A CORRELATION BETWEEN THE HETEROGENEITY OF HYPERVARIABLE REGION 1 OF E2 GLYCOPROTEIN OF HEPATITIS C VIRUS (HCV) AND HCV ANTIBODY PROFILE: A CASE STUDY

D. KMIECIAK[1*], J. BIERNACKA-ŁUKANTY[1], P. MIGDALSKI[2], J. TUREK-PLEWA[1], A. WIERZBICKI[1], J. JUSZCZYK[2], W.H. TRZECIAK[1]

[1]Department of Biochemistry and Molecular Biology, Karol Marcinkowski University of Medical Sciences, 6 Święcickiego St., 60-781 Poznań, Poland; [2]Department of Infectious Diseases, Karol Marcinkowski University of Medical Sciences, 61-003 Poznań, Poland

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Summary. – A correlation between the heterogeneity of hypervariable region 1 (HVR1) of E2 glycoprotein (gp) and Hepatitis C virus (HCV) antibody profile was investigated. Of 6 patients studied two were in acute phase, two in chronic phase and two showed signs of long-time HCV infection, i.e. liver cirrhosis. All the patients exhibited a vigorous antibody response to viral proteins C, NS3, NS4 and NS5. An antibody response to HVR1 of E2 was found in one patient in acute phase and in one or two patients in chronic phase. Such a response was not found in the two patients with liver cirrhosis. Single-stranded conformation polymorphism (SSCP) and sequence analyses of HVR1 of E2 showed the lowest HVR1 heterogeneity in patients in acute phase and the highest one in those in chronic phase, while the long-time carriers of the virus showed an intermediate heterogeneity. This may reflect a specific interplay between the virus and immune system. The HVR1 heterogeneity may rise in the course of infection as a means of evading the immune pressure. Then, when an organism is unable to clear the virus, because the responses to HVR1 epitopes are weakened or exhausted, a population of less heterogeneous HVR1 variants may be established.

Key words: E2 glycoprotein; enzyme immunoassay; Hepatitis C virus; hypervariable region 1; immune pressure; RT-PCR; sequence analysis; SSCP analysis

Introduction

HCV was first identified in 1989 (Choo *et al.,* 1989). Since then, it has become a global health problem affecting over 170 million people (Heintges and Wands, 1997). In a large proportion of individuals, the primary infection with HCV is asymptomatic and later on develops into a chronic phase in which patients are at high risk of liver disease that may lead to hepatitis, cirrhosis and hepatocellular carcinoma (Alter *et al.*, 1989; Saito *et al.*, 1990; Alter *et al.*, 1992). HCV elicits both cellular and humoral immune responses, important for control of the infection. These responses may persist in both acute and chronic phase without any predictable effect on virus replication as well as final outcome. In spite of the entire spectrum of antibodies directed against virtually all viral antigens, namely C (core), E1, E2, NS3-5 (antigens encoded by non-structural regions 3' to 5'), a majority of them are irrelevant to the process of immune containment of the infection. Only a portion of antibodies, directed against envelope glycoproteins E1 and E2, seems to be important in the clearance of the virus (Ishii *et al.*, 1998). Neutralizing anti-E2 antibodies (E2 antibodies) have been reported to block the entry of the virus into a cell by interfering with the binding of E2 to the human cell surface protein CD81 (tetraspanin 1, TAPA-1) (Hadlock *et*

*E-mail: dkmiec@am.poznan.pl; fax: +4861 8546510.

Abbreviations: C = core; E1, E2 = envelope proteins 1, 2; gp = glycoprotein; HBV = Hepatitis B virus; HCV = Hepatitis C virus; HVR1 = hypervariable region 1; IFN-α = interferon alpha; PAGE = polyacrylamide gele electrophoresis; SSCP = single-stranded conformation polymorphism

al., 2000), an entry co-receptor for HCV (Cormier *et al.*, 2004). Although a high titer of E2 antibodies plays a role in successful natural clearance of the virus in a chronic phase (Ishii *et al.*, 1998), their presence in the plasma of HCV-positive individuals in acute phase does not correlate directly with a good prognosis. For example, a study of patients in acute phase showed that both the group resolving the infection and that progressing towards chronicity (slow and rapid progressors) had E2 antibodies (Farci *et al.*, 2000).

