GB virus C quasispecies detected in plasma and lymphocyte subsets in a natural human infection

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Genomic heterogeneity and quasispecies composition of GB virus C (GBV-C) within plasma and lymphocyte subsets in a naturally infected blood donor were investigated. For this purpose, fragments from the 5’ untranslated region (5’ UTR) and the E2 gene recovered from plasma, B and T lymphocytes, were cloned and sequenced. A total of 63 clones was analysed: 95.2% of them (n=60) – obtained from plasma and cells – were assigned to genotype 2b, while only three derived from plasma corresponded to genotype 3. The G215A transition within this region was present in 90.9% of the clones from B lymphocytes, but absent in the remaining cell compartments (P<0.01). Apparently, most of the circulating GBV-C quasispecies in this blood donor were related to the viral population infecting CD8+ T cells, and B cells to a lesser extent. This is the first report showing the quasispecies nature of GBV-C in lymphocyte subsets within peripheral blood mononuclear cells.

GB virus C (GBV-C) has worldwide distribution and its infection is very common, with approximately 2% of healthy USA blood donors actively viraemic at the time of blood donation (Alter, 1997; Linnen et al., 1996; Stapleton et al., 2004). In Argentina, molecular epidemiological studies demonstrated that the prevalence of GBV-C ongoing infection among blood donors is 5.5% (Oubiña et al., 1999).

GBV-C RNA has recently been identified in purified CD4+ and CD8+ T lymphocytes (TL), as well as in CD19+ B lymphocytes (BL) removed ex vivo from infected donors and it has been found that GBV-C replicates in vitro in these peripheral blood mononuclear cell (PBMC) subsets, which suggests that GBV-C is a panlymphotropic virus (Fogeda et al., 1999; George et al., 2003, 2006).

The existence of GBV-C variants with different tropism has been demonstrated by Fogeda et al. (2000). This group observed tissue compartmentalization of the GBV-C 5’ untranslated region (5’ UTR) sequences from PBMC, liver and serum samples simultaneously obtained from chronic GBV-C carriers co-infected by hepatitis C virus. However, the specific characterization of GBV-C variants in different lymphocyte subsets, where the virus replicates, has not been investigated yet.

In the present study, we have analysed the genomic heterogeneity and quasispecies composition of GBV-C by studying two genomic regions: the conserved 5’ UTR and the E2 gene recovered from plasma, B and T lymphocytes, and serum samples simultaneously obtained from chronic GBV-C carriers co-infected by hepatitis C virus. However, the specific characterization of GBV-C variants in different lymphocyte subsets, where the virus replicates, has not been investigated yet.

Lymphocyte populations were purified as described previously (Gamberale et al., 2003) and the purity of each population was found to be >95%. CD19+ B cells were further cultured with phorbol-myristate-acetate (PMA) (50 nM) and ionomicine (5 µg ml⁻¹) for 48 h with the aim of amplifying this minor cell type circulating in the peripheral blood. CD4+ and CD8+ T cells as well as...
CD19+ B cells were washed with PBS and treated with trypsin in order to remove viral particles potentially adsorbed to the cell surface. After centrifugation, four further washes with PBS were performed. The last obtained supernatant was amplified by reverse transcription-nested PCR (RT-nested PCR) followed by Southern blot hybridization with a 32P-labelled internal probe. No amplification was detected in such supernatants after performing the fourth PBS washing (post-typsin treatment) of purified lymphocyte subsets, suggesting that viral particles were no longer absorbed to cellular membranes.

The conserved 5′ UTR and the amino-terminal coding region of the viral envelope protein E2 (Takahashi et al., 1997) were amplified from P and cellular pellets (C) by RT-nested PCR. For the 5′ UTR, RT-nested PCR was performed as described previously (Ruiz et al., 2006). The expected amplicon size (corresponding to nt 41–366 of PNF2161; Linnen et al., 1996) was analysed by ethidium bromide staining and Southern blot hybridization with the above-mentioned internal probe.

For the E2 region, a 248 bp fragment was amplified by RT-nested PCR (nt 1149–1396 of PNF2161), containing the sequence initially proposed for coding the presumed antigenic region (nt 1221–1304 of PNF2161) in the centre (Takahashi et al., 1997).

PCR fragments were cloned into pGEM-T Easy (Promega) and bidirectionally sequenced. The potential Taq-dependent DNA misincorporation rate was investigated by bidirectional sequencing of the PCR products derived from a GBV-C clone, as described previously (Mathet et al., 2003).

