

Introduction

Worldwide, approximately 170 million people are chronically infected with hepatitis C virus (HCV), which frequently progresses to serious liver disease. The current approved therapy involves the combination of pegylated (peg)-IFN α and ribavirin and has response rates for sustained viral clearance of 42% and 82% for genotypes 1 and 2/3, respectively (Manns et al., 2001; Fried et al., 2002). However, a significant proportion of the population still develops serious disease as a consequence of HCV infection. HCV infection is the leading cause for liver transplantation in the US (Thomas and Seeff LB, 2005; Alter HJ and Seeff LB, 2005), and liver cancer due to HCV infection is one of the most rapidly increasing types of cancer in the US (Kim et al., 2005). Little is understood regarding the factors leading to successful or failed viral clearance during IFN α therapy. The only animal model of HCV infection is the chimpanzee. Studies in chimpanzees using pegylated IFN α , traditional IFN α therapy with and

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without ribavirin, and adenovirus-based IFN α gene therapy, all failed to induce a reduction in viral load despite high levels of circulating IFN α for extended times (Demers et al., 2002; Lanford et al., 2000) (Lanford et al., 2006). Total genome microarray analysis in HCV chronically infected chimpanzees revealed high pretreatment IFN stimulated gene (ISG) expression in the liver and no further induction of ISG transcripts in the liver following IFN α dosing, while the response in PBMC was similar to that in uninfected animals. The IFN α response in HCV chronically infected chimpanzees may be mechanistically similar to the null response in the human population (Lanford et al., 2007). Thus, chimpanzees are not well suited for studies on the mechanism IFN α induced viral clearance in HCV infected individuals and additional animal models are needed.

GBV-B is a hepatotropic virus that causes hepatitis in tamarins and is the virus phylogenetically most closely related to HCV (Muerhoff et al., 1995; Robertson et al., 1998; Ohba et al., 1996), and as such, GBV-B represents an important small primate surrogate model for HCV infections. The history of the GB agent originates with the inoculation of tamarins with serum obtained from a surgeon with hepatitis (for review see Beames et al., 2001); however, there is little doubt that GBV-B is a tamarin virus (Lanford et al., 2003a; Bukh et al., 2001; Weatherford et al., 2009).

The GBV-B model overcomes a number of limitations encountered working with HCV (Beames et al., 2001; Beames et al., 2001; Lanford and Bigger, 2002). In tamarins, GBV-B replicates to levels several logs higher than what is observed in HCV-infected humans and chimpanzees. GBV-B replication in marmosets is typically lower than that observed in tamarins, and reproducible infection profiles in the marmoset requires adaptation to the new host (Weatherford et al., 2009)(Bright et al., 2004). Although robust replication of HCV *in vitro* is now possible using derivatives of the Huh7 cell line (Blight et al., 2002) and a limited number of viral isolates (Lindenbach et al., 2005; Zhong et al., 2005; Cai et al., 2005; Yi et al., 2006; Wakita et al., 2005), the use of a primary hepatocyte culture system for GBV-B (Beames et al., 2000) may be more suitable for some studies, especially those involving certain aspects of virus-host interactions.

The utility of the GBV-B model as a surrogate for HCV has been demonstrated in a number of studies. HCV and GBV-B polyproteins possess approximately 25-30% homology at the amino acid level (Muerhoff et al., 1995). The GBV-B NS3 protease correctly processes the HCV polyprotein (Scarselli et al., 1997), HCV/GBV-B chimeric NS3 proteins are enzymatically active (Butkiewicz et al., 2000), and HCV NS3 protease inhibitors are active in GBV-B infected marmosets (Bright et al., 2004). Infectious cDNA clones of GBV-B have been produced that induced hepatitis upon intrahepatic inoculation of tamarins and marmosets with *in vitro* transcribed RNA (Bukh et al., 1999; Sbardellati et al., 2001; Martin et al., 2003). Further modification of the infectious clones was required to render them infectious in marmosets (Weatherford et al., 2009).

In this study, marmoset and tamarin IFN α and IFN γ were cloned, expressed and examined for antiviral activity in the GBV-B primary hepatocyte culture model, as a first step in developing a small primate model for analysis of the mechanism of viral clearance by IFN in a system relevant to HCV. Induction of host gene expression by IFN α was monitored using human total genome microarrays, demonstrating the utility of human long-oligo arrays in a New World primate.

Results

Cloning and expression of tamarin and marmoset IFN genes

IFN α genes were cloned from tamarin liver, primary tamarin hepatocytes, marmoset liver and marmoset spleen. The same IFN α type was cloned regardless of the tissue source of the RNA. Phylogenetic analysis with all 13 human IFN α genes revealed that the tamarin and marmoset IFN α genes were most closely related to human IFN α 2 (Figure 1) and will be referred to as IFN α 2 hereafter. Alignment of the tamarin and marmoset IFN α 2 amino acid sequences with human IFN α 2 and IFN α 5 revealed that the marmoset IFN α 2 gene differed from the tamarin at 10 residues. Human IFN α 2 and IFN α 5 differ from each other by 32 residues and differ from marmoset IFN α 2 by 32 and 33 residues, respectively. Analysis of sequence pair distances by Clustal using PAM250 residue weights indicated that human IFN α 2 and IFN α 5 have 84.1 percent identity, while human IFN α 2 had 83.1 and 81.5 percent identity to tamarin and marmoset IFN α 2.

Marmoset and tamarin IFN α 2 were expressed in the human liver cell line Huh7, secreted into the serum free medium, and concentrated by ultrafiltration. The level of production was estimated using an ELISA for human IFN α that employed a polyclonal antibody that cross-reacted with both marmoset and tamarin IFN α . Tamarin and marmoset IFN α 2 production in Huh7 cells was estimated at 2000 and 700 ng/ml, respectively. Since the ELISA was calibrated with human IFN α 2, the values for marmoset and tamarin IFN α 2 are approximations. The specific activity was determined in a bioassay using inhibition of EMCV cytopathic effect in human A549 cells. The specific activity of human IFN α 2b in the assay was 1.25 units (U)/pg, which is comparable to the 2-5 U/pg normally attributed to purified human IFN α 2. Tamarin and marmoset IFN α 2 exhibited high activity in human cells with approximately 1.6 U/pg and 0.25 U/pg, respectively (Table 1). In contrast, inhibition of EMCV cytopathic effect on primary hepatocytes required high levels of IFN α 2. Human IFN α 2 had 30,000-fold decrease in activity on tamarin hepatocytes, while tamarin and marmoset IFN α 2 decreased by only 500- and 300-fold, respectively. EMCV cytopathic effect in marmoset hepatocytes could not be effectively inhibited even with marmoset IFN α 2. As an alternative to the EMCV assay, the biological activity of marmoset IFN α was tested on marmoset hepatocytes for stimulation of a promoter containing multiple copies of an ISRE (interferon stimulated response element). Primary marmoset hepatocytes were transfected with an ISRE-luciferase reporter construct and treated with various concentrations of marmoset IFN α for 24 hr. Luciferase activity increased by 59% at 0.2 ng/ml IFN α 2 (equivalent to 50 u/ml based on activity in A549 cells; Table 1) and by 515% at 100 ng/ml (Figure 3).

