Virus Research 179 (2014) 93–101

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Changes in immune cell populations in the periphery and liver of GBV-B-infected and convalescent tamarins (*Saguinus labiatus*)☆

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ARTICLE INFO

Article history:
Received 5 July 2013
Received in revised form 3 November 2013
Accepted 7 November 2013
Available online 15 November 2013

Keywords:
Acute viral hepatitis
Immune cell
GBV-B

ABSTRACT

Flaviviruses related to hepatitis C virus (HCV) in suitable animal models may provide further insight into the role that cellular immunity contributes to spontaneous clearance of HCV. We characterised changes in lymphocyte populations in tamarins with an acute GBV-B infection, a hepatitis virus of the flaviviridae. Major immune cell populations were monitored in peripheral and intra-hepatic lymphocytes at high viraemia or following a period when peripheral virus was no longer detected. Limited changes in major lymphocyte populations were apparent during high viraemia; however, the proportions of $CD3^+$ lymphocytes decreased and $CD20^+$ lymphocytes increased once peripheral viraemia became undetectable. Intrahepatic lymphocyte populations increased at both time points post-infection. Distinct expression patterns of PD-1, a marker of T-cell activation, were observed on peripheral and hepatic lymphocytes; notably there was elevated PD-1 expression on hepatic $CD4^+$ T-cells during high viraemia, suggesting an activated phenotype, which decreased following clearance of peripheral viraemia. At times when peripheral vRNA was not detected, suggesting viral clearance, we were able to readily detect GBV-B RNA in the liver, indicative of long-term virus replication. This study is the first description of changes in lymphocyte populations during GBV-B infection of tamarins and provides a foundation for more detailed investigations of the responses that contribute to the control of GBV-B infection.

1. Introduction

Hepatitis C virus (HCV) establishes a chronic infection in approximately 70% of infected individuals (Bowen and Walker, 2005; Chisari, 2005). The mechanisms by which the remainder eliminate detectable infection are not fully understood, though a vigorous multispecific $CD4^+$ and $CD8^+$ T-cell response is thought to contribute to viral clearance (Chang et al., 2001; Lechner et al., 2000; Thimme et al., 2001, 2002). Understanding the mechanisms underlying natural clearance is important both in designing immunotherapies and prophylactic vaccines. An inability to clear virus may, in part, be due to a failure of $CD4^+$ T-cells to support a sufficiently robust virus-specific $CD8^+$ T-cell response, implying that both T-cell subsets should be targeted in any therapeutic strategy (Chang et al., 2001). One obstacle to establishing the precise

Abbreviations: HCV, hepatitis C virus; GBV-B, GB virus B; PD-1, programmed death receptor-1; PD1-L1, programmed death receptor-1 ligand; CTLA4, cytotoxic T lymphocyte antigen-4; NS, non-structural; vRNA, viral ribonucleic acid; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; ge, genome equivalents; IVT, in vitro transcription; HBSS, Hank's balanced salt solution; EGTA, ethylene glycol tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RPMI, Roswell Park Memorial Institute medium; DMSO, dimethyl sulphoxide; FITC, Fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin; IFN, interferon; MFI, median fluorescence intensity; IHL, intrahepatic lymphocytes; NK, natural killer; MHC, major histocompatibility complex.

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http://dx.doi.org/10.1016/j.virusres.2013.11.006

mechanism(s) of clearance is that many infections may be asymptomatic, hence the identification, monitoring and acquisition of samples from acutely HCV-infected patients is difficult.

Chimpanzees are susceptible to HCV however numerous constraints preclude their use. The infection of tamarins and marmosets with GBV-B represents an attractive surrogate model for the study of HCV infection in man (Beames et al., 2000, 2001; Lanford et al., 2003; Jacob et al., 2004). GBV-B is a flavivirus closely related to HCV and causes an acute self-limiting hepatitis: high viraemia is typically followed by viral clearance from the periphery within 18 weeks of infection (Beames et al., 2000, 2001; Bright et al., 2004). This model is applicable to the investigation of acute HCV pathogenesis, antiviral drug development and pre-clinical evaluation of candidate prophylactic vaccines (Butkiewicz et al., 2000; Beames et al., 2001; Bright et al., 2004; Jacob et al., 2004; Rijnbrand et al., 2005). Since GBV-B is most likely to have arisen in tamarins (Stapleton et al., 2011) we studied GBV-B-induced acute hepatitis in red-bellied tamarins to better understand changes in lymphocyte populations in both the periphery and liver, associated with the spontaneous resolution of the flavivirus infection. We explored changes in key cell populations, $CD4^+$, $CD8^+$ and $CD20^+$ cells, in the blood and the liver, between animals with high peripheral viraemia and those in a convalescence phase in which virus was undetectable in the periphery. Furthermore, since the mechanism of T-cell exhaustion has been implicated in persistence of several viruses, including HCV (Hofmeyer et al., 2011), we characterised the presence of the cell surface protein Programmed Death-1 (PD-1) on T-cells. Dysfunctional HCV-specific CD8 T-cells displaying up-regulated PD-1 and PD-1 ligand expression have been identified in the blood and liver of chronically infected individuals (Yao et al., 2007) and the reversal of T-cell exhaustion, evidenced by reduced PD-1 expression, has been associated with spontaneous clearance of chronic HCV infection (Raghuraman et al., 2012). In HCV-infected chimpanzees differences have been reported with higher intrahepatic PD-1 mRNA levels reported in chronically infected individuals by Rollier et al. (2007), while others have reported that PD-1 mRNA levels are not predictive of acute/chronic outcome of infection (Shin et al., 2013). We showed that in the convalescent phase of GBV-B infection a decrease in $CD3^+$ and a corresponding increase in $CD20^+$ lymphocytes occurred. Intrahepatic T- and B-lymphocyte populations were greater at high viraemia and convalescence than pre-infection.