DNA alignments of the nt sequences were generated with the CLUSTAL W program from the BioEdit package (version 5.0.6; Tom Hall, 1997). Phylogenetic trees were constructed using the neighbour-joining (NJ) analysis included within CLUSTAL W program from the BioEdit package (version 5.0.6; Tom Hall, 1997). Phylogenetic trees were constructed using the neighbour-joining (NJ) analysis included within the CLUSTAL W program from the BioEdit package (version 3.5c, J. Felsenstein, Department of Genetics, University of Washington, Seattle, 1993).

In order to study the quasispecies composition of each sample type, the heterogeneity index (HI) was calculated as the proportion of GBV-C clones not bearing the predominant sequences, i.e. HI = 1 – (proportion of clones bearing majority sequences) (Mathet et al., 2007).

For the detection of GBV-C negative-strand RNA, cDNA synthesis was performed at high temperature with the thermostable enzyme, Tth, as described previously (Ruiz et al., 2006). GBV-C 5′ UTR amplification product was confirmed by Southern blot hybridization for P, CD8+ TL and BL. Cellular RNA integrity was checked by RT-PCR of β-actin mRNA. The fact that the 5′ UTR could not be specifically amplified from CD4+ TL was possibly due to primer selection, which allowed more efficient non-specific amplification from a different template. Nevertheless, these primers have been used taking into account their ability to amplify all known GBV-C genotypes from P (Quarleri et al., 1999; Ruiz et al., 2006).

By using the Tth-based strand-specific assay, the GBV-C negative-strand was not detected in any lymphocyte subset, even after Southern blot hybridization with the above referred internal probe. Considering that the replication intermediate (RI) is generally present in titres which are 1–2 logs lower than genomic RNA (Laskus et al., 1997, 1998; Radkowski et al., 1999) or it is even undetectable (Mellor et al., 1998), and taking into account that positive-strand titre was very low in the cellular pellets analysed, the possibility that RI would have been present in lymphocytes, but below the sensitivity threshold of our strand-specific assay (10^3 genomic equivalents; see Ruiz et al., 2006) cannot be formally ruled out.

The genomic region coding for the amino-terminal end of GBV-C E2 protein was amplified from P, TL (CD4+ and CD8+ and BL RNAs, thus confirming GBV-C RNA in these cells, although it does not prove that the cells were productively infected.

In order to investigate the genetic diversity potentially associated with selective tropism for these cells, we searched for the composition of viral quasispecies recovered from both P and lymphocytes in vivo.

To study the GBV-C 5′ UTR sequence heterogeneity, a total of 33 clones (12 from P, 10 from CD8+ TL and 11 from BL) was analysed. Sequences from all GBV-C 5′ UTR 33 clones were compared with the consensus sequence obtained from P (conP, the consensus sequence is the sequence that occurs with the highest frequency for each nt position; Fig. 1a). Of the 12 clones from P, five (41.6 %) were identical to the consensus sequence, two were identical between them but different from conP and the remaining five were distinct, accounting for an HI = 0.58 (Table 1). Three insertions and 29 substitutions were observed throughout, with transitions accounting for 58.6% of the total. The finding of a common set of three insertions together with 22 substitutions present in three clones simultaneously (P8, P10 and P15) suggests the presence of a viral subpopulation within the P. Although

**Fig. 1.** Nucleotide alignment of GBV-C 5′ UTR (a) and E2 gene (b) sequences recovered from individual clones isolated from plasma (P), CD4+ TL (CD4), CD8+ TL (CD8) and BL (B) and their respective consensus sequences (ConP, ConCD4, ConCD8 and ConB). Dots represent identical bases and dashes indicate deletions. Numbers depicted in parentheses indicate the number of clones corresponding to a given sequence. Boxed regions correspond to already known polymorphic regions within the 5′ UTR (V-1, V-2 and V-3). Numbers following P, CD4E, CD8E or BE identify a given E2 clone from the corresponding compartment.
GBV-C quasispecies in plasma and lymphocytes

(a)