Antiviral activity of IFN α in GBV-B infected cultures

To examine the antiviral activity of tamarin IFN α 2 in the GBV-B model, cultures of primary tamarin hepatocytes were treated with five-fold dilutions of IFN α (8, 40 and 200 ng/ml) from 24 hr prior to infection through 72 hr post-infection. At 8 ng/ml of IFN α 2, GBV-B cell-associated RNA was suppressed by over 2000-fold, while secreted virus was reduced by 5000-fold (Figure 4A). No further decrease in viral RNA was observed with higher levels of IFN α , suggesting that the residual values for cell-associated RNA probably reflect non-replicating virus adhering to the cultures from the inoculum. Secreted virus was near the limits of detection in all IFN α 2 treated cultures and was undetectable at 40 ng/ml of IFN α 2 but again detectable at 200 ng/ml of IFN α 2. A second experiment was conducted to extend the lower IFN α range using 0.040 to 5.0 ng/ml. No decrease in viral RNA was detected at 0.04 ng/ml of IFN α 2, while viral RNA was decreased by 3.1- and 6.0-fold in cells and media at 0.2 ng/ml of IFN α 2, and over 2-log reductions of viral RNA were observed in both cells and media at 5.0 ng/ml (Figure 4B). The antiviral activity of marmoset and tamarin IFN α 2 were examined in marmoset hepatocytes as well. At 4 ng/ml of marmoset IFN α , cell-associated and secreted GBV-B RNA

were suppressed by 4.5-fold and 5.0-fold, respectively, while at 100 ng/ml IFN α 2, the cell-associated RNA decreased 357-fold and the secreted virus was undetectable (Figure 5). These data are in agreement with the data from EMCV in which higher levels of IFN α were required to observe inhibition on marmoset hepatocytes. Tamarin IFN α exhibited similar GBV-B antiviral activity as marmoset IFN α on marmoset cells, with 238-fold and 1136-fold suppression of cell and media viral RNA at 200 ng/ml, respectively.

Tamarin hepatocytes were examined in a time course experiment by pre-treating cells at 200 ng/ml for 24 hr prior to infection and then extending treatment for 3, 7 and 11 days post-infection. Cell-associated GBV-B RNA progressively declined over 11 days from 1533-fold at day 3 to 8632-fold at day 11 (Figure 6A). Again, the remaining cell-associated viral RNA probably represents residual inoculum adhering to the cultures, rather than replication, since secreted viral RNA was undetectable at all time points. No apoptosis or cytopathic effect was noted in the cultures exposed to high levels of IFN α for 11 days and for greater times in other studies. Since primary hepatocytes are non-dividing cultures, it was not possible to examine the effects of IFN α on proliferation. To examine the antiviral efficacy of IFN α 2 in an established infection, replicate cultures were infected with GBV-B for 3 days and then treated with IFN α 2. A marked reduction in the efficacy of the IFN α treatment was noted with only 51- to 97-fold decline in the cell-associated RNA after 3 and 11 days of treatment, respectively (Figure 6B). However, a more pronounced antiviral effect was observed for secretion of viral RNA with a 156-fold decline on day 3 of treatment and no detectable virus secretion on days 7 and 11. Although it was more effective to inhibit GBV-B using pre-infection treatment with greater than 3 logs of inhibition in secreted virus on day 3 (Figure 4A, 5, 6A), treatment of established infections still achieved more than a 2 log decrease in viral secretion by day 3. We repeated the experiment using 5 ng/ml of tamarin IFN α 2 to determine if lower levels of IFN α 2 could effectively inhibit an established infection. IFN α treatment was initiated 3 days post-infection and cultures were harvested after 1 to 7 days of treatment. Cell-associated viral RNA declined by more than 1 log (12-fold) after 24 hr of treatment with a 42-fold reduction by day 7 (Figure 6C). Secreted virus was reduced to below the levels of detection by day 7. Thus, a clear dose response was observed between experiments conducted with 5 and 200 ng/ml IFN α 2 in cultures with an established infection.

We also examined cultures derived from an infected animal to compare the antiviral activity of IFN α in cultures with infections established *in vivo*. Surprisingly, the reduction in cell-associated RNA was minimal over the 11 days of treatment at 200 ng/ml IFN α with a 14-fold reduction on day 3 in comparison to untreated cells and no substantial increase in the reduction over 11 days (Figure 6D). Day 11 data were difficult to interpret, since the levels of virus in untreated cultures declined over time in this set of cultures, presumably due to the less robust nature of primary cultures obtained from an animal during acute viral infection. The inhibition of viral secretion was of lesser magnitude than that observed with *in vitro* infected cultures as well, with a 14-fold reduction on day 3 of treatment (in comparison to 3 logs with pre-infection treatment on day 3).

Antiviral Activity of IFN γ in GBV-B infected cultures

The tamarin and marmoset IFN γ genes were amplified and cloned from PBMC stimulated with phorbol myristate acetate (PMA) and ionamycin. Marmoset and tamarin IFN γ differed by only 3 amino acids (Figure 7). Human IFN γ differed from marmoset and tamarin IFN γ by 18 and 16 residues, respectively, and thus the New World monkey IFN γ gene had diverged less than IFN α 2 in comparison to human. IFN γ was produced in Huh7 cells using the same procedure as described for IFN α . ELISA values indicated that marmoset and tamarin IFN γ were secreted in transfected Huh7 cells at 200 and 80 ng/ml, respectively. In the EMCV inhibition assay using A549 cells, tamarin and marmoset IFN γ exhibited specific activities of 1 U/pg and 0.5

U/pg, respectively. Tamarin IFN γ was compared with commercially obtained human IFN γ (R&D Systems; produced in bacteria) in GBV-B infected hepatocytes. Cultures were pretreated with 0.04 to 5.0 ng/ml and harvested 3 days post infection. Inhibition of GBV-B was first detected for tamarin IFN γ at 0.2 ng/ml with 2.6- and 5.1-fold reduction in the cell and media viral RNA, respectively. These values are remarkably close to the inhibition with 0.2 ng/ml of tamarin IFN α 2 (Fig. 4B). A dose response was observed with higher concentrations of IFN γ with 5.0 ng/ml reducing GBV-B RNA by 67-fold and 90-fold in the cells and media, respectively (Figure 8A). Human IFN γ exhibited no antiviral effect in tamarin hepatocytes at the concentrations tested (data not shown). The specific activity of the human IFN γ (0.002 U/pg) was much lower than the tamarin IFN γ when calibrated in the same ELISA and bioassay, and this alone could explain the lack of activity in the GBV-B assay even if no species difference in activity were present.

A time course experiment was performed in cultures with established infections. Cultures were treated at 5.0 ng/ml for 1 to 7 days beginning 3 days after infection. At day 3 of treatment, the fold reduction in cells and medium was only 3-fold lower than that observed in pre-treated cultures. A maximum reduction in viral RNA occurred at 7 days of treatment, with 120-fold inhibition of cell-associated RNA and undetectable secreted virus. The data suggest that GBV-B may have a more limited ability to block the antiviral activity of exogenous IFN γ in cultures with established infections. The antiviral activity of marmoset IFN γ in marmoset hepatocytes was examined in a titration experiment with pre-treatment for 24 hr prior to infection. The values were similar to that observed with tamarin IFN γ with a 221-fold reduction in cell-associated RNA on day 3 at 5.0 ng/ml (data not shown).

Antiviral activity of marmoset and tamarin IFN α and IFN γ in HCV replicon cells

The inhibition of EMCV cytopathic effect in human A549 cells suggested that both tamarin and marmoset IFNs should have antiviral activity against the HCV replicon in Huh7 cells. We examined tamarin and marmoset IFN α in the replicon model by treating a cell line containing a genotype 1b replicon for 72 hrs. Tamarin IFN α 2 (200 ng/ml) and marmoset IFN α 2 (100 ng/ml) reduced replicon RNA levels by 114- and 96-fold, respectively. Human IFN α 2 was used as a positive control at 1000 U/ml (0.8 ng/ml), a level we had previously observed to have potent antiviral activity in the replicon model (Lanford et al., 2003b). Human IFN α 2 reduced replicon levels by 37-fold and was probably less active due to the concentration difference. The values for marmoset and tamarin IFN α and IFN γ were comparable to the inhibition of GBV-B in cultures with established infections treated at the same level of IFN. IFN α 2 induction of transcripts for ISG12 and MX1 were monitored in the replicon cell line as well. Tamarin IFN α 2 yielded the highest induction of ISG12 (596-fold) and MX1 (1012-fold), while human IFN α 2 induced the transcripts by 276- and 112-fold.

