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Antiviral Research 46 (2000) 157–170

Antiviral Research

www.elsevier.com/locate/antiviral

Genetic evolution of GB virus C/hepatitis G virus (GBV-C/HGV) under interferon pressure

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Received 1 July 1999; accepted 28 February 2000

Abstract

The epidemiology and clinical features of chronic GBV-C/HGV infection have largely been explored, but there is little information about the mechanisms enabling GBV-C/HGV to cause persistent infection. Since analysis of the genomic variation of GBV-C/HGV under interferon pressure might provide some insight into this issue, we analyzed the nucleotide sequence variation of the 5′NC and NS3 regions in GBV-C/HGV isolates obtained sequentially from seven patients co-infected with HCV and treated with interferon. A reduction of GBV-C/HGV-RNA serum level below the detection limit of the RT-PCR assay was observed during treatment in all patients, but upon interferon withdrawal, viral RNA remained undetectable in only two patients. Among the five patients who did not clear GBV-C/HGV-RNA, viral strains emerging after treatment were identical to those present at baseline in three cases. In a further case, in whom GBV-C/HGV-RNA re-emerged during therapy (breakthrough episode), several mutations appeared in relapse samples. In the remaining patient, with a mixed infection before therapy, only one of the two GBV-C/HGV strains present at baseline was detected upon treatment withdrawal. These data raise the possibility that positive selection may act over GBV-C/HGV genome during interferon therapy, and contribute to persistence of infection with this virus.

Keywords: Viral persistence; Mixed infection; Positive selection; Breakthrough; Quasispecies

1. Introduction

The GB virus C (GBV-C) (Simons et al., 1995; Leary et al., 1996), or hepatitis G virus (HGV) (Linnen et al., 1996), is a member of the Flaviviridae family (Leary et al., 1996), that may cause

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0166-3542/00/$ - see front matter
PII: S0166-3542(00)00079-6

transient or long lasting infection in humans (Linnen et al., 1996; Alter HJ et al., 1997; Alter MJ et al., 1997). GBV-C and HGV are isolates of the same virus (Zuckerman, 1996). They are closely related to GB viruses A and B (Muerhoff et al., 1995). Chronic infection with this virus is highly prevalent in patients with chronic liver diseases of different etiologies, particularly in patients with chronic viral hepatitis B or C (Simons et al., 1995; Leary et al., 1996; Linnen et al., 1996; Tanaka et al., 1996; Alter HJ et al., 1997; Saiz et al., 1997; Enomoto et al., 1998; Guilera et al., 1998; Pawlotsky et al., 1998), but its pathogenic significance is unclear (Alter HJ et al., 1997; Alter MJ et al., 1997; Guilera et al., 1998; Mushahwar and Zuckerman, 1998).

Capability to cause persistent infection is a remarkable feature of GBV-C/HGV. In general, viral persistence depends on the interaction between host determinants, aimed to control the infection, and a variety of viral mechanisms, aimed to maintain persistent infection (Domingo et al., 1998). In chronic hepatitis C virus (HCV) infection, a good example of viral persistence, sequential analysis of the hypervariable region 1 (HVR-1) has shown that selection of host immune escape variants (Weiner et al., 1992; Farci et al., 1994; van Doorn et al., 1995), leading to inability of the host to produce protective antibodies, appears to be one of the most important mechanisms of HCV persistence.

Little is known about the mechanisms that enable GBV-C/HGV to establish persistent infection. Although GBV-C/HGV and HCV belong to the same Flaviviridae family (Leary et al., 1996), their different pathogenicity suggests that these viruses have different interactions with the host. Analyses of GBV-C/HGV isolates of diverse origin have shown a different evolutionary pattern for these viruses (Erker et al., 1996; Khudyakov et al., 1997; Muerhoff et al., 1997; Pickering et al., 1997; Wang et al., 1997; Giménez-Barcons et al., 1998; Ibáñez et al., 1998). Neither a viral hypervariable region, nor putative immune escape variants, closely involved in HCV persistence, have been detected in chronic GBV-C/HGV infection (Erker et al., 1996; Muerhoff et al., 1997; Tacke et al., 1997b; Wang et al., 1997; Bukh et al., 1998; Kato et al., 1998). Furthermore, humoral immune response to viral proteins is usually associated with clearance of viral RNA from serum in GBV-C/HGV infection (Feucht et al., 1997; Tacke et al., 1997a,b), but not in chronic HCV infection.

Although GBV-C/HGV is sensitive to interferon, as shown mainly in patients co-infected with HCV, persistence of the infection after interferon administration is frequently observed (Berg et al., 1996; Tanaka et al., 1996; Alter HJ et al., 1997; Saiz et al., 1997; Enomoto et al., 1998; Pawlotsky et al., 1998). In chronic hepatitis C, selection of variants during interferon therapy is a well documented potential mechanism of viral resistance (Okada et al., 1992; Enomoto et al., 1994; Polyak et al., 1998). In contrast, the mechanisms conferring interferon resistance to GBV-C/HGV, which might also be involved in the naturally occurring chronic course of this infection, have not been fully investigated. To date, no relationship between any specific region, or nucleotide position, of GVB-C/HGV and resistance to interferon activity has been established. Analysis of the E2 and NS5A regions of GBV-C/HGV sequential samples from patients treated with interferon did not show differences between pre- and post-treatment isolates (Kato et al., 1998, 1999) and fixation of mutations in the E2 gene of GBV-C/HGV, as a consequence of immune escape, have not been detected in chronic GBV-C/HGV infection (Tacke et al., 1997a; Bukh et al., 1998; Kato et al., 1998). It is possible that, due to the pleiotropic character of the antiviral activity of interferon, mutations in different regions of the viral genome should arise to confer resistance to this drug (Novella et al., 1996).