It still remains unknown what are the mechanisms that allow the virus to persist and replicate during infection and how to predict the final outcome. It is thought that a specific interplay is established between the virus and the host immune system (Weiner *et al.*, 1992). One of the ways of evading the immune pressure is the development of a number of distinct but closely related variants of the virus, called quasispecies (Weiner *et al.*, 1992; Farci *et al.*, 2000).

The failure of immune system in clearing the virus could be at least partially explained by epitope specificity of E2 antibodies. E2 gp, the primary target for neutralizing antibodies contains HVR1 at the N-terminus (Weiner *et al.*, 1991). HVR1 has been shown to contain B cell epitopes (Kato *et al.*, 1993, 1994). If E2 antibodies in an infected individual are developed against HVR1 but not against conserved regions, then often escape mutants containing changes in HVR1 and capable of evading the immune pressure appear in the course of infection (Weiner *et al.*, 1992). HCV variability is due to the poor fidelity of its RNA polymerase, which lacks proofreading activity and thus allows natural generation of virus variants. Variants with mutations in HVR1 and other regions of E2 gp are also called quasispecies (Weiner *et al.*, 1992; Farci *et al.*, 2000). These variants are subjected to selection pressure of the host immune system (Weiner *et al.*, 1992; Farci *et al.*, 2000; Kato *et al.*, 1993, 1994). An effective immune response might result in the elimination of primary variant and a recovery. However, when the response is not effective enough, there is a burst in the number of new variants and, therefore, increased heterogeneity (Weiner *et al.*, 1991, 1992; Kato *et al.*, 1993, 1994).

In this case study, we attempted to find correlation between the profile of HCV antibodies, in particular E2 antibodies and HVR1 heterogeneity in different phases of the infection.

Materials and Methods

Patients. The patients were admitted at the Department of Infectious Diseases, University of Medical Sciences, Poznań, Poland. The Ethical Committee of the University approved the study and a written consent was obtained from each patient.

HCV RNA was detected in patients' sera using commercial Roche v2.0 Amplicor Assay (Roche, Switzerland).

RNA was extracted from 0.2 ml of patient's serum according to Chomczynski and Sacchi (1987) using the TRIzol Reagent (Invitrogen Life Technologies, USA).

RT, primary PCR and nested PCR were directed to HVR1 of E2 gp. The positions of the designed primers were based on a complete HCV nucleotide sequence (GenBank Acc. No. D50480) (Enomoto *et al.*, 1995). In RT, the primer A (5'-GCCGAAACG GTCGGTCGT-3', nts 1898–1881) was used. Briefly, 10 µl of RNA and 1 µl (50 moles) of primer A were mixed, denatured for 5 mins at 65°C and chilled on ice. The reaction was carried out in a 30 µl volume consisting of 10 µl RNA, 1 µl of the primer, 6 µl of the 5x buffer, 1 µl (28 U) RNAzin (Promega), 3 µl 0.1 mol/l dithiothreitol, 1.5 µl of 2.5 mmol/l dNTPs (Sigma-Aldrich), 6.5 µl of H_2O and 1 µl (200 U) of Moloney murine leukemia virus reverse transcriptase. The mixture was incubated for 1.5 hr at 37°C, boiled for 5 mins and chilled on ice. The reagents used for RT, unless otherwise indicated, were purchased from Invitrogen Life Technologies, USA