Analysis of Interferon Stimulated Gene Expression in Marmoset Hepatocytes

One of the obstacles of working with GBV-B in tamarins and marmosets is the lack of reagents for New World primates. The marmoset genome has recently been sequenced, but at this time, it has not been annotated. The genetic divergence between humans and marmosets presents difficulties in the use of quantitative RT-PCR and microarrays for analysis of gene expression during GBV-B infections. We designed an experiment to test whether array platforms using the long-oligo approach may offer an opportunity to examine GBV-B infections with human arrays. We have previously utilized human microarrays to examine changes in liver gene expression in HCV acute and chronic infections in chimpanzees and during IFN α therapy (Bigger et al., 2001; Bigger et al., 2004; Lanford et al., 2006; Lanford et al., 2007). In one of these studies, primary human and chimpanzee hepatocytes were induced with IFN α 2 and changes in gene expression were determined at 4 and 8 hr post treatment (Lanford et al., 2006). Thus, a database of human genes verified to be induced by IFN α 2 in our primary

hepatocyte culture system was available for comparison. The identical experiment was conducted in marmoset hepatocytes using marmoset IFN α 2. Cultures were treated with or without 20 ng/ml of marmoset IFN α 2 and were harvested at 4 hr post treatment. Total cell RNA was derived from 18 cultures with pools of 3 cultures each used to probe 6 arrays, 3 untreated and 3 IFN α treated. The ABI long-oligo arrays representative of the entire human genome were utilized. Our previous studies in human and chimpanzee hepatocytes were performed with Affymetrix microarrays which utilize shorter oligos and were found to be unsuitable for analysis of marmoset hepatocytes under standard hybridization conditions. In contrast, the ABI arrays scored a large number of transcripts as present and significantly induced by IFN α 2.

Our previous studies in chimpanzee and human hepatocytes detected 384 ISGs (positively induced genes), while our studies in the chimpanzee detected 500 ISGs in the liver and over 1000 total ISGs detected in combined data from PBMC and liver (Lanford et al., 2006). Of the ISGs detected in chimpanzee hepatocytes, only 227 were detected at 4 hr post-treatment, the only valid time point for comparison to the marmoset study, and of these 83 (37%) were detected and induced in the marmoset hepatocytes. ISGs detected in both human/chimpanzee hepatocytes and marmoset hepatocytes represented many of the commonly recognized ISGs (Supplement Table 1), and 30 of the genes with the largest fold induction in the chimpanzee/human data set are shown in Table 2. An additional 21 ISGs detected in the marmoset had been detected in chimpanzees under conditions other than the 4 hr time point (either in hepatocytes at 8 hr post-IFN or in liver of IFN α treated chimpanzees) confirming their ISG designation. Thus, 104 ISGs were detected by the marmoset array. Reproducibly down-regulated transcripts were detected as well that were common to the marmoset and chimpanzee/human data sets (Supplement Table 1). There were 366 ISGs in the chimpanzee/human data set that were not detected in the marmoset hepatocytes (Supplement Table 2), but only 144 of these ISGs were detected at 4 hr in the in vitro chimpanzee/human hepatocytes experiments. The lack of detection of many ISGs with the ABI arrays is likely due to sequence divergence of the marmoset. Thirty of the most highly induced genes in chimpanzee and human hepatocytes not detected in marmosets are shown in Table 3.

There were 1660 genes detected using the ABI arrays that were not detected in our previous studies with Affymetrix arrays in chimpanzees (Supplement Table 3), 675 of these have no gene symbol and are denoted as nulls. Genes that were only detected using the ABI array with marmoset hepatocytes were for the most part not known ISGs, and their presence may reflect a less stringent selection criteria and an under-powering of the experiment using 3 treated and 3 untreated arrays. Since many of these transcripts are not represented on the Affymetrix array, the reason for their detection with the ABI array using marmoset hepatocytes is uncertain. Resolution of this issue would require repetition of the chimpanzee and human in vitro experiments using the ABI arrays. The detection of a large set of ISGs in marmoset cells under conditions known to induce this set of genes suggests that human long-oligo arrays are suitable for pathway analysis in GBV-B infections, even though many genes will be missed.

Discussion

The GBV-B model provides a powerful surrogate system for HCV. GBV-B is the most closely related virus to HCV phylogenetically, and it displays a liver tropism similar to HCV. The use of small non-human primates as the animal model for GBV-B, in contrast to chimpanzees for HCV, has distinct advantages. However, the fact that tamarins and marmosets are New World monkeys presents challenges as well, since they have not been as extensively characterized as models for infectious disease, and immunological and molecular reagents are limited. This deficiency is being overcome rapidly due to an increased interest in the marmoset as a model for numerous biomedical studies, and the recent sequencing of the marmoset genome. In this

report, we have cloned and expressed IFN α and IFN γ from the tamarin and marmoset to advance studies on the mechanism of antiviral response to GBV-B as a surrogate for HCV, and to compare and contrast the effects of type I and II IFNs in these closely related models.

The IFN α genes cloned from several tissues from both marmosets and tamarins were most closely related to human IFN α 2 showing 17 to 18% divergence from the human sequence. Surprisingly, the marmoset and tamarin clones differed by 5% at the amino acid level. Both marmoset and tamarin IFN α 2 displayed a high level of cross-species functionality in human cells when tested in the EMCV inhibition assay and for the inhibition of the HCV replicon in Huh7 cells. High levels of IFN α 2 were required to inhibit EMCV in primary hepatocytes from marmosets and tamarins, suggesting that hepatocytes may have a reduced sensitivity to IFN α . Inhibition of EMCV in marmoset hepatocytes required 1 million-fold more human IFN α 2 in comparison to human cells. This was not simply a lack of cross-reactivity of human IFN α to marmoset cells, since over 30,000-fold more marmoset IFN α 2 was required in marmoset cells in comparison to human cells. The A549 cells have been selected for their sensitivity to IFN in this assay, and this may account for much of the differences observed. The different sensitivities of the cell lines to IFN α may be a reflection of differences in the levels of various components of the Jak-STAT pathway. The enhancement of the IFN α response following priming with IFN γ demonstrates the impact of levels of Jak-STAT signaling proteins on the intensity of the IFN α response (Levy et al., 1990).

Actual estimation of the levels of IFN α and the specific activity were complicated by the lack of an immunological assay specific for marmoset and tamarin IFN α and appropriate standards. Although the dilution of each IFN possessing 1 unit of activity (inhibition of EMCV cytopathic effect on human A549 cells) is easily measured, the concentration of IFN α represented by 1 unit could only be estimated using an ELISA for human IFN α and human IFN α standards. The fact that tamarin IFN α had higher activity than human IFN α on human A549 cells could be due to an underestimation of the tamarin IFN α levels in the ELISA, or an increase in the specific activity of IFN produced and secreted in mammalian cell culture in comparison to protein synthesized in bacterial culture (human IFN α). Nonetheless, pretreatment of primary hepatocytes with tamarin and marmoset IFN α 2 revealed potent antiviral activity with complete inhibition of GBV-B secretion and reduction of cell-associated viral RNA to levels approaching the background from viral particles from the inoculum adhering to the cultures. In contrast, the antiviral activity in cultures with established GBV-B infections was reduced.

The innate immune response of the host recognizes a variety of pathogen-associated molecular patterns. Viral RNA can interact with a number of cellular proteins to trigger the innate response through the toll-like receptors TLR3 and TLR7/8, and the intracellular sensing molecules RIGI and MDA5 (Yoneyama et al., 2004; Yoneyama et al., 2005; Foy et al., 2005) which lead to downstream activation of IRF3 and NF- κ B and induction of Type I IFN synthesis. Most viruses have evolved mechanisms to block activation of the innate responses. The HCV NS3/4a protease blocks this pathway (Foy et al., 2005; Foy et al., 2003; Li et al., 2005a) via cleavage of the mitochondrial adapter protein (CARDIF, IPS-1, MAVS, VISA) (Xu et al., 2005; Seth et al., 2005; Kawai et al., 2005; Meylan et al., 2005). The GBV-B NS3 protein has been shown to similarly cleave the tamarin IPS protein (Chen et al., 2007). HCV inhibits the innate response by other mechanisms that are likely shared by GBV-B as well. However, further studies will be required to determine the mechanism by which GBV-B interferes with the antiviral activity of exogenously added IFN α .