To evaluate the possible role of genomic variation in maintaining persistent GBV-C/HGV infection, we analyzed the temporal nucleotide sequence changes that occurred in the 5′ non-coding (5′NC) and NS3 regions of GBV-C/HGV isolates from a series of patients with chronic hepatitis related to HCV and GBV-C/HGV co-infection who received a single course of interferon therapy.

2. Patients and methods

2.1. Patients and treatment

Serial serum samples were obtained before, during and after interferon treatment from seven patients with chronic hepatitis who were co-infected with HCV and GBV-C/HGV. Four of them were infected with genotype 1b HCV and three with HCV of genotype 3a. The main clinical, epidemiological and biochemical characteristics of the patients have been described elsewhere (Saiz et al., 1997). Treatment consisted of a 24-week course of interferon α-2b (3 MU t.i.w.) and patients were followed for at least 6 months on no therapy. Response to treatment was evaluated on biochemical and virological terms according to changes in serum ALT activity and to detectability of HCV-RNA and GBV-C/HGV-RNA by nested PCR. Normalization of ALT values and undetectability of viral RNA during therapy, lasting up to the end of the 6-month follow-up period, was considered as sustained response (SR). Normalization of ALT level and undetectability of viral RNA during therapy, lasting up to the end of treatment, followed by a rebound upon treatment withdrawal was considered as a transient response (TR). Persistence of abnormal ALT values and/or detectable viral RNA at the end of treatment was considered as no response (NR). Initial responders who presented a biochemical and virological breakthrough (BT) during treatment were considered as non-responders. All patients gave their informed consent and the study was approved by the Ethics Committee of our institution.

2.2. GBV-C/HGV-RNA analysis

RNA was extracted from a volume of 140 μl of serum and specific sequences from two GBV-C/HGV regions, 5′NC and NS3, were separately reverse transcribed and amplified using a one-tube RT-PCR system as described (Saiz et al., 1997). Briefly, the RT-PCR mixture contained 10 pmol sense and antisense outer oligonucleotides, 200 μM dNTPs, 1.5 mM $MgCl_2$, 10 mM Tris–HCl pH 8.3, 50 mM KCl, 0.01% gelatin, 10 U Rnase inhibitor (Promega, Madison, WI), 2.5 U AMV reverse transcriptase (Promega) and 1.5 U *Taq* DNA polymerase (Gibco-BRL, Gaithersburg, MD) in a total reaction volume of 50 μl. The samples were incubated for 30 min at 42°C followed by one cycle of denaturation at 94°C for 2 min and 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 8 min. A 5-μl aliquot was reamplified in a 50-μl reaction mixture containing 10 pmol sense and antisense inner oligonucleotides and 1.25 U *Taq* DNA polymerase. Cycling parameters were as described above. All PCRs were run with negative controls and measures to prevent sample contamination were carefully observed. To verify amplification and to estimate product yield, 1/20 of the nested PCR mixture was run on a 1.5% agarose gel. Specific 5′NCR (outer 5′UTR1 and 5′UTR4; inner 5′UTR2 and 5′UTR3) and NS3 (outer GNS3-1 and GNS3-2; inner GNS3-3 and GNS3-4) oligonucleotides have been reported (Giménez-Barcons et al., 1998; Ibáñez et al., 1998).

The concentration of GBV-C/HGV-RNA in serum was estimated semiquantitively by testing 10-fold serial dilutions of extracted RNA, as previously reported (Saiz et al., 1997). The relative concentration of GBV-C/HGV-RNA was expressed as the log of the reciprocal of the highest dilution of extracted RNA in which GBV-C/HGV-RNA was detectable. Values are expressed as the titer per ml serum (Masuko et al., 1996).

2.3. Molecular cloning and DNA sequencing

PCR products were purified by using the Qiaquick spin PCR purification kit (Qiagen, Hilden, Germany). Approximately 50 ng of NS3 purified DNA was ligated with the TA cloning plasmid pGEM T (Promega). Competent *Escherichia coli* XL-1 cells where then transformed and screened for white colonies on ampicillin-isopropyl-β-D-thiogalactopyranoside (IPTG)-5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) agar plates. A small-scale plasmid preparation was carried out to recover bacterial DNA. Isolated plasmid DNA was screened for NS3 region by PCR using oligonucleotides GNS3-3 or GNS3-4. PCR

products of both GBV-C/HGV regions (5′NC and NS3) obtained from sequential samples from the patients analyzed, as well as plasmid colonies, were sequenced with the ABI PRISMTM Rhodamine Terminator Cycle Sequencing kit (Applied Biosystems, Westerstad, Germany). The products of the reaction were then analyzed on an Applied Biosystem 310 DNA sequencer (Applied Biosystems, Warrington, UK). Sense and antisense inner primers from each region were used in the sequencing reactions. Sequence editing was performed using the Sequence Navigator program (Applied Biosystems).

