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RELATIONSHIP BETWEEN DIVERSITY OF HEPATITIS C QUASISPECIES AND HISTOLOGICAL SEVERITY OF LIVER DISEASE

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Abstract The aim of this work was to assess if the diversity of hepatitis C virus (HCV) quasispecies is related to histological severity and duration of infection in a cohort of untreated patients with an estimated onset of the disease. A total of 27 patients with diagnosis of chronic liver disease and history of blood transfusion (n = 16) or intravenous drug use (IDU) (n = 11) were included. All were anti-HCV positive and had detectable serum HCV-RNA. The onset and the duration of the disease were estimated from the time of the transfusion or the first drug injection. Patients who consumed drugs for more than 2 years, or were coinfected with HBV or HIV were excluded. History of alcohol intake (> 80 g/day), ALT level and age at infection were recorded. Histological assessment of grading and staging was performed according to Knodell score. The quasispecies diversity was investigated by single strand conformation polymorphism (SSCP) targeted to HVR-E2 region and SSCP pattern was evaluated as a single or multiple bands. The number of quasispecies did not correlate with the estimated duration of the disease. Patients who acquired hepatitis C by blood transfusion did not differ in number of bands from patients who were IDU. There was no correlation between the heterogeneity of HCV quasispecies and age, serum ALT, Knodell score, HAI and fibrosis. In conclusion the cuasiespecies diversity of E2 had no correlation with grade and stage of chronic HCV infection and the presence of quasispecies was independent of the duration of the disease.

Resumen ***Relación entre la diversidad de* cuasiespecies *del virus de la hepatitis C y la severidad de las lesiones hepáticas.*** El objetivo del presente estudio fue evaluar si la diversidad de cuasiespecies del virus de la hepatitis C (HCV) se relaciona con la severidad de las lesiones histológicas y con la duración de la infección en un grupo de pacientes no tratados en quienes se pudo estimar el inicio de la enfermedad. Fueron incluidos en este estudio 27 pacientes con diagnóstico de hepatitis crónica e historia de transfusiones (n: 16) o de consumo de drogas intravenosas (n: 11). Todos fueron anti-HCV positivos y presentaron el ARN del HCV detectable por técnica de PCR. El inicio y duración de la infección fueron estimados desde la fecha de la transfusión o de la primera inyección de drogas. Aquellos pacientes que consumieron drogas por un período mayor de 2 años o que presentaron co-infección con el virus de HIV o HBV fueron excluidos del estudio. Se analizaron los niveles de ALT, edad al tiempo de infección y consumo de alcohol. El grado de actividad histológica y el estadio fueron evaluados a través del *score* de Knodell. La diversidad de cuasiespecies fue investigada por técnica de SSCP dirigida hacia la región hipervariable E2 y el patrón de bandas obtenido fue evaluado como banda única o múltiples bandas. El número de cuasiespecies no correlacionó con la duración estimada de la enfermedad ni con la vía de infección (transfusiones o drogas intravenosas). No existió correlación entre el número de bandas y la edad, el nivel de ALT, el *score* de Knodell, el índice de actividad histológica ni la fibrosis. En conclusión, la diversidad de cuasiespecies no tuvo relación ni con el grado ni con el estadio, ni con la duración de la infección.

Key words: HCV-quasispecies, HCV-heterogeneity, hepatitis C, HCV variability, HCV infection, SSCP-HCV

Hepatitis C virus (HCV) is a human pathogen that frequently causes chronic liver disease, including hepatocellular carcinoma[1]. The HCV viral genome is a positive-sense, single-stranded RNA molecule approximately 9.4 kb in length which encodes a polyprotein of about 3100 amino acids. This polyprotein is cleaved into functional proteins by cellular and viral proteases. Analysis of individual isolates demonstrates that there are 2 hypervariable regions located within the virion structural proteins. The continuous evolution of new variant glycoproteins is a major mechanism of viral evasion.

HCV, like other RNA viruses exists within its host as pools of genetically distinct but closely related variants referred to as quasispecies[2]. The term "quasispecies" refers to the genetic diversity of a virus population that can be observed in a single infected individual owing to changes that have taken place during the course of the infection[2]. Selection of HCV quasispecies is related to many factors such as viral replication efficacy,

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immunomodulation of the host, therapeutic interventions and coinfection with others viruses[3]. The extensive genetic variability of HCV is likely to have important implications for diagnosis, pathogenesis, treatment and vaccine development[4, 5].

HCV infection is characterized by a slow indolent progression to liver failure and the period of infection prior to liver failure can range from 10-40 years[6, 7]. The gold standard for staging liver damage associated with viral hepatitis is liver biopsy. Disease progression is defined according to grade and stage, with grade referring to the degree of inflammation and stage referring to the degree of fibrosis/cirrhosis.

The rate of disease progression in hepatitis C infection is influenced by both virus- and host-related factors. Virus-related factors include size of initial inoculum at infection, quasispecies diversity, and genotype. It was also described that transfusion-associated infection has a more rapid progression to active liver disease than needlestick-associated infection[8, 9], presumably it is related to the smaller viral burden at exposure in the case of the latter. Interesting to note, the clinical importance of HCV diversity remains under research and there is disagreement about the role of hepatitis C quasispecies as a prognostic tool for disease progression.