In primary PCR, the primers B (5'-TCAAGCTTTGGCATGG GATATGATGATG-3', nts 1282–1301, the *Hind*III site underlined) and C (5'-GGCTGGGAGTGAAGCAATA-3', nts 1866–1848) were used (Manzin *et al*., 1997). PCR mixture contained 10 µl of cDNA, 4 µl of 2.5 mmol/l dNTPs (Sigma-Aldrich), 1 µl (20 pmoles) of each primer, 5 µl of the 10x buffer, 0.5 µl (1.5 U) of high fidelity pfu DNA polymerase (Promega) and 28.5 µl H_2O. The cycling parameters were as follows: 94°C/1 min, 35 cycles of 94°C/1 min, 45°C/2 mins, and 72°/3 mins, and final extension step of 94°C/ 7 mins.

In semi-nested PCR, the primers B and D (5'-TGCTGCAG-GTCATTGCAGTTCAGGGC-3', nts 1622–1605, the *PstI* site underlined) were employed (Bukh *et al.* (1993). PCR mixture for the second round of amplification contained the same reagents, except one primer and a template, which was 10 µl of 20-fold diluted DNA obtained from primary PCR. The conditions of amplification were the same as in primary PCR. The fragment of app. 340 bp amplified by the semi-nested PCR, contained the C-terminus of E1 and N-terminus of E2 nucleotide sequences including HVR1.

SSCP analysis of HVR1 heterogeneity. The products of the semi-nested PCR were subjected to SSCP analysis (Orita *et al.*, 1989). Five to ten µl of the products were mixed 1:1 with a loading buffer (4% ficoll, 0.02% Bromophenol Blue, 80% deionized formamide in the 0.5x Tris-borate-EDTA buffer), denatured at 95°C for 10 mins and chilled on ice. The samples were subjected to polyacrylamide gel electrophoresis (PAGE) by loading onto a 10% non-denaturing polyacrylamide gel and electrophoresed at 300 V for 30 mins at 30°C, for another 30 mins at 15°C and finally for 30 mins at 5°C. A standard silver staining was performed.

Cloning and sequencing. The DNA fragments obtained from semi-nested PCR were purified, digested with *Hind*III and *Pst*I (Roche Diagnostics, Germany) and inserted into pBluescript SK+/– (Stratagene, USA). After transforming competent *E. coli* cells of XL1-Blue strain (Stratagene), the recombinant colonies were amplified in 2 ml of LB broth (Invitrogen Life Technologies) for plasmid isolation. Five to ten clones were subjected to sequencing using the fmol Sequencing System (Promega) and standard CY5-labeled T7 primer. Electrophoretic separation and detection of la-

beled fragments was carried out in 6% polyacrylamide gel containing 8 M urea at 1,500 V using an ALF Express Sequencer (Amersham-Pharmacia Biotech, Sweden). The data obtained were subjected to amino acid sequence analysis using the OMIGA 2.0 Program (Genetic Computer Group, Oxford Molecular Company, USA). Representative, i.e. most often occurring sequences in the clones for the patients Nos. 1–6 were deposited at the GenBank Database under Acc. Nos. AY212808, AY234212, AY212809, AY234214, AY234213 and AY234215, respectively.

Enzyme immunoassay (EIA) of HCV antibodies. Serum HCV antibodies were assayed using commercial LiaTek HCV III Test (Organon Teknika N.V., Belgium) according to the instructions of the manufacturer. Briefly, nylon strips, covered with separated bands of HCV antigens (C1, C2, HVR1 E2 1b, NS3 1b, NS4 and NS5) were incubated with 1:100 dilutions of patients' sera, washed, and incubated with alkaline phosphatase-conjugated anti-human Ig antibody. The reaction was visualized with BCIP/NBT. The intensity of brown colour measured against negative and positive controls was proportional to the amount of specific antibody present in the sample.