2.4. Sequence alignment and phylogenetic analysis

Sequences were aligned using CLUSTAL W (Thompson et al., 1994). Phylogenetic reconstructions were generated by using neighbor-joining in the Phylogeny Inference Package (PHYLIP) (Felsenstein, 1988, 1995), with Kimura two-parameter distance matrix and a ratio of transition to transversion of 2.0 (programs DNADIST and NEIGHBOR). Bootstrap resampling (Felsenstein, 1985) was applied to the neighbor-joining trees (programs SEQBOOT and CONSENSE) to assign approximate confidence limits to individual branches. The final graphic output was created with the program TREEVIEW (Page, 1996).

A phylogenetic analysis of all sequences obtained in our laboratory was routinely carried out to rule out possible cross-contamination. The GBV-C/HGV sequences have been submitted to GenBank under accession number AF156317-AF156366.

2.5. HCV-RNA analysis

For detection of HCV-RNA reverse transcription and nested PCR were carried out as previously described (Saiz et al., 1997) using specific oligonucleotides from the well-conserved 5′NC region.

3. Results

GBV-C/HGV-RNA was detected in serum from eight of 143 HCV-infected patients with chronic hepatitis who subsequently received treatment with interferon (Saiz et al., 1997). Serum samples from seven of the eight patients co-infected with HCV and GBV-C/HGV were available for study.

The presence of GBV-C/HGV-RNA and HCV-RNA was investigated in serial samples from the seven patients immediately before interferon treatment (baseline, time point 0), during treatment (week 12, time point 1), at the end of treatment (week 24, time point 2) and at the end of the post-treatment follow-up (week 48, time period 3, and week 72, time period 4, when available). Based on the results of the detection of HCV-RNA and ALT levels, patients were classified as follows: patient 1 as sustained responder (SR); patients 2, 3 and 4 as transient responders (TR); patient 5 as a breakthrough responder (BT); and patients 6 and 7 as no responders (NR). Patients 1–5 presented the same pattern of response for both viruses, HCV and GBV-C/HGV. Patient 6 was a SR for GBV-C/HGV and a NR that presented a BT episode for HCV, and patient 7 was a TR for GBV-C/HGV and a NR for HCV (Fig. 1).

3.1. Sequential analysis of the 5′NCR of GBV-C/HGV

The nucleotide sequences of the 5′NCR amplificates obtained from all patients at the different time-points are shown in Fig. 2. Such analysis was not possible in patients 1 and 6, who had a sustained GBV-C/HGV-RNA response. Nucleotide changes were not observed in isolates obtained before and after treatment from the three patients with a transient GBV-C/HGV response to interferon (patients 2, 3 and 4). In patient 5, with a BT GBV-C/HGV response, four nucleotide mutations were detected between samples 5.0 and 5.2, and these changes were fixed in the next time point analyzed, 5.3. In patient 7, a NR for HCV and TR for GBV-C/HGV, two GBV-C/HGV strains that differed in four nucleotide positions were detected at baseline. In order to verify this unusual fact, and to rule out cross-contamination of samples in the laboratory, an additional serum

sample (sample 7.0.-1), which was obtained 2 weeks before the baseline sample, was amplified and sequenced. The two GBV-C/HGV strains detected at baseline (sample 7.0) were found in sample 7.0.-1. As deduced from the intensity of the peaks on the electropherogram, one of the variants was present in a higher proportion (7.0M) than the other (7.0m). After post-treatment follow-up, only one of the infecting isolates (7.0m) was found at time point 7.3, and no mutations were detected between samples 7.0m and 7.3.

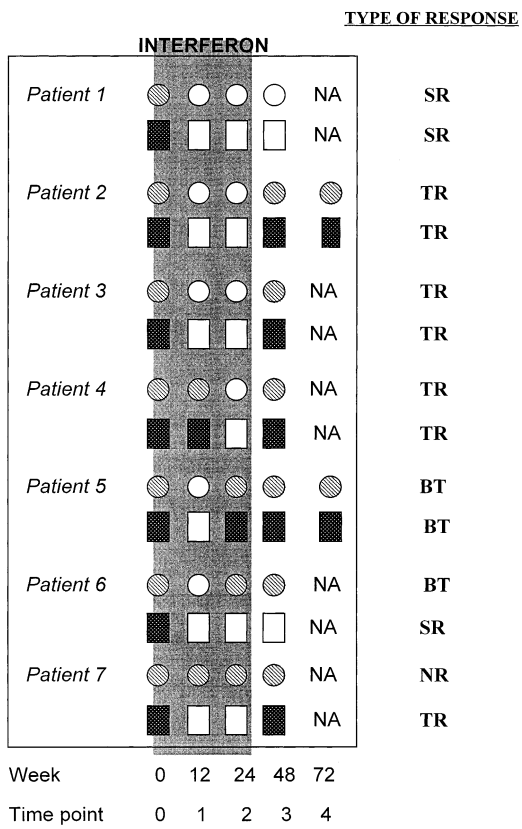

Fig. 1. Detection of serum HCV-RNA and GBV-C/HGV-RNA in the seven patients sequentially analyzed during interferon treatment. Squares represent GBV-C/HGV-RNA, circles represent HCV-RNA. Filled symbols, detectable viral RNA in sera. Unfilled symbols, undetectable viral RNA in sera. GBV-C/HGV-RNA became undetectable at some time during therapy in all patients. NA, not available. SR, sustained response; TR, transient response; NR, no response; and BT, breakthrough.