The aim of the present study was to assess if the diversity of HCV quasispecies is related to histological severity and duration of infection in a cohort of untreated patients with a defined parenteral route of transmission in whom we could estimate the onset of the disease.

Patients and Methods

Patients

Twenty seven patients with histological diagnosis of chronic liver disease and history of blood transfusion (n = 16) or intravenous drug use (IDU) (n = 11) were included. There were 17 males and 10 females ranging in age from 15 to 69 years (median age 32 years, mean age 36.25 years). All were positive for anti-HCV and had detectable serum HCV RNA by reverse transcriptase polymerase chain reaction (RT-PCR) using primers from the 5'noncoding region. All the patients had abnormal alanine aminotransferase levels (ALT) at the time of the study. The onset and the duration of the disease was estimated from the time of the transfusions or the first time of intravenous drug injection. Patients who received transfusions in more than one opportunity or consumed intravenous drugs for more than 2 years were excluded. Patients coinfected with HBV or HIV were not included. History of alcohol intake (> 80 g/day), ALT level and age at infection were recorded. Liver biopsy specimens from all the studied patients were analysed by assessing the grade of necroinflammatory process and the stage of fibrosis. Grading score: sum of the histological activity index (HAI) component I/II and III of Knodell score[10], and staging score: fibrosis component, IV of Knodell score. Clinical and epidemiological profile of the patients according to the route of transmission of HCV infection are showed in Table 1. Written informed consent was obtained from all patients and the study protocol was approved by the local ethical committee in accordance with the 1975 Declaration of Helsinki.

Serological analysis

Serum antibodies to HCV were detected with the second-generation HCV enzyme-linked immunosorbent assay (ELISA-2) Abbot Laboratories, North Chicago, USA.

PCR detection of HCV-RNA

Total RNA was extracted by the acid guanidinum-phenol-chloroform method as previously described[11]. Briefly: 150 µl serum were mixed with 500 µl of denaturing solution 4 M guanidium thicyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, 50 µl of 2 M NaAc (pH 4.0), 500 µl of phenol and 100 µl of chloroform. After centrifugation aqueous phase was recovered and precipitated over night at – 20 °C with 650 µl of isopropanol and 20 µg dextran T500. The resulting pellet was washed with 70% ethanol and resuspended in 9 µg of water. RNA obtained was denatured at 78 °C for 5 min and primed with 0.4 µg of random hexamers. Reverse trnascription conditions were 50 mM TrisHCl (pH 8.3), 25 mM KCL, 3 mM $MgCl_2$), 0.1 mM DTT, 1 mM dNTPs, 18 U of ribonuclease inhibitor (Promega) and 100 U of M-MLV reverse transcriptase (Gibco), reaction was performed for 90 min at 37 °C. After heat inactivation at 95 °C for 5 min and chilling on ice, the cDNA was amplified. The 50 µl PCR reaction contained: 20 mM TrisHCl, 50 mM KCl, 50 pmoles of each primer for the 5UT region of HCV genome, 5UT1 (5' CCTGTGAGGAACTACTGTCTTCACGC 3') and 5UT2 (5' AGGTCTCGTAGA CCGTGCACC 3') and 1.25 U of Taq. The PCR reaction consisted of 40 cycles each with denaturing at 94 °C for 30 sec, annealing at 55 °C 30 sec, and polymerisation at 72 °C for 45 sec. Nested PCR was done with 2 µl of PCR product as template, using internal primers 5UT3 (5' TCT AGC CAT GGC GTT AGT GCG AGT GT 3) and 5UT4 (5' CAC TCG CAA GCA CCC TAT CAG GCA GT 3), in the same conditions of the first round. PCR products were analysed by ultraviolet fluorescence after ethidium bromide staining.

Detection of HCV quasispecies

Sera were stored at – 70 °C within 4 hours of venesection. Viral quasispecies was determined using single strand conformational polymorphism (SSCP) analysis of the HCV hypervariable region (HVR2). SSCP pattern was defined as a single band or as multiple bands (two or more than two bands).

SSCP analysis

Hypervariable region contained in the N-terminal E2 region was amplified by PCR with the protocol above described, using 50 pmoles of each primer; HVR1 (5' GC CAT ATA ACG GGT CAC CGC ATG GC 3); and HVR2 (5' TCT CAG GAC AGC CTG AAG MGT TGA A 3) for the first round, and primers HVR3 (5' GCA TGG GAT ATG ATG ATG AAC TGG TC 3); and HVR4 (5' GGT GTT GAG GCT ATC ATT GCA RTT 3') for the nested. Three microliters of the nested PCR product were mixed with 7 µl of SSCP loading buffer (0.05% xylene cyanol, 20 mM EDTA and 95% formamide), denatured at 95 °C for 10 minutes and chilled on ice water, then 5 µl were applied to a 6% nondenaturating polyacrylamide gel (acrylamide: N, N'-bisacrylamide, 29: 1). After electrophoresis at room temperature for 20 h at a constant voltage of 350 V, gel was developed by silver stain.