(b)

http://vir.sgmjournals.org
Table 1. Heterogeneity of 33 5’ UTR and 30 E2 GBV-C nt sequences observed in P and lymphocytes in vivo.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>No. of clones</th>
<th>No. of different sequences</th>
<th>HI*</th>
<th>Frequency of sequences†</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ UTR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>12</td>
<td>7</td>
<td>0.58</td>
<td>1 (5), 1 (2), 5 (1)</td>
</tr>
<tr>
<td>CD8+ TL</td>
<td>10</td>
<td>4</td>
<td>0.30</td>
<td>3 (1), 1 (7)</td>
</tr>
<tr>
<td>BL</td>
<td>11</td>
<td>4</td>
<td>0.27</td>
<td>3 (1), 1 (8)</td>
</tr>
<tr>
<td>E2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>9</td>
<td>9</td>
<td>1.00</td>
<td>9 (1)</td>
</tr>
<tr>
<td>CD4+ TL</td>
<td>9</td>
<td>2</td>
<td>0.11</td>
<td>1 (8), 1 (1)</td>
</tr>
<tr>
<td>CD8+ TL</td>
<td>5</td>
<td>4</td>
<td>0.60</td>
<td>3 (1), 1 (2)</td>
</tr>
<tr>
<td>BL</td>
<td>7</td>
<td>1</td>
<td>0.00</td>
<td>1 (7)</td>
</tr>
</tbody>
</table>

*HI, Proportion of GBV-C clones not bearing the predominant sequences, i.e. HI=1−(proportion of clones bearing majority sequences).
†The number preceding the parentheses represents a sequence, or sequences; the number within the parentheses is the number of clones in which that sequence or those sequences appear. For instance, for P, one sequence appears in five clones, one sequence appears in two clones and five sequences appear in one clone.

Thirty specific clones corresponding to the GBV-C E2 gene were amplified from P (n=9), CD4+ TL (n=9), CD8+ TL (n=5) and BL (n=7) and analysed. When nt sequences within each compartment were compared, it was observed that all clones recovered from P were different from each other (HI=1, Table 1; Fig. 1b) with substitutions encompassing 14 nt positions, 13 of which (92.8%) corresponded to transitions. Eight of nine clones from CD4+ TL were identical (HI=0.11), but none of these nine clone sequences was 100% identical to any sequence derived from P. The C1286T substitution was present in all clones amplified from CD4+ TL, but proved to be absent in clones derived from P. When analysing clones from CD8 TL, seven substitutions with respect to the conPE sequence were observed. C1371T was the only one also present in one clone from the plasmatic compartment. The seven clones amplified from BL were identical (HI=0), showing three substitutions with respect to the conPE sequence (C123T, C1238T and C1310T). These mutations were also present in at least one clone amplified from P (PE104; Fig. 1b). The phylogenetic tree constructed with GBV-C E2 gene sequences showed that clones amplified from BL clustered together (Fig. 2b). The consistent clustering of BL nt sequences (with bootstrap values of 68 and 91% for the 5’ UTR and E2 regions, respectively) strongly suggests the compartmentalization of these viral quasispecies. A similar event might occur with those sequences derived from CD4+ TL, although without reaching a significant bootstrap value.

As mentioned above, some of the E2 quasispecies observed in clones amplified from P were also present in clones derived from CD8+ TL. However, none of the clones amplified from CD4+ TL or BL showed 100% identity with any quasispecies from P. This might suggest a dissimilar contribution of such cell populations to the bulk of viral load.

The E2 deduced amino acid sequence showed a high level of identity, since most of the recorded substitutions were synonymous. H75Y non-synonymous substitution was observed in two of four clones derived from CD8+ TL and one from P (Supplementary Fig. S3, available in JGV Online).

Since the 5’ UTR could not be specifically amplified from CD4+ TL, the possibility that the plasmatic subpopulation had originated in this cellular subset cannot be ruled out. Alternatively, other sources, such as monocytes/macrophages or vascular endothelial cells (Handa & Brown, 2000) might have accounted as the source of the plasmatic genotype 3 subpopulation. Taking into account that E2-derived sequences amplified from CD4+ TL were absent in clones amplified from P, the latter possibility seems more plausible. In order to confirm that 5’ UTR nt sequences from the plasmatic subset (the most divergent among 33 analysed clones) were truly related to GBV-C, an alignment with a different GBV-C genotype 3 isolate was performed that clustered properly (data not shown).