A prerequisite to optimal exploitation of this model is a clear definition of the pathogenesis and naturally occurring host immune response against GBV-B in tamarins. We identified significant immune infiltration into the liver at high viraemia as well as in two of the four animals in the convalescent phase, concomitant with flares in serum ALT levels and vRNA was isolated from the livers of all animals, including those with undetectable peripheral viraemia. Antibodies against Core have only been reported for two tamarins (Nam et al., 2004; Bukh et al., 2008); our data on the limited immune responses to GBV-B NS3 are broadly consistent with the production of such antibodies in tamarins and marmosets (Beames et al., 2000; Lanford et al., 2003; Martin et al., 2003; Woollard et al., 2008).

This is the first description of the changes in a marker of T-cell activation/exhaustion on key immune cell populations in acute GBV-B infection of tamarins and the first report of an infection in the liver persisting when virus in no longer detected in the blood. These data provide a foundation for further immunological and pathological studies to dissect fully the mechanisms underlying natural viral clearance in this valuable surrogate model of acute HCV infection.

2. Materials and methods

2.1. Animals and virus inoculum

Eight purpose-bred red-bellied tamarins (*Saguinus labiatus*) were used. Animals were housed and maintained in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and Home Office guidelines for care and maintenance of non-human primates. All animals were inoculated intravenously with 1×10^7 genome equivalents (ge) of GBV-B in serum. One group of four animals (animals W1, W2, W4, W11) was terminated at 6 weeks post-infection (wpi) and one group (animals W3, W5, V7, V8) was terminated following clearance of detectable virus from the periphery (approximately 24 wpi).

2.2. Quantification of GBV-B vRNA

To quantify GBV-B vRNA from the periphery, total RNA was extracted from 140 μl serum using the QIAamp Viral RNA Mini kit (Qiagen, UK). Core sequences were quantified in duplicate using the RNA Ultrasense One-step quantitative RT-PCR system. Primers 558F and 626R (Beames et al., 2000) were used at a concentration of 400 nM and 900 nM, respectively. The probe (5′ FAM-AGC GCG ATG CTC GGC CTC GTA AT-BHQ1 3′) was used at a concentration of 200 nM. Reverse transcription was performed at 50 °C for 15 min followed by amplification for 40 cycles (95 °C, 60 s; 62 °C, 30 s; 72 °C, 30 s). Standards to determine ge were derived from synthetic GBV-B RNA *in vitro* transcribed (IVT) from a plasmid (MEGAscript SP6; Ambion, USA). Serially diluted IVT RNA was quantified using a Poisson distribution; the limit of quantification was 10^2 ge/ml serum.

To quantify GBV-B vRNA from liver tissue, total RNA was extracted from a 0.5 cm^3 frozen section of liver in 1 ml RLT buffer (Qiagen RNeasy Mini Kit; Qiagen, UK). The tissue was homogenised using a 50 μM sterile Medicon unit (BD Biosciences) attached to a Medimachine (Dako), following the manufacturers' instructions. RNA was purified from the homogenate using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA was quantified and the concentration adjusted to 0.2 μg/μl. vRNA was quantified as described for serum vRNA levels and titres expressed per 400 ng total RNA (equating to approximately 10,000–15,000 cells). The limit of quantification was 7.6×10^{-2}/400 ng total RNA.

2.3. Quantification of serum liver enzymes

To indirectly assess liver damage, serum levels of alanine aminotransferase (ALT) and glutamate dehydrogenase (GLDH) were measured using a Kodak Ektachem automated analyser (Kodak Ltd. UK Suppliers, Orthochemical Diagnostics, Amersham, UK). Pre-infection samples were also assessed for each animal.

2.4. Isolation of intrahepatic lymphocytes (IHL)

Isolation of IHL from the liver retrieved at termination was performed on fresh tissue using adapted methods (Heydtmann et al., 2006; Nakamoto et al., 2008). The liver was washed at 37 °C by perfusion with 1 × HBSS (Life Technologies, UK) supplemented with 0.5 mM EGTA, 10 mM HEPES and 50 μg/ml gentamycin. Hepatocytes were disaggregated by perfusion with collagenase solution (1 mg/ml collagenase type II [Life Technologies] in 1× HBSS). The capsule was removed and the liver finely diced and incubated in collagenase solution containing 1 μg/ml DN*ase*I (Sigma–Aldrich) at 37 °C for 1 h. IHL were gravity filtered through a 100 μm nylon mesh and purified over a Ficoll-Paque Plus (GE Healthcare, UK) density gradient. IHL were cryopreserved in 80% foetal calf serum (FCS) and 10% dimethylsulfoxide (DMSO) in 1× RPMI. IHL were available from one uninfected tamarin from a parallel study.

2.5. Analysis of T-cell and B-cell populations by flow cytometry

PBMC and IHL (available at termination only) were washed in 1× RPMI containing 1% FCS, pelleted and resuspended in CellWash (BD Biosciences, Oxford, UK) containing 1% FCS. Cells (3.5×10^5 cells