Results

Clinical data

All the 6 patients under study tested positive for HCV antibodies (data not shown) and serum HCV RNA (Table 1). Earlier clinical information, liver biopsy and histology tests allowed us to conclude that the patients 3 and 4 were in chronic phase. The patients 5 and 6 showed liver cirrhosis and overall signs characteristic of long-time HCV infection. Interviews and examinations (e.g. serum γ-glutamyltranspeptidase) excluded other possible causes of liver damage, e.g. Hepatitis B virus (HBV) or alcoholism. The patients 1 and 2 were admitted initially at the hospital from other reasons and tested negative for HCV antibodies, but, 2–3 months later, they showed symptoms resembling an HCV infection and turned HCV-seropositive. This was confirmed by the presence of HCV RNA in their sera and their status was considered an acute phase. The patient 2 was pregnant at the time of acute infection and after delivery, it was difficult to obtain any follow-up information about her, but, judging from the clinical data and reviews, she recovered presumably from the infection in natural way. The patient 3 was subjected to treatment with a combination of interferon alpha (IFN-α) and ribavirin for six months and then reported recovered, as confirmed by disappearance of HCV RNA from the serum at that time and 24 weeks later.

For all the patients, the same serum sample was used for the HVR1 sequence and HCV antibody analyses.

SSCP analysis of HVR1 heterogeneity

To assess the heterogeneity of HVR1 of E2 gp of HCV the PCR products obtained from the patients' sera were subjected do SSCP analysis (Fig. 1). The number of bands after PAGE is thought to reflect the number of mutations occurring in to the analyzed region. A distinct band pattern was obtained for each patient. The two patients (1 and 2) in acute phase showed no increase in the number of bands compared to the control. The two patients (3 and 4) in chronic phase exhibited a higher number of bands compared to the control, while those (5 and 6) with liver cirrhosis exhibited a diminished number of bands resembling the control.

Sequence analysis of HVR1 heterogeneity

To confirm the above assumption that the number of bands in SSCP analysis (PAGE) reflects the number of mutations in the given region and to gain a closer look at the changes within HVR1 of E2 gp, 5–10 clones from each

Table 1. Characteristics of the patients

Patient	Age/Sex	Serum HCV RNA	Phase/Histology treatment	IFNα + ribavirin
1	53/F	+	Acute, biopsy G2, S1	No
2	23/F	+	Acute	No
3[a]	48/M	+	Chronic, biopsy G3, S3	Yes
4	52/M	+	Chronic, biopsy G1, S2	No
5	51/M	+	Marked cirrhosis	No
6	48/F	+	Marked cirrhosis	No

[a]The patient recovered. G = grading. S = staging.

All blood sample withdrawals were performed 3–6 months after liver biopsy, except for the patient 1, whose blood was taken directly after the biopsy and the patient 2, in whom the biopsy was not performed. The blood from the patient 3 was taken a few days before starting the treatment.

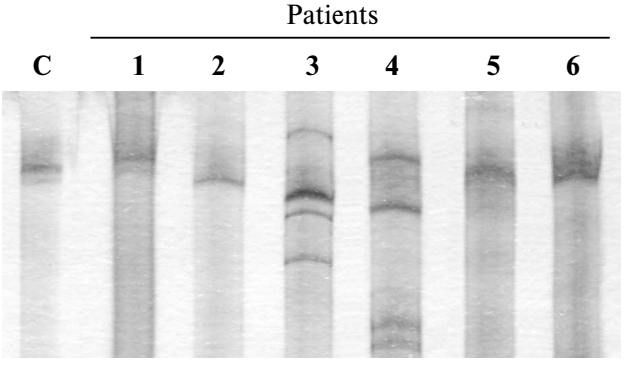

Fig. 1

SSCP analysis of HVR1 heterogeneity of HCV RNA detected in the patients' sera

PAGE. Control, the HVR1 region amplified from pBluescript SK+/– containing HCV E1/E2 cDNA clone (lane C); sera from the patients in acute phase (lanes 1 and 2); sera from the patients in chronic phase (lanes 3 and 4); sera from the patients with liver cirrhosis (lanes 5 and 6).