the small number of clones and large number of polymorphisms in the plasmatic viral variants might preclude provision of strong evidence of such subpopulations – as mentioned above – these three clones (9.1%) were ascribed to genotype 3, instead of 2b, in clear contrast with the remaining GBV-C population (n=30, 90.9%) (Supplementary Fig. S1, available in JGV Online). This genotype 3 sequence was not observed among clones recovered from cellular compartments (Supplementary Fig. S2, available in JGV Online), and these three clones clustered as a separate branch within the phylogenetic tree with a bootstrap value of 100% (Fig. 2a). Seven of 10 clones recovered from CD8+ T cells were identical to their consensus sequence (conCD8) and to conP (HI=0.3), while the remaining three contained a single (but dissimilar) transition with respect to conP. Eight of 11 clones from BL had sequences identical to the one corresponding to conB, and the remaining three were different from each other (HI=0.27). No GBV-C clone amplified from BL was 100% identical to any sequence recovered from P. Transition G215A, alone or together with another mutation, was observed in 10 of 11 clones (90.9%) from this cellular compartment although it does not provide strong evidence of a specific-adaptive quasispecies (Fig. 1a). When analysing all sequenced clones, it was observed that most of the substitutions occurred within the three regions associated with specific polymorphisms in the 5’ UTR, strongly correlated with phylogenetic clustering of variants (Hsieh et al., 1997; Smith et al., 1997), herein named V-1, V-2 and V-3 (Fig. 1a).
E2 nt sequences obtained from lymphocyte subsets showed the presence of ‘marker’ positions, also potentially associated with GBV-C tropism, like silent transitions C1286T in CD4\(^+\) TL, and C1223T, C1238T and C1310T in BL, which were present in 100% of the respective cell-derived clones. The detection of one clone amplified from P (PE104), which showed the same three substitutions observed in BL, suggests that this plasmatic sequence could have originated in that cellular subset.

The majority of nt changes observed in the amino terminus of the GBV-C E2 gene were transitions, most of them (19/22) being placed at the third codon position, and not associated with amino acid substitutions. The significance of these nt mutations could be related to the concept of variability reservoirs (Mathet et al., 2007). It is reportedly known that substitutions at the second nt position in a given codon are more restricted than changes at the first one, and the latter more than those at the third location. Thus, synonymous replacements could mean a potential (intermediate) source of subsequent drastic non-synonymous nt changes that might rapidly appear when selection pressures are evidently operative.

Strikingly, as shown in Table 1, a lower HI was apparent among E2 nt sequences derived from CD4\(^+\) TL and BL, as compared with those derived from CD8\(^+\) TL and P. This may suggest that GBV-C does not evidently evolve inside CD4\(^+\) TL and BL cells, which could be related – among several possibilities – to (i) a low replication rate within them; (ii) a more recent infection event; or alternatively (iii) a sequence constraint to allow crucial interactions between viral RNA and cellular protein(s).

When comparisons were made between HIs, a significant difference was evident between values recorded from CD8\(^+\) TL and BL E2 sequences (0.6 versus 0.0), in contrast to similar values obtained from the same cells at 5\(^\prime\) UTR sequences (0.3 versus 0.27) (Table 1). This finding might also support the speculation that E2 nt sequences constraint in BL could be assigned to a GBV-C envelope

**Fig. 2.** Phylogenetic tree generated by NJ constructed with GBV-C 5\(^\prime\) UTR (a) and E2 gene (b) sequences corresponding to clones isolated from plasma (P), CD8\(^+\) TL (CD8) and BL (B). Clones isolated from CD4\(^+\) TL were obtained only for the E2 gene (see the text). All clones were classified as genotype 2b, except 5\(^\prime\) UTR P8, P10 and P15, assigned to genotype 3, as shown in Supplementary Figs S1 and S2. Consensus sequences from each subset are also included (Con). Most significant bootstrap values are shown. Numbers following P, CD8 or B indicate the identification of a given 5\(^\prime\) UTR clone from the corresponding compartment. Numbers following PE, CD4E, CD8E or BE denote the identification of a given E2 clone from the corresponding compartment.
interaction with putative cellular receptor(s) or another sort of host cellular protein(s), requiring a specific and conserved amino acid sequence.

Although the depicted virological data were obtained from a single GBV-C-infected subject – and thus we may not be able to generalise – this study reached several important conclusions: (i) genomic quasispecies showing differential tropism for distinct cell subsets were observed during the course of a natural human infection; (ii) GBV-C quasispecies exhibited genomic characteristics that could be associated with the notion of compartmentalisation within cell populations; (iii) at least at the time of the study, in the selected blood donor, the maximal viral contribution to the plasmatic viral load was provided by the CD8⁺ TL; and (iv) two main subpopulations of (5' UTR sequenced) viral quasispecies assigned to distinct genotypes were detected in P (one of which without being represented among the studied cellular subsets), not surprisingly showing variants derived from – even dead – infected cells, irrespective of the cell type.

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