Studies with GBV-B not only offer the advantage of a small primate model, but the GBV-B model also offers the advantage of an in vitro culture system using primary hepatocytes. Although the use of primary cultures is a disadvantage in routine virological studies, continuous

cell lines are subject to many alterations that affect viral-host interactions. Recent studies with in vitro cultivation of HCV in Huh7.5 cells have provided new opportunities to examine aspects of HCV replication and viral-host interactions (Lindenbach et al., 2005; Zhong et al., 2005; Cai et al., 2005; Yi et al., 2006; Wakita et al., 2005), but this model is limited in its potential for use in some aspects of the interaction of the virus with the innate immunity. Our early studies in the replicon demonstrated that Huh7 cells were defective in the response to dsRNA (poly-IC) in comparison to primary hepatocytes and other hepatoma cell lines (Lanford et al., 2003b). Others have found Huh7 to be defective in innate responses, as well (Li et al., 2005b; Preiss, 2008) and more recent studies have suggested that Huh7.5 cells are even further defective in these pathways, thus perhaps partially explaining their uniquely high permissiveness to JFH1 infection and replication (Saito et al., 2007). Thus, improved in vitro models are needed for analysis of the myriad of viral-host interactions that appear to be regulating the innate response to HCV infection. Primary hepatocytes may also reveal viral-host interactions involved in replication and pathogenesis beyond the innate immunity that are not represented in the current established cell lines.

Although, the GBV-B model offers distinct advantages for analysis of some viral-host interactions, as mentioned above, the analysis of viral-host interactions in GBV-B is limited by the availability of reagents. In this study, we examined human microarrays for use in the marmoset model. Using long-oligo arrays, we were able to detect increases in many of the same genes detected in human and chimpanzees hepatocytes in response to IFN α . This will provide an opportunity to initiate studies on the host response to GBV-B both in vivo and in vitro, until the marmoset genome has been annotated and a marmoset specific array has been generated. It has only recently become possible to examine pre- and post-infection changes in gene expression with HCV using the JFH1-Huh7.5 culture system. However, for reasons still not appreciated, the JFH-1 virus replicates well in vitro but poorly in vivo in chimpanzees (Lindenbach et al., 2006), while GBV-B exhibits robust replication both in vivo and in vitro, allowing direct comparison to changes in gene expression under both conditions. Again, despite the current limitations of working in New World primates, the GBV-B model offers distinct advantages over HCV for certain types of studies.

The mechanism of the antiviral response to IFN α has been highly characterized in a number of model systems, yet the reasons for failed IFN α therapy in humans infected with HCV are still poorly understood. Recently, a correlation was observed between failed response to IFN α and increased pretreatment levels for some hepatic ISG transcripts (Chen et al., 2005). Studies in HCV chronically infected chimpanzees demonstrated that high pretreatment levels of liver transcripts for ISGs correlated with a lack of antiviral response to IFN α therapy and a lack of transcriptional response of the liver to IFN α , while PBMC in the same chronically infected animals responded to IFN α in a manner similar to uninfected animals (Lanford et al., 2006; Lanford et al., 2007). The recent use of paired biopsies in humans undergoing IFN α therapy demonstrated that individuals responding to IFN α therapy have low pretreatment levels of ISGs and undergo further activation of hepatic ISGs upon dosing with IFN α , while individuals that failed therapy were similar to chimpanzees and had high pretreatment levels of hepatic ISG transcripts and no further induction upon dosing with IFN α (Sarasin-Filipowicz et al., 2008). A number of factors have been shown to correlate with response to IFN α therapy including the HCV genotype, NS5A ISDR sequence, viral load, age, race, weight and fibrosis. Presently, the interaction of each of these factors with the antiviral response initiated by exogenous IFN α is incompletely understood. Although the marmoset small size lends itself well to studies on the antiviral response to IFN α , currently it is not possible to examine chronic infections in the GBV-B model. Persistent infections have been rarely observed and eventually resulted in viral clearance (Martin et al., 2003; Nam et al., 2004). Ongoing efforts to generate chronic infections in this model may overcome this limitation in the near future, whereas the

lack of chronic infection in the GBV-B model may offer insight into the roles of the innate and adaptive immune response required for viral clearance and persistence.

Materials and Methods

Animals and Hepatocytes

Tamarins (*Saguinus mystax*) and marmosets (*Callithrix jacchus*) were housed at the Southwest National Primate Research Center at the Southwest Foundation for Biomedical Research. The animals were cared for 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. Liver and spleen samples were obtained by either open biopsy or at the time of sacrifice. Total cell RNA was purified from tissues using RNeasy RLT (Qiagen, Crawfordsville, IN, USA) following the manufacturer's protocol and quantified by optical density. Primary hepatocytes were isolated by collagenase perfusion and were maintained in a hormonally defined, serum free medium (Lanford et al., 2003a) (Beames et al., 2000; Lanford and Estlack, 1998). Cells were grown in 6 well dishes for 3 days prior to infection. Inoculations were performed with 5 μ l of GBV-B containing serum (approximately 2×10^5 to 4×10^6 GE) in 1 ml of serum free medium for 6 hr at 37 °C followed by two washes to remove residual inoculum. Culture medium was changed three times per week.

Interferon cloning and sequence analysis

Primers for cDNA cloning of IFN α were designed by aligning all human IFN α genes and the tamarin IFN α sequence (12). The forward primer included a KpnI site and the first 22 nucleotides of the IFN α open reading frame. The reverse primer included an EcoRI site and the last 27 nucleotides of the anti-sense strand. cDNAs from primary tamarin hepatocytes, tamarin liver, marmoset liver, and marmoset spleen were amplified using the Superscript One-Step RT-PCR for Long Templates kit (Invitrogen, Carlsbad, CA, USA) and cloned into the mammalian expression vector pcDNA4/TO (Invitrogen, Carlsbad, CA, USA). The IFN γ sequence was cloned from tamarin and marmoset PBMC using a similar design. PBMC from marmoset and tamarins were cultivated 24 hr in RPMI with 10% FBS and then treated for 24 hr with 10 ng/ml phorbol myristate acetate and 1 μ g/ml ionomycin to induce IFN γ transcription. The primers were based on the sequence of the human IFN γ gene. The forward primer was 5'-GGGGTACCACAATGAAATATACAAGTTATATCTTGGC-3' and the reverse primer was 5'-GGAATTCTTATTGGGACGCTCTTCGACCTCG-3'. GenBank accession numbers for tamarin and marmoset consensus sequences are: Tamarin IFN α 2 = **FJ598590**, Marmoset IFN α 2 = **FJ598591**, Tamarin IFN γ = **FJ598592**, Marmoset IFN γ = **FJ598593**.

Production and Assay of recombinant interferon

A single clone was selected for expression of marmoset and tamarin IFN α and IFN γ . Huh7 cells were transfected using Mirus II Trans-It transfection kit (Mirus Corp., Madison, WI, USA) following the manufacturer's protocol. Huh7 cells were transfected in the same manner using the vector without an insert as a negative control. At 24 hours post transfection, the medium was replaced with the serum-free medium formulated for primary hepatocytes (Lanford et al., 2003a), and the culture medium was harvested at 48 hr post transfection. Secreted IFN α was concentrated by ultrafiltration (10,000 molecular weight cut-off; Millipore, Bedford, MA), and the final concentration was determined by ELISA (Human Interferon Alpha ELISA Kit; R&D Systems, Minneapolis, MN, USA). The specific activity of IFN α 2 was determined using a bioassay for inhibition of EMCV induced cytopathic effect in the A549 human cell line. A549 cells growing in 96 well plates were pretreated for 24 hr with 3-fold dilutions of IFN α 2, cells were infected with 10^4 TCID $_{50}$ of EMCV per well, and monolayers were stained with crystal violet at 24 hr post-infection. One unit (U) was the amount required to reduce cytopathic effect by approximately 50%. The absence of non-IFN α contaminants with ISG inducing activity

was demonstrated by immuno-depletion of ISG-inducing activity with rabbit anti-IFN α (data not shown) and production of media lacking ISG-inducing activity following transfection of the same cells with the vector without an insert. IFN γ was also quantified using an ELISA based on a polyclonal antiserum that cross-reacted with tamarin and marmoset IFN γ (Quantikine; Human IFN γ Immunoassay; R&D Systems, Minneapolis MN).

RT-PCR for GBV-B

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).