3.2. Sequential analysis of the NS3 region of GBV-C/HGV

The nucleotide sequences of the NS3 region amplificates obtained from all patients at the different time-points are shown in Fig. 3. Among patients with transient GBV-C/HGV response, a single nucleotide change (C to T at position 4537) between sample 2.0 and 2.3 was found in patient 2, but this change reverted later to time point 2.4; no mutations were observed between different time points in patients 3 and 4. Probably, the C4537T mutation detected in patient 2 was not caused by enzymatic misincorporation because it was also detected after repeated amplification and sequence analysis. In isolates from patient 5, who had a breakthrough response for GBV-C/HGV-RNA, five synonymous nucleotide changes were detected between samples 5.0 and 5.2, that persisted in the follow-up sample 5.3. In patient 7, who had a transient response for GBV-C/HGV but no response for HCV, the two GBV-C/HGV strains found in the baseline sample (7.0), that differed in 31 nucleotide positions in the NS3 region, were also detected in sample 7.0-1. As shown above for 5′NCR sequences from this patient, sequence analysis of NS3 region in sample 7.3 did not show mutations with respect to baseline subpopulation 7.0m, and 7.0M subpopulation was not detected at that time.

3.3. Mixed infection by two GBV-C/HGV strains

To analyze further the features of the mixed infection by two GBV-C/HGV strains disclosed by direct sequencing of 5′NCR and NS3 region in patient 7, PCR products of the NS3 region from samples 7.0 and 7.3 were cloned, and 10 randomly selected clones from each sample were re-amplified and sequenced. Two viral populations were identified in sample 7.0. One of these (7.0M) was more represented, as deduced from the 7/10 clones with sequences very closely related to the 7.0M consensus sequence deduced from the mixed global PCR product. The other population (7.0m) was less represented, and only 3/10 clones had sequences closely related to the consensus sequence of the 7.0m strain deduced after analysis