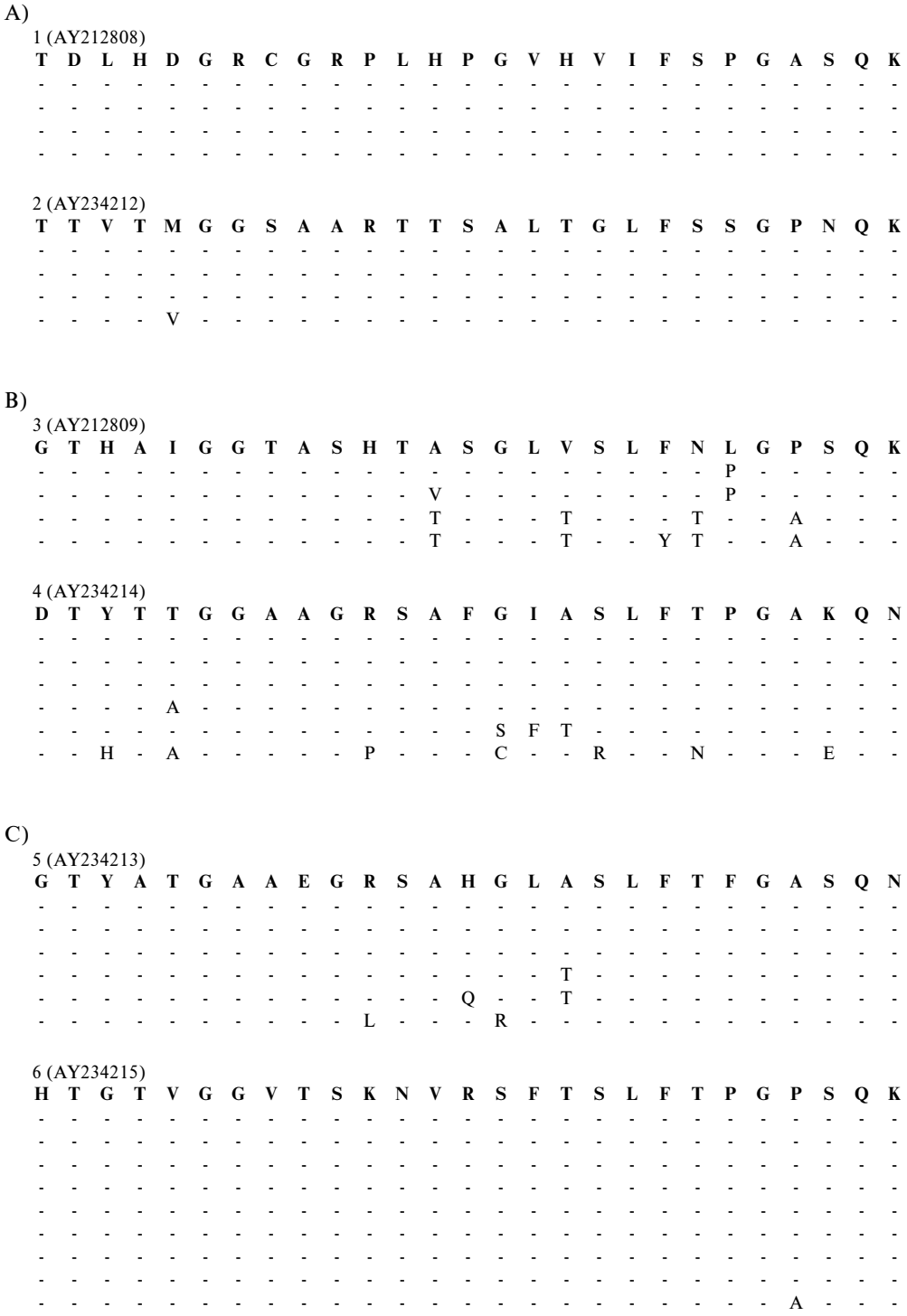

Fig. 2

Amino acid sequences of HVR1 detected in the patients' sera

Sequences of clones shown in bold letters were submitted to GenBank and their Acc. Nos are given in parentheses. For each clone under comparison, only mutated amino acids are indicated, the same amino acids are marked by a dash. The patients in acute phase (A); the patients in chronic phase (B); the patients with liver cirrhosis (C).