Microarray analysis

Primary marmoset hepatocytes were cultivated in serum free medium in 6 well dishes. Nine wells each were untreated or treated with 1000 U/ml of marmoset IFN α for 4 hr prior to harvest for total cellular RNA. Each sample for array analysis was comprised of a pool of three wells for a total of 6 arrays, 3 treated and 3 untreated. Raw data was processed using the AB 1700_Algorithm. The data was normalized using ABI's quantile-based normalization, and the normalized signal intensities were used to generate fold changes between the treated and untreated samples. Each of the 3 treated arrays was compared to each of the control arrays, and genes that differed by 2-fold or more in 6 of 9 comparisons were included in the comparison with chimpanzee and human hepatocytes. Analysis of primary chimpanzee and human hepatocytes using Affymetrix arrays has been previously published (Lanford et al., 2006). The chimpanzee hepatocytes were cultivated under identical conditions as used for marmoset hepatocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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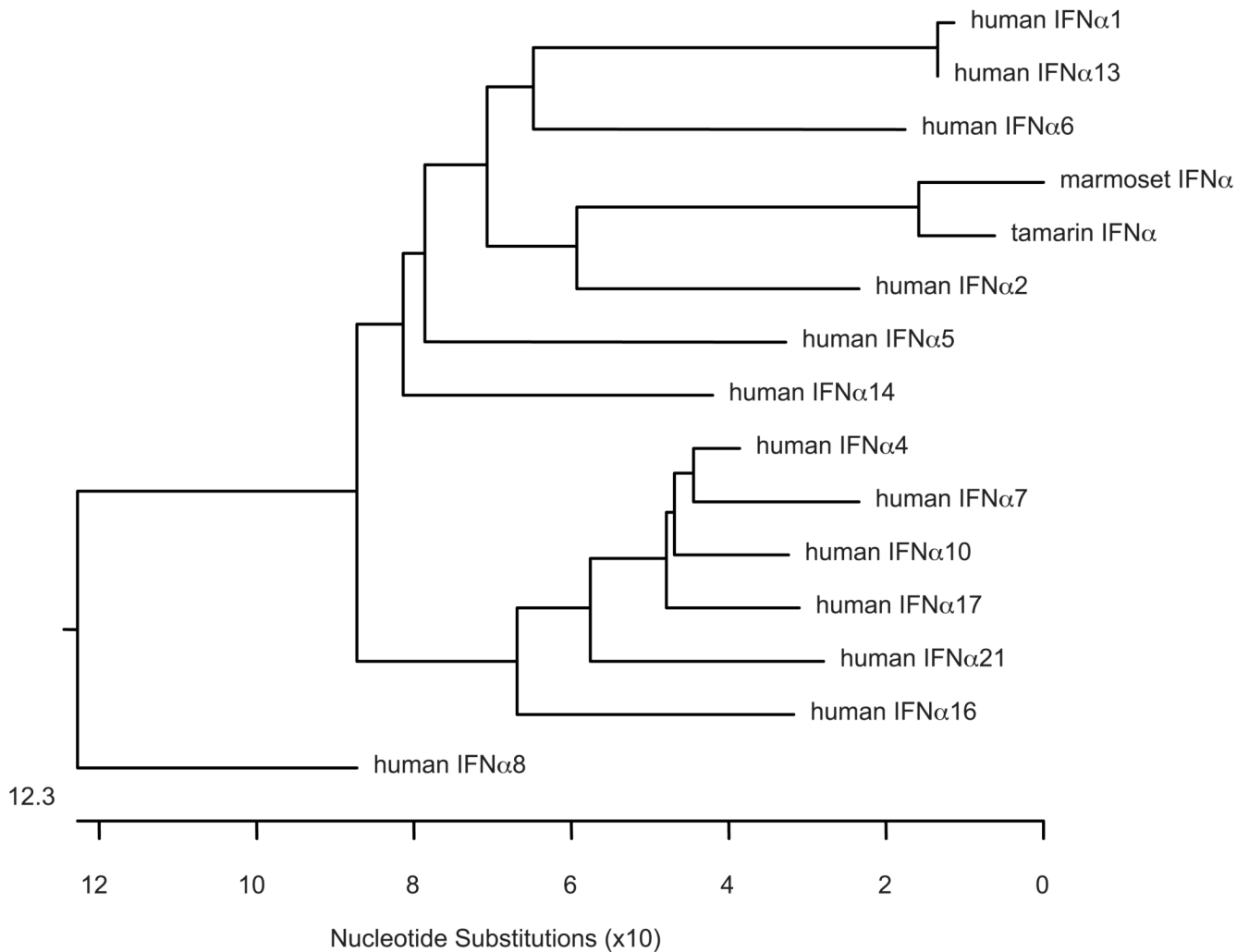


Figure 1.

Phylogenetic analysis of IFN α genes. A phylogenetic tree comparing the thirteen human IFN α genes with the marmoset and tamarin IFN α genes was compiled using software programs in LaserGene. Marmoset and tamarin IFN α cDNAs cloned in this study were most closely related to human IFN α 2. Accession numbers for the IFN α genes are: IFN α 1 (J00210), IFN α 2 (V00549), IFN α 4 (X02955), IFN α 5 (X02956), IFN α 6 (X02958), IFN α 7 (M34913), IFN α 8 (K01900), IFN α 10 (V00551), IFN α 13 (X75934), IFN α 14 (NM_002172), IFN α 16 M28585, IFN α 17 (V00532) and IFN α 21 (NM_002175). The tamarin IFN α 2 (**FJ598590**) and marmoset IFN α 2 (**FJ598591**) have been deposited with GenBank.

	10	20	30	40	50	
1	MALTFRLLVALLVLSCKSSCSVGCGLPQTHSLGSRRTLMLLAQMRKISLF					human IFN α 2
1	...P.V..M..V..N...I..L.....SN.....IM...GR..P.					human IFN α 5
1	.T...P.....V...Y.....L.....Q.N.....R..P.					tamarin IFN α
1	.T...P..M..T...Y.....LD.N.....N.....R..P.					marmoset IFN α
	60	70	80	90	100	
51	SCLKDRHDFGFPQEEF-GNQFQKAETIPVLHEMIQQIFNLFSTKDSSAAW					human IFN α 2
51D.....QA.S.....T.....T.					human IFN α 5
51R..E.....D.....RA.F.....T.....					tamarin IFN α
51E.....D.....RA.F...I..T.....					marmoset IFN α
	110	120	130	140	150	
100	DETLKDKFYTELYQQLNLEACVIQGVGTETPLMKEDSILAVRKYFQRI					human IFN α 2
101MM.E...ED...NV...T.....					human IFN α 5
101R.....E.....M.E...D..VLN...T.....					tamarin IFN α
101R.....E.....M.E...D..LN...T.K.....					marmoset IFN α
	160	170	180	190		
150	TLYLKEKKYSPCAWEVVRAEIMRSFSLSTNLQESLRSKE					human IFN α 2
151	...T.....A...R..R..					human IFN α 5
151A.....KG...K					tamarin IFN α
151A.....S...KG...K					marmoset IFN α

Figure 2.

Alignment of amino acid sequence of IFN α genes. Human IFN α 2 and IFN α 5 amino acid sequences were aligned with the marmoset and tamarin IFN α 2 sequences. The human IFN α 2 was used as the consensus sequence with sequence identity to this sequence indicated by a dot. The full length intact proteins are 189 amino acids (188 amino acids for human IFN α 2, note internal deletion) with the first 23 amino acids being the cleaved signal sequence. The marmoset and tamarin IFN α 2 genes differ at 10 residues.