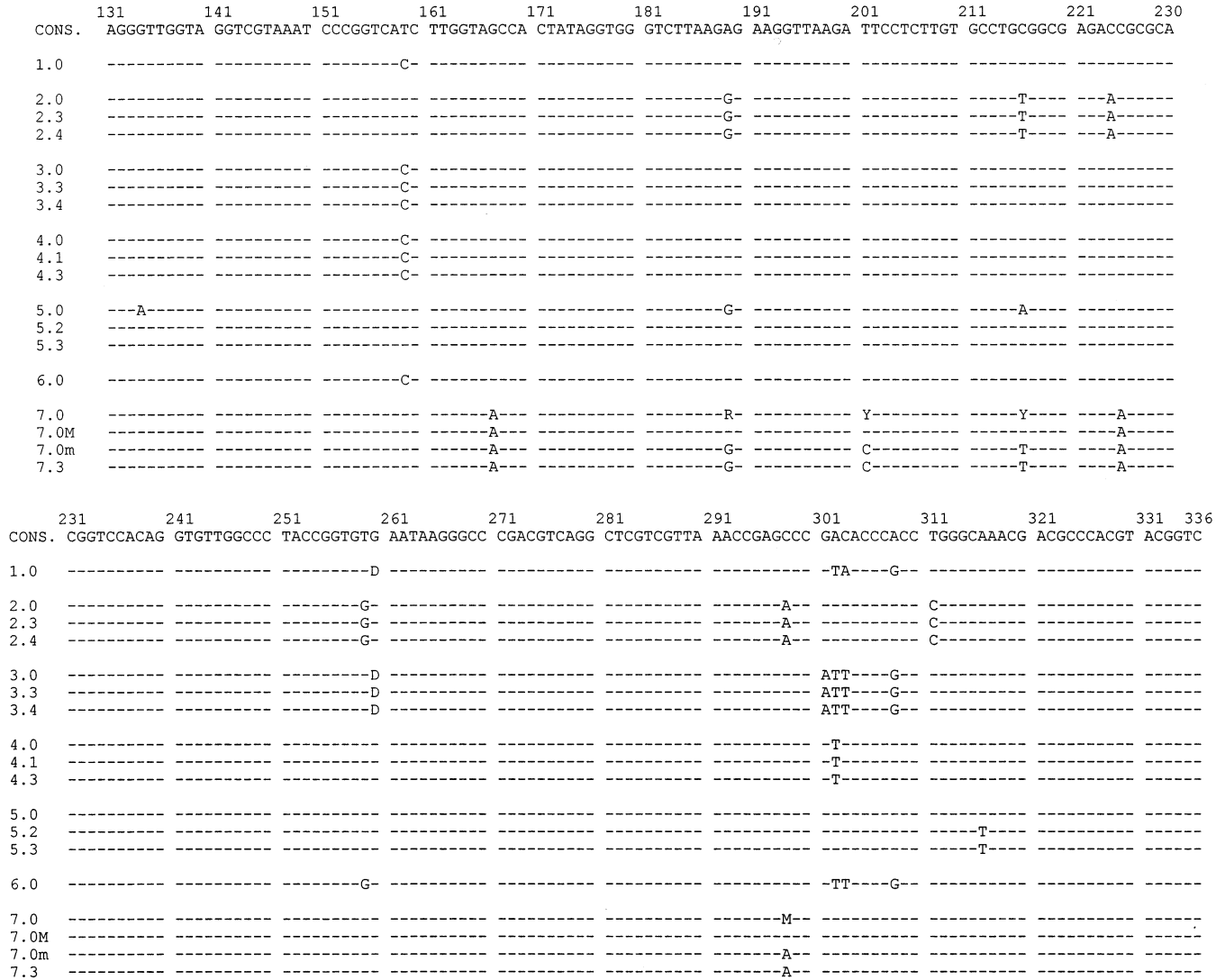

Fig. 2. Alignment of sequences from the 5′NC region of GBV-C/HGV isolates sequentially obtained from the seven patients (1–7) analyzed. The second digit denotes the time at which the serum sample was obtained. 0, baseline (before treatment); 1, week 12 of treatment; 2, week 24 of treatment (end of treatment); 3, week 48 (24 weeks of follow-up); and 4, week 72 (42 weeks of follow-up). 7.0M and 7.0m denote the major and minor sequences, deduced from the intensity of the peaks on the electropherogram, obtained after sequencing of the mixed global PCR product of serum sample 7.0. CONS denotes the deduced consensus sequence after alignment of all sequences. D indicates nucleotide deletions. The nucleotide sequences were described by using standard IUPAC ambiguity code where R = A/G, W = A/T, Y = C/T, S = C/G, K = G/T, M = A/C. Nucleotide positions are according to that described by Linnen et al. (1996).

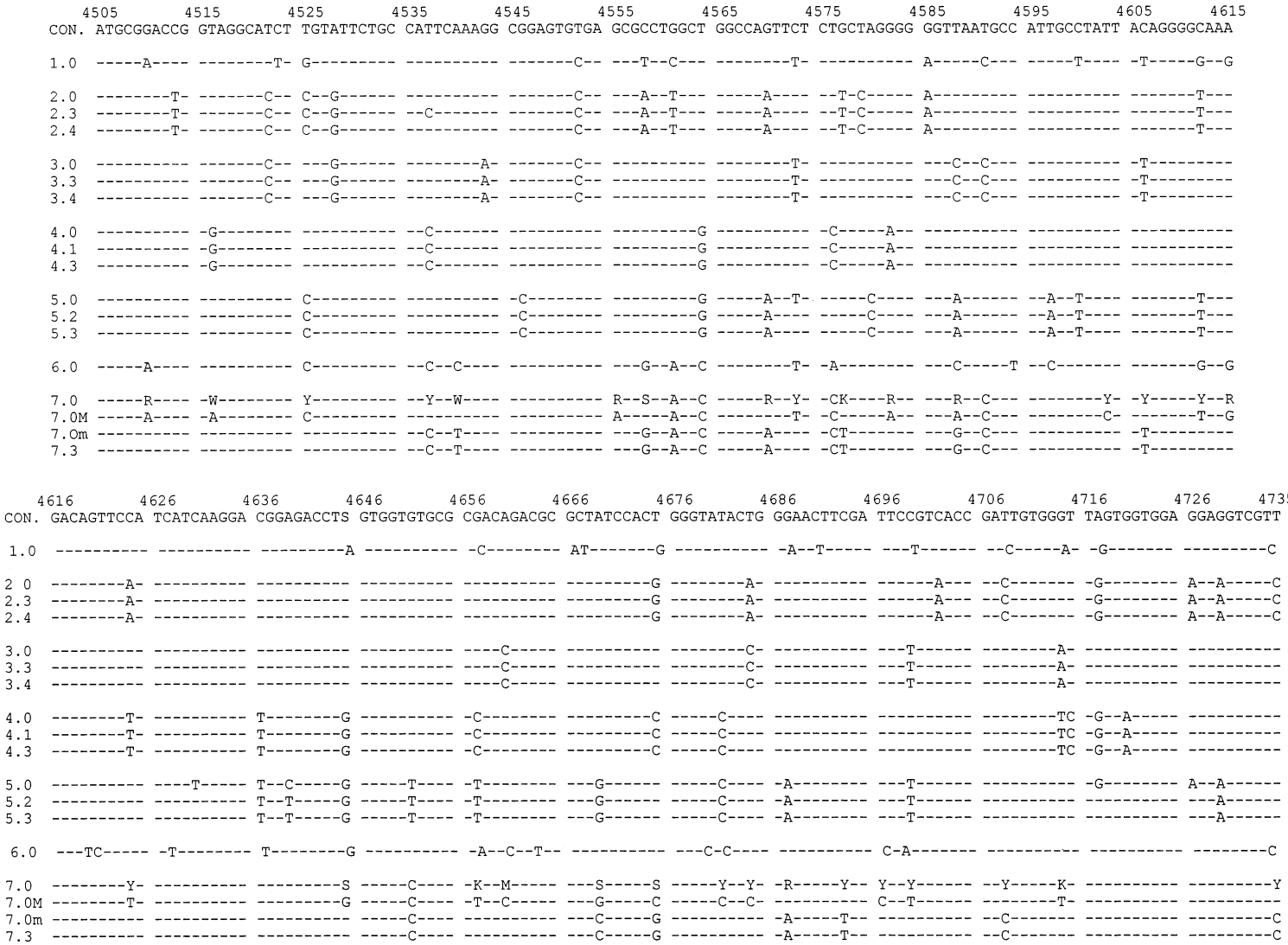

Fig. 3. Alignment of the sequences from the NS3 region of GBV-C/HGV isolates sequentially obtained from the seven patients (1–7) analyzed. Nomenclature and symbols are as in Fig. 2.