patient were sequenced. Fig. 2 shows deduced amino acid sequences of interest. In general, the changes in amino acid sequence confirmed the heterogeneity observed by SSCP analysis. The patients Nos. 1 and 2 did not show any mutations within the sequence analyzed, except for a single substitution in the latter patient.

For the patients Nos. 3 and 4 with the highest number of SSCP bands we could expect mutations not only in the 81 bp long HVR1 region but also in the 259 bp long 5'- and 3'-flanking regions. However, this was not the case, the flanking regions were free of mutations (data not shown). Within the 27 amino acid sequence of HVR1 (Fig. 2B), changes were restricted to particular regions, while certain amino acids at certain positions could be regarded as conserved (e.g. 2T, 6G, 7G and 20F) (Casino *et al.*, 1999; Penin *et al.*, 2001). From the sequences of 5'-flanking regions (data not shown) we assessed the genotype of the HCV detected as 1b in accordance with the classification of Bukh *et al.* (1993).

Serum HCV antibodies

A common feature of the sera from all the 6 patients was a high level of antibodies directed to NS3 1b and NS4 antigens. The abundant presence of NS3 1b antibodies confirmed the results of sequencing, indicating that the sole or prevailing genotype of the virus is 1b. Also, the antibodies directed to the most conserved HCV core protein antigens C1 and C2, were present in a high quantity, except for the patient 1. The NS5-specific antibodies were abundant in the patients 3, 4 and 5, while they were undetectable in others. The medium levels of E2 antibodies were found in the patients 2 and 4, while the data for the patient 3 was dubious (Table 2).

Discussion

We investigated a correlation between the HVR1 heterogeneity of HCV and HCV antibody profile. The LiaTek HCV III test, based on recombinant and synthetic HCV antigens, proved reliable, highly reproducible and a sensitive for the detection of specific HCV antibodies.

An active immune response, directed against HCV C, NS3 and NS4 antigens appeared in all the phases of the infection, indicating no direct influence on the HVR1 heterogeneity. E2 antibodies were detected in the patient 4 in chronic phase and in the patient 2 in acute phase. It is known that not all the patients in an acute phase with serum E2 antibodies clear the virus (Farci *et al.*, 2000). However, the presence of such an antibodies in a patient in acute phase coupled with a lack of mutations in HVR1 correlates with good overall outcome of the disease, because it reflects a broader and more effective activity of the immune system at early stage of infection (Farci *et al.*, 2000). The clinical picture of the patient 2 displaying no changes in HVR1 and presence of E2 antibodies seemed to match this situation in contrast to that of the patient 1, who was also in acute phase without changes in HVR1, but lacked E2 antibodies.

A different situation is represented by chronic phase of HCV infection (the patient 4), in which the virus is well established in the affected individual, HVR1 contains mutations and E2 antibodies are present; however, all this does not imply recovery. This is so despite the fact that antibodies specific to HVR1 are assumed to block virus attachment to cell (Zibert *et al.*, 1995). In chronic as well as acute phase, the presence of such antibodies might indicate a more vigorous and broader immune response. However, in chronic phase, a spontaneous recovery occurs rather rarely and the mechanisms responsible for this phenomenon include the inefficiency of exhausted and/or impaired immune system, which is unable to pursuit new emerging quasispecies (Kato *et al*., 1994; Wedemeyer *et al*., 2002).