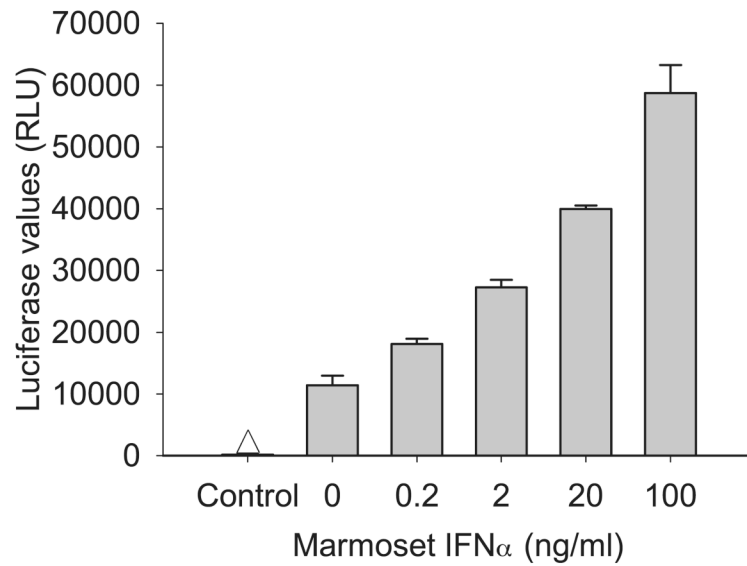


Figure 3.

Transcriptional activation of ISRE promoter by marmoset IFN α 2. Primary marmoset hepatocytes were transfected with a pISRE-Luc (Stratagene) which contains five copies of the IFN-stimulated response element (ISRE) derived from the ISG54 gene upstream of a basic promoter element (TATA box) and the firefly luciferase gene. Cells were treated with various concentrations of marmoset IFN α 2 and harvested 24 hr later for luciferase assay. Error bars indicate the variation in values from duplicate cultures. The level of variation was not sufficient to yield visible error bars on those where none appear.

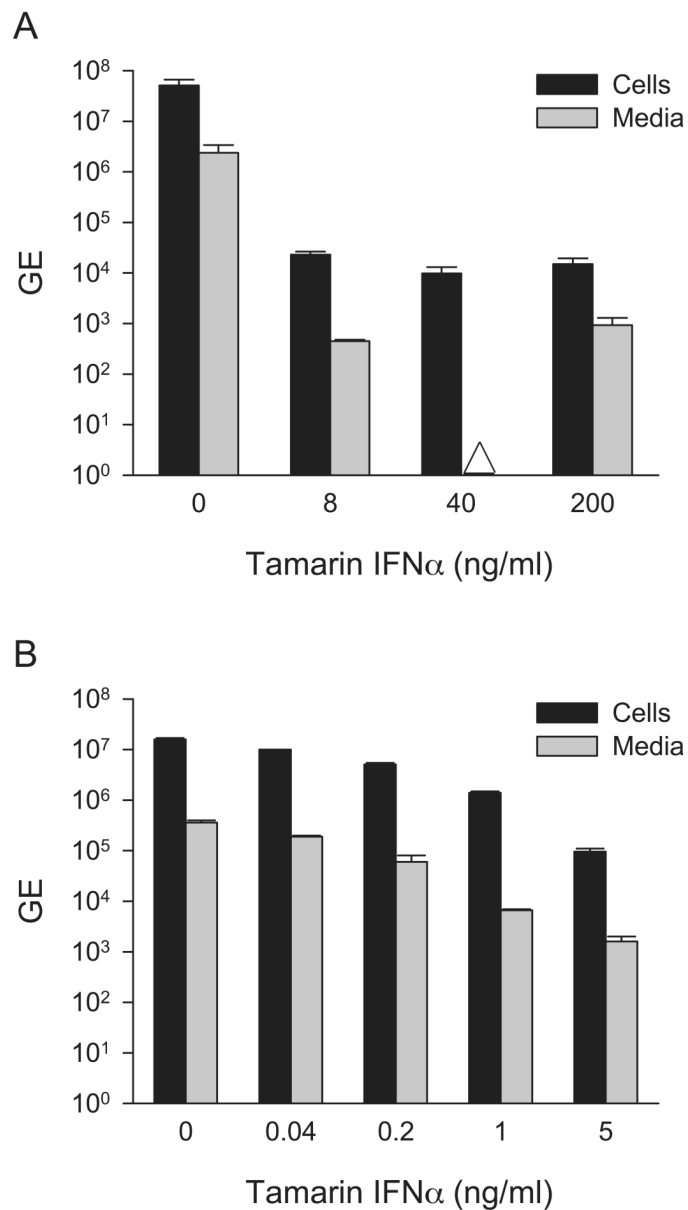


Figure 4. Titration of antiviral activity of tamarin IFN α 2 on tamarin hepatocytes infected with GBV-B. 4A. Tamarin primary hepatocytes were treated with various concentration of tamarin IFN α 2 (0 to 200 ng/ml) for 24 hr prior to infection with GBV-B and for an additional 72 hr after infection. Total RNA was purified from cells and media and analyzed for GBV-B RNA genome equivalents (GE) by quantitative RT-PCR. 4B. Tamarin primary hepatocytes were treated with 0 to 5 ng/ml of tamarin IFN α 2 and infected with GBV-B as in 4A. Values are expressed as GE per culture based on 6 well dishes. Black bars = GBV-B GE in cell-associated viral RNA, grey bars = GE of GBV-B secreted into media. The Δ symbol denotes that the sample was below the level of detection. Error bars indicate the variation in values from duplicate cultures. The level of variation was not sufficient to yield visible error bars on those were none appear. Comparison of the values for cell associated viral RNA in the treated and untreated cultures in 4A and 4B using a Student's t-Test yielded p values of $p=0.0002$ and 0.002 , respectively.

Comparison of the values for the media associated viral RNA in the treated and untreated cultures in 4A and 4B using a Student's t-Test yielded p values of $p=0.002$ and $p=0.0007$.