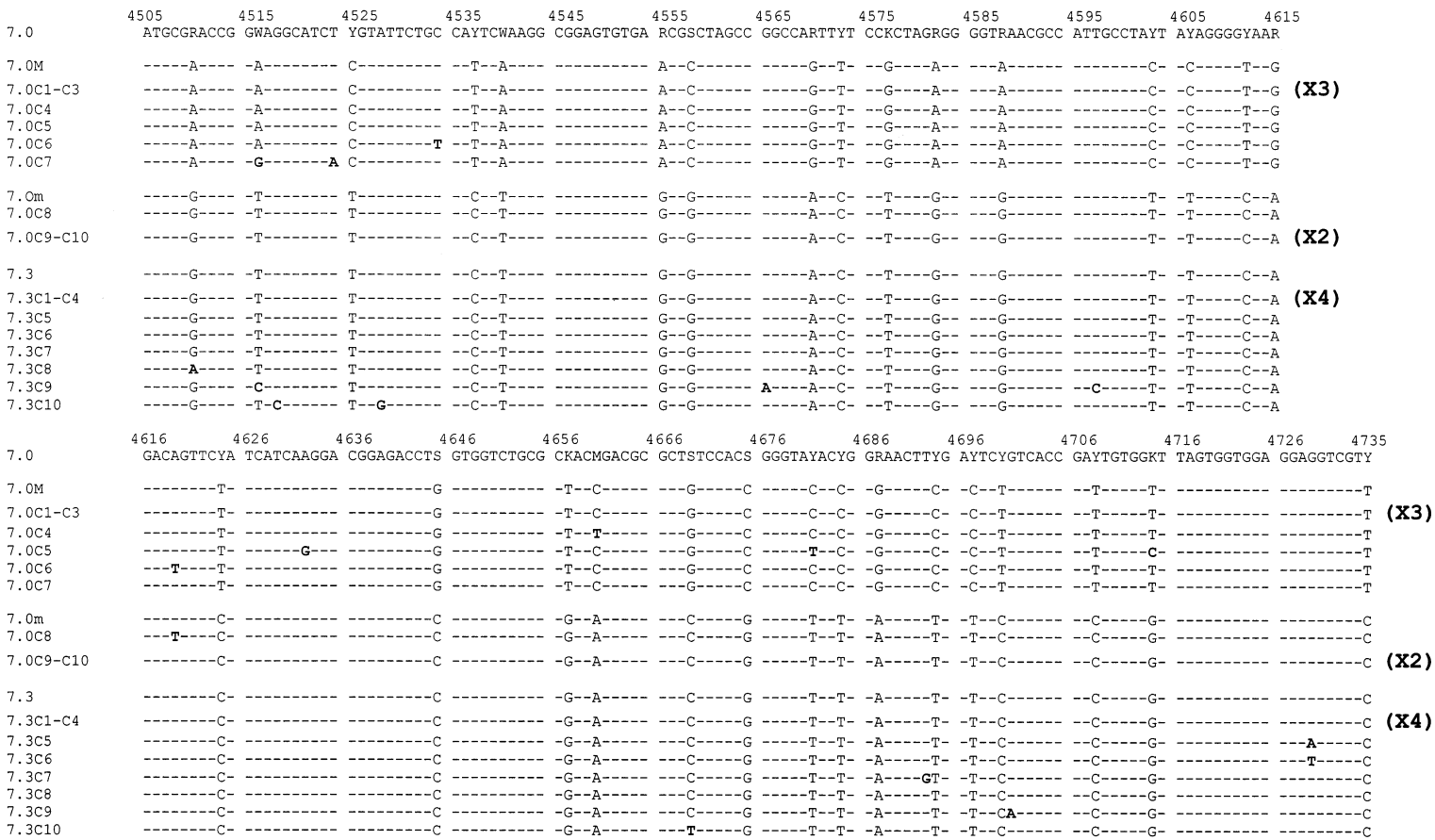

Fig. 4. Alignment of the sequences from the NS3 region of GBV-C/HGV clones obtained from serum samples 7.0 and 7.3 of patient 7. C1–C10 indicate individuals clones from samples 7.0 and 7.3. Bold letters denote nucleotide changes in relation to the sequence deduced from the global PCR population of sample 7.0. Time points nomenclature is as in Fig. 2. The number of clones with the same sequence is indicated at the end of each line.

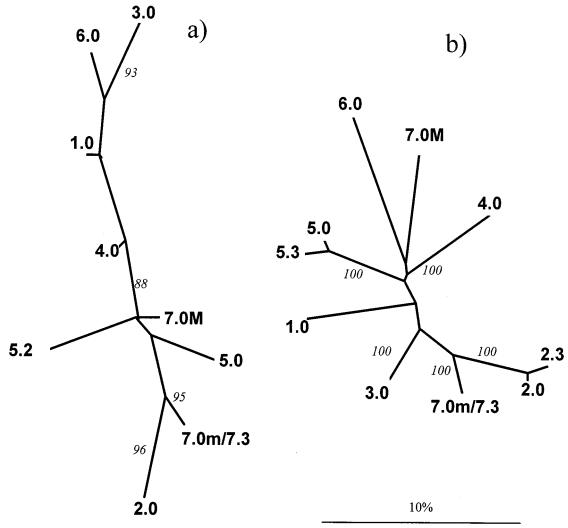

Fig. 5. Unrooted phylogenetic trees of the 5′NC and NS3 regions. (a) GBV-C/HGV 5′NCR and (b) NS3 region. Number in italics represent bootstrap proportions in support of the adjacent node based on 100 resampling iterations, only bootstrap values greater than 80% are shown. Time-point nomenclature is as in Fig. 2.

of the mixed global PCR product (Fig. 4). These proportions correlated with the intensity of the peaks observed in the electropherogram obtained by direct sequence analysis of the mixed global PCR product. Analysis of 10 clones from sample 7.3 confirmed that only viral population 7.0m was present, because all the clones analyzed were closely related to 7.0m strain and none with 7.0M (Fig. 4). Thus, of the two variants present at baseline only the minor one (7.0m) was detectable after interferon therapy.