Our data from SSCP and sequence analysis showed that, as expected, the greatest HVR1 variability occurred in chronic phase (the patients Nos. 3 and 4). This finding indicates in accordance with those of other studies that the emergence of new mutations within HVR1 together with the presence of E2 antibodies help the virus to evade the immune pressure (Weiner *et al.*, 1991, 1992; Kato *et al.*, 1993, 1994; Farci *et al.*, 2000; Fernandez *et al.*, 2004).

In this study, because of the lack of available sera, we were unable to screen the patients 3 to 6 for the variability of HVR1 regions and HCV antibody profile in various phases of infection. Instead, we chose to analyze the HVR1 heterogeneity and HCV antibody profile in relation to the degree of infection advancement. The analysis of patients in an acute phase, shortly after infection, showed no or little changes in HVR1 compared to those in chronic phase, suggesting that at this stage single prevailing clone was present in the host. This is in agreement with an assumption that, as a result of specific interaction between the virus and host shortly after infection, only some quasispecies present in the inoculum are able to infect and multiply, thus starting a new population (Lin *et al.*, 2001).

Another interesting situation was disclosed by the study of the patients Nos. 5 and 6 with advanced cirrhosis and without serum E2 antibodies. Namely, the number of mutations in HVR1 of these patients was lower compared to that of the patients in chronic phase. If this decrease were the result of the duration of infection, then the best fitted population of variants facing little if any effective immune defense would be established, while some old and new variants unfitted to the unchanging environment would not gain an evolutionary advantage. This assumption needs support from new data, because the existing ones do not

Table 2. HCV antibody profiles of the patients

Patient	Antibody					
	C1	C2	E2	NS3	NS4	NS5
1	++	+	–[a]	+++	+++	–
2	+++	+++	++	+++	+++	–
3	+++	+++	+/–	+++	+++	+++
4	+++	+++	++	+++	+++	+++
5	+++	+++	–[a]	+++	+++	+++
6	+++	+++	–[a]	++	++	–

(+), (++) and (+++) = different levels of antibodies. (+/–) = dubious result. (–) = negative result.

[a]As the assay was based on use of the HCV E2 1b antigen only, a negative result (–) did not exclude the presence of antibodies directed to HCV E2 antigens of subtypes other than 1b.

necessarily confirm it. For example, a study of HVR1 heterogeneity of patients with mild, moderate or severe form of chronic HCV 1b hepatitis, liver cirrhosis and superimposed hepatocellular carcinoma revealed a similar number of SSCP bands (López-Labrador *et al.*, 1999).

It should be stressed that our investigation presented here should rather be considered a case study not allowing statistical analysis and generalization. The data obtained from a higher number of patients could give different results. Anyway, our results seem to correspond to those of Fernandez *et al. (*2004), who have examined the molecular evolution of HCV over a 4-year period in two chimpanzees infected with a virus consisting of a single clone of single sequence as a starting population. They have reported (i) accumulation of mutations in virus genome but a decline in the mutation rate at the amino acid level over time in both animals, suggesting a lower immune pressure, possibly due to a weaker T-cell response compared to that in acute infection and (ii) increase in E2 antibody titers in the course of infection in correlation with higher number of amino acid substitutions in E2 gp, indicating their accumulation due to selection pressure rather than a consequence of long-term HCV replication (Fernandez *et al.*, 2004). Although the animal model of HCV infection may not fully reflect the mode of infection in humans, this observation indirectly supports our results concerning the relationship between the presence of neutralizing E2 antibodies and variability of HVR1. Namely, the patients (Nos. 5 and 6) with liver cirrhosis and without E2 antibodies showed a diminished HVR1 heterogeneity compared to those (Nos. 3 and 4) in chronic phase. This situation may be interpreted as a result of weakened or exhausted immune systems, unable to block the multiplication of a major quasispecies but still strong enough to eradicate some minor quasispecies.

Further studies are certainly required to verify this reasoning. Anyway, the data presented in this study seem to indicate that the knowledge of the level of serum E2 antibodies could be an important information determining the therapy and its possible outcome.

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