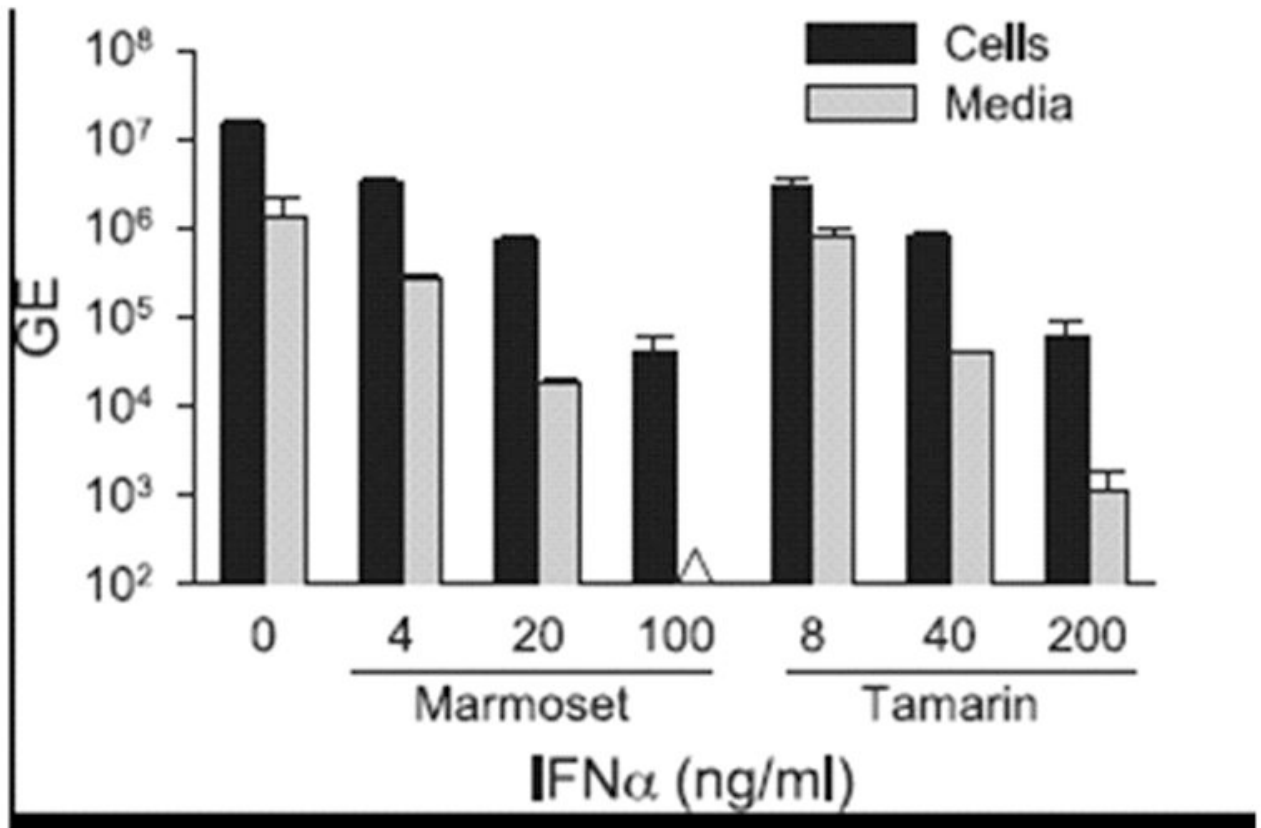
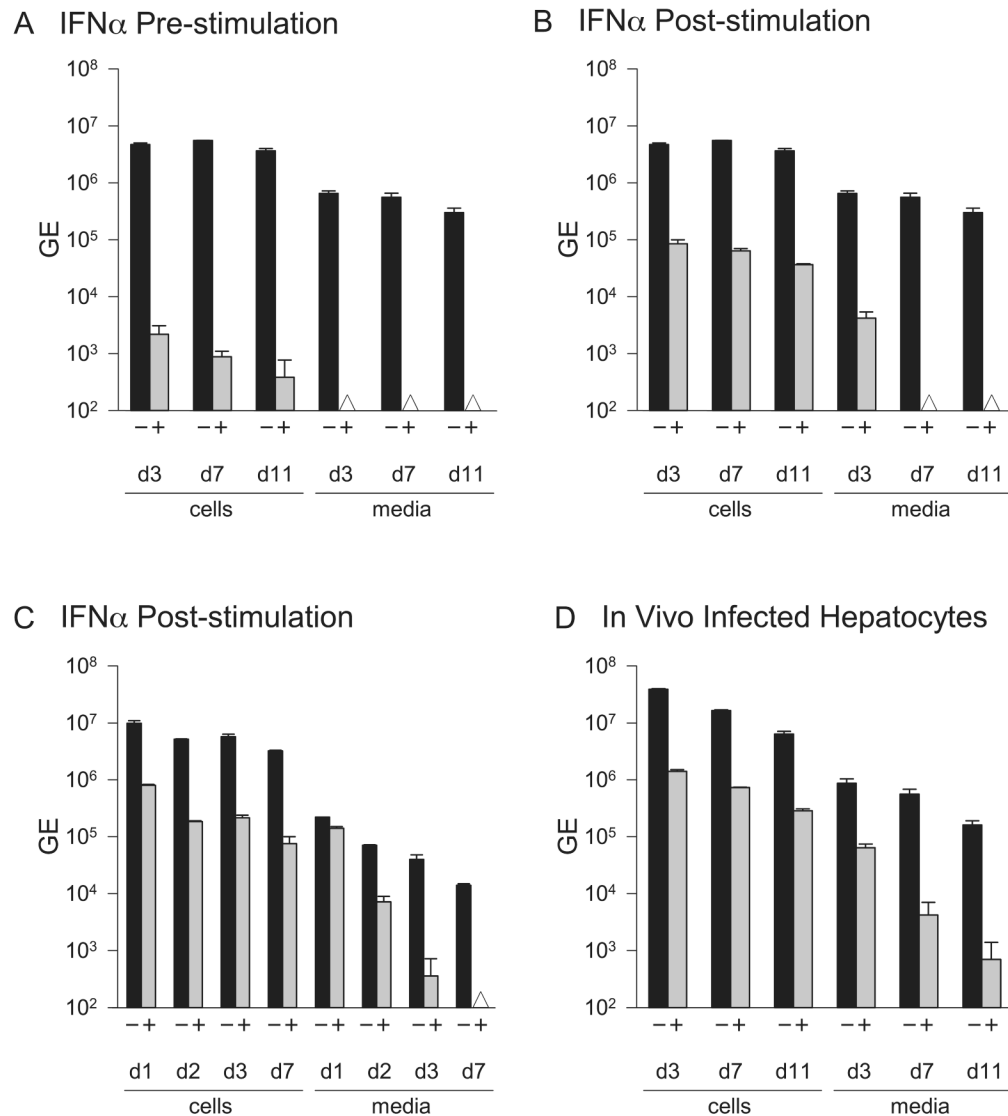


Figure 5.

Comparison of antiviral activity of marmoset and tamarin IFN α 2 on marmoset hepatocytes infected with GBV-B. Primary marmoset hepatocytes were treated with either marmoset or tamarin IFN α 2 at concentrations of 0 to 200 ng/ml for 24 hr prior to infection with GBV-B and for another 72 hr post-infection. Cell and media were analyzed for GBV-B RNA as described for Figure 4. Black bars = GBV-B RNA GE in cell-associated viral RNA, grey bars = GE of GBV-B secreted into media. The Δ symbol denotes that the sample was below the level of detection. Error bars indicate the variation in values from duplicate cultures. The level of variation was not sufficient to yield visible error bars on those where none appear.

**Figure 6.**

Time course of antiviral activity of tamarin IFN α 2 in GBV-B infected tamarin hepatocytes. A. Pre-infection IFN α treatment. Tamarin hepatocytes were either untreated (-) or treated (+) for 24 hr with 200 ng/ml of tamarin IFN α 2 prior to infection with GBV-B, and for an additional 3, 7 or 11 days prior to harvest. B. Post-infection IFN α treatment. Tamarin hepatocytes were infected with GBV-B for 3 days to establish the infection and then treated (+) with 200 ng/ml tamarin IFN α 2 for 3, 7 or 11 days before harvest. C. Post-infection IFN α treatment. Tamarin hepatocytes were infected with GBV-B for 3 days prior to treatment (+) with 5 ng/ml of tamarin IFN α 2 for 1, 2, 3 or 7 days before harvest. D. Post-infection IFN α treatment. Tamarin hepatocyte cultures were prepared from a tamarin during the acute phase of infection with GBV-B to obtain cells with an *in vivo* established infection. Three days after plating, the hepatocytes were treated with 200 ng/ml of tamarin IFN α 2 for 3, 7 or 11 days before harvest. Black bars = GE of cell-associated GBV-B RNA, grey bars = GE of GBV-B secreted into media. The Δ symbol denotes that the sample was below the level of detection. Error bars indicate the variation in values from duplicate cultures. The level of variation was not sufficient to yield visible error bars on those were none appear.

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      10      20      30      40      50
1  MKYTSYILAFQLCIVLGSISLGCYCQDPYVKEAENLKKYFNAGHSDVADNGT human IFN $\gamma$ 
1  .....D..... tamarin IFN $\gamma$ 
1  .....V.....D..... marmoset IFN $\gamma$ 

      60      70      80      90      100
51 LFLGILKNWKEESDRKIMQSQIVSFYFKLFKNEFKDDQSIQKSVETIKEDM human IFN $\gamma$ 
51 ...D..RT..R..G.....I.....N.....M..... tamarin IFN $\gamma$ 
51 ...D..RT..R..G.....I.....N.....M..... marmoset IFN $\gamma$ 

      110     120     130     140     150
101 NVKFFNSNKKKRDDFEKLTINYSVIDLNVQRKAIHELIQVMAELSPAAGTG human IFN $\gamma$ 
101 .....R.Q...R....K.....P.I. tamarin IFN $\gamma$ 
101 .....R.Q...R....N.....P.I. marmoset IFN $\gamma$ 