3.4. Intersubject sequence variation

By pairwise comparisons of isolates from different subjects, the degree of similarity was 93–100% (mean 97%) for 5′NC and 78–93% (mean 85.5%) for NS3. Mutant frequencies, relative to the corresponding consensus (average values) sequence, were calculated for both sets of sequences. The NS3 region displayed a mutant frequency (mean 0.9×10^{-2} substitutions per nucleotide) four times greater than the 5′NCR (mean 2.0×10^{-2} substitutions per nucleotide). Furthermore, substitutions within the 5′NCR were only found in 16 positions out of 206 sequenced. Therefore, although a substantial genetic diversity between samples from different patients was observed in both regions, it was more extensive in the NS3 region, although only 6% of these mutations were nonsilent changes.

3.5. Intrasubject sequence variation

Pairwise comparisons between different time points of the two regions analyzed showed a 99.5–100% similarity (mean 99.9%) for 5′NC and a 98–100% similarity (mean 99.7%) for NS3. A drastic reduction of serum GBV-C/HGV-RNA titer, below the limit of detection of our assay, was observed in all patients during interferon therapy. Only in patient 5 nucleotide changes were detected between baseline sample and the variant arising just after the breakthrough episode. The calculated rate of GBV-C/HGV evolution under interferon pressure in this isolate was 3.8×10^{-2} substitutions per nucleotide per year (s/nt per year) and 4.3×10^{-2} s/nt per year for the 5′NC and NS3 regions, respectively.

3.6. Phylogenetic analysis

A phylogenetic tree of each of the two regions studied was constructed including all the sequences amplified from all the patients (Fig. 5). In the NS3 region, distinct clusters were observed with high bootstrap values (100%), corresponding to each group of sequential samples from each patient. Similar results, but with slightly lower bootstrap values (88–96%), were obtained in the 5′NCR. Phylogenetic analysis showed that variant 7.0M was not more closely related to the co-infecting variant 7.0m than to any other of the samples analyzed, confirming that they represent different isolates and not different subpopulations of the same strain. GBV-C/HGV isolates cleared during interferon therapy did not cluster together; thus, no information regarding interferon sensitivity of a specific strain could be deduced from sequence analysis of the two amplified regions used in the present report.

4. Discussion

Several studies have analyzed the sensitivity of GBV-C/HGV to interferon treatment (Berg et al., 1996; Tanaka et al., 1996; Saiz et al., 1997; Enomoto et al., 1998; Pawlotsky et al., 1998), but few data are available regarding the genetic evolution of GBV-C/HGV during interferon therapy. In the present report the interpatient and intrapatient genetic diversity of GBV-C/HGV has been studied in serum samples obtained sequentially from patients co-infected by HCV and treated with interferon.

Confirming previous observations (Berg et al., 1996; Tanaka et al., 1996; Saiz et al., 1997; Enomoto et al., 1998), sustained clearance of GBV-C/HGV-RNA from serum following interferon therapy was observed in two of the seven (28.5%) patients analyzed in this study. As previously described (Saiz et al., 1997), clearance of GBV-C/HGV-RNA correlated with a low pretreatment level of viremia. Failure of interferon therapy to suppress GBV-C/HGV infection in highly viremic patients could be due to a 'threshold' effect of viral replication (Domingo et al., 1995), which may be critical for determining the emergence of mutants capable of providing selective advantage to environmental pressure, as it was suggested in patients with chronic HCV infection (Saiz et al., 1998).

The degree of intersubject and intrasubject sequence diversity of GBV-C/HGV found in this study was very similar to that described in patients from other parts of the world (Erker et al., 1996; Khudyakov et al., 1997; Muerhoff et al., 1997; Viazov et al., 1997) as well as in other patients from our geographical area (Giménez-Barcons et al., 1998; Ibáñez et al., 1998). The mutant frequencies in the 5′NCR were lower than in the NS3 region. Mutations in the 5′NCR were restricted to a few positions, probably reflecting a tight functional requirement of RNA secondary structures (Giménez-Barcons et al., 1998; Ibáñez et al., 1998) due to the presence of an IRES element (Simons et al., 1996). Although the mutations observed in the NS3 region were more widely distributed, most of these nucleotide changes were silent mutations. This pattern of mutation might be due either to a strong selection against non-synonymous mutations or to an absence of positive selection during viral propagation (Erker et al., 1996; Muerhoff et al., 1997; Pickering et al., 1997; Viazov et al., 1997; Giménez-Barcons et al., 1998).

The pattern of genomic variation, both in coding and non coding regions, of HCV and GBV-C/HGV is very different (Erker et al., 1996; Khudyakov et al., 1997; Muerhoff et al., 1997; Ibáñez et al., 1998), and the number of nonsynonymous mutations found throughout the genome is much lower in GBV-C/HGV than in HCV isolates (Muerhoff et al., 1997). Although these observations may suggest that GBV-C/HGV and HCV have a different rate of evolution, we previously described (Giménez-Barcons et al., 1998) that the intrapatient rate of evolution of GBV-C/HGV ($1.3–2.4 \times 10^{-3}$ s/nt per year in 5′NCR and $1.3–9.4 \times 10^{-3}$ s/nt per year in NS3) is not different from that of HCV (Ogata et al., 1991). Similar rates of evolution have been reported from experimentally GBV-C/HGV-infected chimpanzees (Bukh et al., 1998), hemodialyzed patients (Masuko et al., 1996), and from interferon-treated patients (Berg et al., 1996), in whom 0 to 13 synonymous nucleotide changes were found in the GBV-C/HGV-NS3 region between pre and post-interferon treatment samples obtained between 2 and 4 years apart.