Statistical methods

Numerical values were expressed as median (25^{th}-75^{th} percentile). Mann-Witney and Fisher exact tests were used for comparisons; coefficient r was used for correlation's. A p level < 0.05 was considered significant.

Results

Clinical and demographics characteristics of the patients as well as histological findings are shown in Table 1. Median age at infection was 21 years and duration of infection was 10 years. Median of ALT was 80 IU/L.

SSCP analysis showed a single pattern in 10 patients and a multiple pattern in 17 patients. The number of observed bands in SSCP analysis did not correlate with the estimated duration of the disease. Patients who acquired hepatitis C by blood transfusion did not differ in SSCP pattern of patients who were IDA. There was not correlation between the heterogeneity of HCV quasispecies and age, serum ALT, Knodell score, HAI and fibrosis (Table 2). There was no significant correlation between age at infection and number of SSCP bands.

Discussion

The clinical course and outcome of hepatitis C are variable and many viral factors may influence the rate of progression to chronic hepatitis such as viral load, genotype and the presence of quasispecies.

In this study, we analysed the relationship between diversity of HCV quasispecies and histological severity and duration of infection in a cohort of untreated patients with a defined parenteral route of transmission in which we could estimate the onset of the disease. Our results indicate that the heterogeneity of HCV quasispecies was

TABLE 1.– *Clinical and histological profile of the patients studied according to the route of transmission of HCV*

	Transfusion acquired n = 16	IDU n = 11	p
Age (years)	38 (28.5-51)	29 (27-36)	0.06
Male/female	8/8	9/2	0.1
Duration of infection (years)	12.5 (8-18.5)	8 (6-16)	0.2
Age at acquisition (years)	24 (18-34)	21 (19-22)	0.2
ALT activity (IU/L)	80 (60-130)	100 (60-120)	0.4
Knodell score	6 (4.5-13)	8 (3-11)	0.6
HAI	5 (4.5-10)	7 (3-10)	0.6
Stage	1 (0-2.5)	1 (0-1)	0.6

IDU: intravenous drug use; ALT: alanine aminotransferase; HAI: histological activity index. Results were expressed as median (25^{th}-75^{th} percentile)

TABLE 2.– *Clinical and histological profile of the patients studied according to the SSCP analysis*

	Single band (n = 10)	Multiple bands (n = 17)	p
Age (years)	42.5 (32-52)	29 (27-38)	0.09
Age at infection (years)	24.5 (20-42)	21 (18-22)	0.07
Duration of infection (yers)	11.5 (8-19)	8 (6-18)	0.6
ALT (IU/L)	80 (60-80)	100 (60-160)	0.2
Knodell score	5.5 (4-15)	8 (4-11)	0.8
HAI	5 (4-11)	7 (4-10)	0.8
Stage	0.5 (0-2)	1 (0-1)	0.8
Transfusion acquired	6	10	1
IDU	4	7	1

Results were expressed as median (25^{th}-75^{th} percentile)

independent of duration of infection and severity of histological lesions. We also noted that it was not associated to host factors such as age of infection, gender or route of infection (transfusion or IDU).

However, other observations suggest that the diversity of HVR quasispecies increases as the disease progresses[12]. It has been shown that patients with liver cirrhosis have more HCV populations and the diversity of HVR quasispecies augment in patients with hepatocellular carcinoma[12]. It is important to note that in our series, only 3 out to 27 patient had cirrhosis at the time of the analysis. One may speculate that this discrepancy with the reported study might be explained by the fact that the duration of the disease in our patients was not long enough to develop end stage liver disease as in the mentioned study. Interestingly, we also note that patients having single or multiple bands in SSCP analysis did not differ in HAI or fibrosis score, showing lack of correlation between aggressive liver disease and HCV genetic complexity.

In agreement with a recent report of Leone et al. in which the studied patients showed a similar clinical and histological profile to that of the patients studied in our series, HCV-HVR1 complexity did not correlate with the severity of liver disease, whatever the biological and virological profile of the viral infection[13].

Finally, it has been postulated that the persistence of HCV is one of the most striking and important consequence of the existence of quasispecies[5]. However, the mechanisms involved in the development of liver injury caused by HCV infection are poorly understood.

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Cita leída en el Museo de Ciencias de Londres*

"...Hic me gravedo frigida et frequens tussis quassavit usque dum in tuum sinum fugi et me recuravi otioque et urtica..."

"...At this point I was laid low by chilly catarrh and frequent coughing until I took refuge with you and cured myself with rest and nettle tea..."

On getting a cold. Catullus (84-54 BC)

"...Entonces yo estaba vencido por los escalofríos, el catarro y la tos frecuente, hasta que me refugié en ti y me curé con el descanso y el te de ortiga..."

Resfriándose. Cátulo, (84-54 a.C.)

*Science Museum, Exhibition Road, London SW72DD, 1999-2000