      160
151 KRKRSQMLFRGRASQ human IFN $\gamma$ 
151 .....T..... tamarin IFN $\gamma$ 
151 ..R...T..... marmoset IFN $\gamma$ 

```

Figure 7.

Alignment of amino acid sequence of marmoset, tamarin and human IFN γ genes. The human IFN γ sequence is used as the consensus sequence with identity to this sequence indicated by a dot. Tamarin and marmoset IFN γ genes differ by 3 residues, while they differ from the human sequence at 16 and 18 residues, respectively. The full length proteins are 166 amino acids with the first 23 residues representing the cleaved signal sequence.

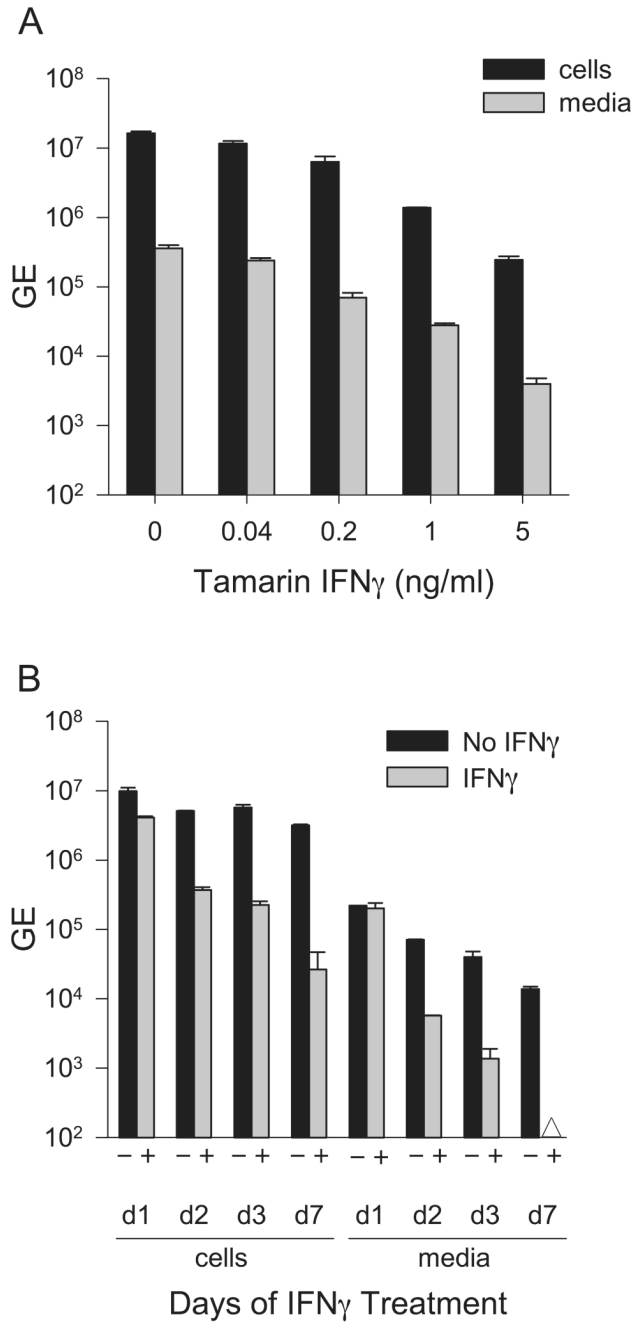


Figure 8. Antiviral activity of tamarin IFN γ in tamarin hepatocytes infected with GBV-B. A. Pre-infection IFN γ treatment. Tamarin primary hepatocytes were treated with various concentration of tamarin IFN γ (0 to 5.0 ng/ml) for 24 hr prior to infection with GBV-B and for an additional 72 hr after infection. Total RNA was purified from cells and media and analyzed for GBV-B RNA genome equivalents (GE) by quantitative RT-PCR. B. Post-infection IFN γ treatment. Tamarin hepatocytes were infected with GBV-B for 3 days to establish the infection and then treated with tamarin IFN γ (+) at 5.0 ng/ml for 1, 2, 3 or 7 days before harvest. The Δ symbol denotes that the sample was below the level of detection. Error

bars indicate the variation in values from duplicate cultures. The level of variation was not sufficient to yield visible error bars on those were none appear.

Table 1Specific activity of IFN α 2 on different cell types^a

IFNα2	Human A549	Tamarin hepatocyte	Marmoset hepatocyte
Human	1.25	0.00004	0.000001
Tamarin	1.60	0.003	0.000004
Marmoset	0.25	0.0008	0.000008

^aValues expressed as U/pg based on inhibition of EMCV.

Table 2
Interferon Stimulated Genes Detected in Marmoset Hepatocytes^a

Gene symbol ^b	ABI Microarray		Affymetrix UI33 Plus 2.0 Array			
	Marmoset hepatocytes		Chimpanzee and human hepatocytes		Uninfected chimpanzee liver	
	4 hr	8 hr	4 hr	8 hr	4 hr	8 hr
CXCL11 (I-TAC)	11	172	161	172	21	25
RSAD2 (Viperin)	117	116	130	116	13	15
IFIT2 (ISG54)	34	16	55	16	38	7
CXCL10 (IP-10)	18	46	50	46	24	6
IFIT1 (ISG56)	55	44	44	44	18	13
GBP5	11	37	37	37	6	
IFIT3 (RIG-G)	112	21	30	21	26	9
PML	3	10	26	10		
SP110	4	10	23	10		4
HK2	2	15	22	15		
LOC129607	11	18	20	18	28	17
DDX58 (RIG-I)	28	13	19	13	14	10
IFIH1 (MDA5)	2	13	14	13	13	8
HERC6	10	16	13	16	30	15
IL28RA	2		13		3	
OASL	132	14	13	14	9	4
ETV7	5	12	13	12		
ZC3HAV1	2	5	12	5	4	
MX1	26	14	12	14	19	18
ISG15	19	14	12	14	20	23
HERC5	6	15	11	15	12	9
TNFSF10 (TRAIL)	3	5	10	5	6	
SECTM1	3	5	10	5	5	4
SAMD9L	11	10	8	10	5	5
DHX58 (LGP2)	11	8	8	8	6	7
EPSTI1	68	12	8	12	7	7

Gene symbol ^b	ABI Microarray		Affymetrix UI33 Plus 2.0 Array			
	Marmoset hepatocytes		Chimpanzee and human hepatocytes		Uninfected chimpanzee liver	
	4 hr	8 hr	4 hr	8 hr	4 hr	8 hr
IL1RN	5		7	4	6	3
NT5C3	4		7	5	7	3
OAS1	30		7	10	12	8
OAS2	17		7	9	11	11

^a Comparison of microarray data from marmoset hepatocytes, chimpanzee and human hepatocytes, and chimpanzee liver following treatment with IFN α 2. Data shown are fold-induction of genes in comparison to untreated cultures or chimpanzees.

^b Common gene names are shown in parenthesis.

Table 3
Interferon Stimulated Genes Not Detected in Marmoset Hepatocytes^a

Gene symbol	ABI Microarray		Affymetrix U133 Plus 2.0 Array			
	Marmoset hepatocytes		Chimpanzee and human hepatocytes		Uninfected chimpanzee liver	
	4 hr	8 hr	4 hr	8 hr	4 hr	8 hr
IFI44L	ND ^b	112	175	24	31	
SOCS1	ND	28				
ANGPTL1	ND	21	34			
CCL8	ND	20	15	16	7	
LAMP3	ND	19	33			
IFIT3	ND	18	14	26	13	
TREX1	ND	17	15	7	7	
LINC1	ND	17				
CX3CL1	ND	16				
---	ND	16		36		
MAB21L2	ND	15		5		
IFI16	ND	15	18	8	5	
SLENS5	ND	14	16			
USP18	ND	13	14	10	4	
ISG20	ND	13	21	28	45	
RNF24	ND	13				
BATE2	ND	12	6	13		
HES4	ND	12	11			
FAM26F	ND	11		5	3	
SAMD9	ND	10	10	10	5	
NOD2	ND	9				
USP42	ND	9				
SDS	ND	9	6			
CYP11B1	ND	9				
PRIC285	ND	9	8	9	5	
CXCL9	ND	8	6			

Gene symbol	ABI Microarray		Affymetrix UI33 Plus 2.0 Array			
	Marmoset hepatocytes		Chimpanzee and human hepatocytes		Uninfected chimpanzee liver	
	4 hr	8 hr	4 hr	8 hr	4 hr	8 hr
SLEF5	ND		8	6	4	
GBP4	ND		8	5	5	3
IFI44	ND		8	12	8	9
GBP1	ND		8	7	8	6

^a Comparison of microarray data from marmoset hepatocytes, chimpanzee and human hepatocytes, and chimpanzee liver following treatment with IFN α 2. Data shown are fold induction of genes in comparison to untreated cultures or chimpanzees.

^b ND, No change greater than two-fold detected.