In the current study, nucleotide changes between baseline and post-treatment samples were not detected in the three GBV-C/HGV transient responders. These observations suggest that, in some patients, interferon therapy reduces the level of viremia below the limit of detection but does no eradicate the infection. Therefore, when interferon pressure is eliminated, GBV-C/HGV variants present at baseline are emerging again.

However, selection of GBV-C/HGV variants may also occur during therapy. In patient 5, who presented a breakthrough episode while being treated, a GBV-C/HGV variant (either newly generated or pre-existing in the pretreatment quasispecies) arose during interferon therapy. In this patient, the evolutionary rate of GBV-C/HGV was found to be higher (3.8×10^{-2} and 4.3×10^{-2} s/nt per year for the two regions analyzed),

since four and five nucleotide changes were detected in the 5′NC and NS3 regions, respectively, between the baseline sample and the variant isolated during GBV-C/HGV breakthrough. Due to the pleiotropic character of the antiviral activity of interferon, RNA viruses may encounter multiple barriers to replicate under interferon pressure, and mutations in different regions of the viral genome may arise to confer resistance to this drug (Novella et al., 1996). The mutations in 5′NC and NS3 regions found in patient 5 may represent a contribution to the mechanism(s) of GBV-C/HGV resistance to interferon, although they may be merely accompanying mutations that arose by a hitch-hiking phenomenon, as described in other RNA viral models (Domingo et al., 1993). The mutation rate in NS3 observed in patient 5 appears to be similar to that deduced from the data reported by Berg et al. in GBV-C/HGV isolates obtained from some patients treated with interferon. These authors sequenced samples taken at baseline and during post-treatment follow-up, and, therefore, no conclusions can be drawn regarding whether or not these particular viral variants were selected while interferon pressure was still ongoing.

The pathogenic mechanisms leading to persistence of GBV-C/HGV infection are at present unclear. One possible explanation is the existence of a low level of host immune pressure over GBV-C/HGV (Pickering et al., 1997; Bukh et al., 1998), as suggested by the apparent absence of immune escape variants (Tacke et al., 1997b; Bukh et al., 1998; Kato et al., 1998) and by the absence of detectable humoral immune response in almost all the patients with ongoing infection (Feucht et al., 1997; Tacke et al., 1997a,b). An alternative explanation is that GBV-C/HGV may have reached an evolutionary stasis (Holland et al., 1992), that makes the virus highly adapted to the environment (Erker et al., 1996; Giménez-Barcons et al., 1998). The low number of non-synonymous mutations observed in this and in previous studies (Erker et al., 1996; Khudyakov et al., 1997; Muerhoff et al., 1997; Pickering et al., 1997; Wang et al., 1997; Giménez-Barcons et al., 1998; Ibáñez et al., 1998) would support this hypothesis. Whatever the reason of persistence, evolutionary mechanisms common to RNA viruses, such as bottle neck transmission (Menéndez et al., 1999) and positive selection, as deduced from the present data, are operating during GBV-C/HGV evolution. However, the absence of a GBV-C/HGV susceptible cell culture hampers the confirmation of these findings.

Some authors have suggested that GBV-C/HGV would have a RNA-dependent RNA polymerase with a novel, less ‘error prone’, proof-reading activity (Erker et al., 1996; Pickering et al., 1997). However, the quasispecies heterogeneity of GBV-C/HGV shown in this study, as well as the high variability of GBV-C/HGV genome, mainly in the third codon positions of the NS3 region, observed in this and in other studies (Erker et al., 1996; Khudyakov et al., 1997; Muerhoff et al., 1997; Viazov et al., 1997; Giménez-Barcons et al., 1998; Ibáñez et al., 1998), indicate that such a novel enzymatic activity is not present in GBV-C/HGV.

A mixed infection with two different isolates of GBV-C/HGV was demonstrated in patient 7. Evidence has been reported suggesting that double infection with GBV-C/HGV may occur in some patients (Muerhoff et al., 1997; Pujol et al., 1998) but, to our knowledge, this is the first time that this possibility is confirmed, at the molecular level, by nucleotide sequence analysis of the quasispecies populations. Interestingly, of the two variants present at baseline, only one remained detectable in post-treatment samples, raising the possibility that co-infecting variants may have a different sensitivity to interferon, and that GBV-C/HGV variants may be selected by treatment.

Acknowledgements

M. Giménez-Barcons is funded by Institut d’Investigacions Biomèdiques Augusto Pi i Sunyer (IDIBAPS), A. Sánchez-Fueyo by Hospital Clinic, S. Ampurdanés and M. Guilera by Fundació Clinic, F. Puig-Basagoiti by Fondo de Investigaciones Sanitarias (FIS), and A. Ibáñez by Fundació IRSI-CAIXA. Work at HC was supported in part by grants from CICYT (SAF 97-0103) and FIS (99-0277), and at HUGTIP by

Fundació IRSI-CAIXA and Fundació Catalana de l'Hemofilia. We thank E. Domingo for valuable discussion during preparation of the manuscript.

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