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RNA-HCV VIRAL LOAD IN SERUM, PERIPHERAL BLOOD MONONUCLEAR CELLS AND LIVER IN CHILDREN WITH CHRONIC HEPATITIS C

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Abstract: The liver is the major site of hepatitis C virus (HCV) infection and replication. However, HCV may infect and replicate in extrahepatic sites as well. Several investigators have demonstrated that peripheral blood mononuclear cells (PBMCs) are the major extrahepatic milieu of infection and viral replication. The aim of the study was to investigate the correlation between RNA-HCV level in serum, PBMCs and liver in children with chronic viral hepatitis C (CHC). The impact of RNA-HCV level on the sustained virological response (SVR) after therapy was also determined. Study was carried out in the group of 10 children with CHC, age 8 to 17 years. Antiviral therapy was implemented in all patients with pegylated interferon α (Peg-IFNα) 2a or 2b and ribavirin during 48 weeks. The following tests were performed prior the therapy: basic laboratory parameters, histology of liver biopsy, RNA-HCV viral load in serum, PBMCs and in liver. The behavior of HCV-RNA viral load in serum, PBMCs and liver in children with CHC did not present strict mutual relations. However, the positive correlation between serum and PBMCs viral load ($r = 0.47$) and negative correlation between PBMCs and liver viral load ($r = –0,47$) was demonstrated. Although no statistically significant results were found, some trends of relationship in viral load between various body compartments were present. Given the aforementioned results, it is clear that more data are needed, mostly more numerous groups of patients, especially those whose influence of RNA-HCV viral load had a major impact on the antiviral treatment.

Keywords: children, chronic hepatitis C, RNA, HCV, viral load

Hepatitis C virus (HCV) has been known for 20 years (1, 2). There are still several studies going on in the attempt to elucidate the pathogenesis of this infection, clinical course and prognostic factors of good response to antiviral treatment. New generations of antiviral compounds are at the stage of research. HCV has a narrow host range and infects only humans and chimpanzees, therefore, studies of HCV rely on the observations made during natural infection in humans. It is well known that HCV progresses insidiously and results in serious complications such as cirrhosis and hepatocellular carcinoma in approximately 20 and 4% of adult patients, respectively (3–5). The data on the natural history of HCV liver disease in children are even more conflicting and unpredictable (6–8).

The liver is the major site of HCV infection and replication. However, HCV may infect other cells and tissues and replicate in extrahepatic sites. Several investigators have shown that peripheral blood mononuclear cells (PBMCs) are the major extrahepatic sites of viral replication.

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Implementation of reverse transcription and polymerase chain reaction (RT-PCR) using Taq enzyme, that is recombined DNA polymerase from *Thermus thermophilus* bacteria, confirmed the replication of HCV in other sites, such as lymphoid tissue, bone marrow and cerebrospinal fluid (9).

At present, attention is being paid to another feature of HCV, namely the survival and mini replication skills, so called occult persistence of HCV infection. The discovery of occult HCV infection has challenged the accepted view that complete resolution of hepatitis C, either spontaneously or therapeutically-induced, would be indicative of eradication of HCV (10). Thus, it became of interest, what is the distribution of viral load in extrahepatic sites of HCV habitat confronted with liver viremia and the clinical course of CHC. Such information might create premises for focused anti-viral therapy, directed toward particular cells or tissue.

MATERIALS AND METHODS

The study was carried out in the group of 10 children (8 girls and 2 boys) with chronic viral hepatitis C (CHC), age 8 to 17 years (mean age 11.8 years). The diagnosis was based on: medical history, clinical examination, evaluation of alanine aminotransferase (ALT) activity, exclusion of the presence of tissue autoantibodies, the exclusion of the infection with hepatitis B virus, cytomegalovirus, Epstein-Barr virus, Herpes viruses, Wilson's disease and a1-antitrypsin deficiency. In all patients, anti-HCV antibodies for at least 6 months and morphologic examination of biopsy specimen of percutaneous liver needle biopsy were done. The histopathology included the evaluation of inflammatory activity – *grading* (G) and the evaluation of the spread of fibrosis – *staging* (S) 0–4 points. The following tests were performed directly before the treatment in all patients:

HCV genotype analysis with the use of HCV II Amplification Procedure INNO-LIPA HCV-II (Innogenetics).

RNA-HCV viral load in serum by Amplicor Monitor Test, Version 2.0 (Roche), sensitivity level 50 IU/mL (quality assessment) and 600 IU/mL (quantity assessment).

RNA-HCV viral load in PBMNCs and liver by RoboGene HBV DNA Quantification Kit (AJROBOSCREEN).

The RNA-HCV viral load in the samples was estimated from the standard curve generated by using five standard concentrations ranging from 10 to 1×10^5 international units (IU)/mL with conversion 3.8 copies = 1.8 IU. Briefly, human PBMCs were isolated from peripheral blood by Ficoll density gradient centrifugation. Residual red blood cells were hypotonically lysed and cells were washed three times with phosphate buffered saline. PBMC preparation consisted of > 98% lymphocytes and monocytes and < 2% granulocytes as determined by morphology of cells stained according to Pappenheim. HCV specific RNA was extracted from 200 μL of serum and from 10^6 cells isolated from blood or liver biopsy specimens. For the detection of HCV specific RNA in PBMC and in the liver, 100 ng of total cellular RNA were subjected to a HCV specific nested RT/PCR procedure.

Directly before treatment the following laboratory tests were carried out:

- ALT activity – by enzymatic method (upper normal limit 40 IU/L).
- Aspartate aminotransferase (AST) activity – by enzymatic method (upper normal limit 42 IU/L).
- Cellular blood count – normal limits:
  White blood cells – WBC ($\times 10^3$/mL) 4.0–12.0
  Red blood cells – RBC ($\times 10^6$/mL) 3.8–5.5
  Hemoglobin – Hb (g/dL) 12.0 – 15.0
  Platelets count – PLT ($\times 10^3$/mL) 130–350
- Neutrophil (Neut) level ($\times 10^3$/μL)
- γ-Globulin (γ-glob) – normal limits 13–21%
- Albumin (alb) –normal limits 53–68%

In all patients the therapy was implemented with Peg-IFNα 2a or 2b and ribavirin during 48 weeks. Parents of all children along with children over 12 years old signed informed consent for the diagnostic procedures and anti-viral treatment. The agreement for the study was obtained from the local University Ethical Committee. Potential conflicts of interest in this research study do not exist. After the end of treatment (T_{72}) SVR was estimated (undetectable HCV-RNA in serum).

Data were analyzed by statistical software – Statistica 8.0 (StatSoft Inc.). Since the patient cohort was very small, only descriptive statistics were used. Because data were not normally distributed variables, Spearrman rang correlation coefficient was applied to check relationship between analyzed variables. Its significance was assessed by Student *t*-test. All tests were analyzed at significance level $p = 0.05$.

RESULTS

Medical history revealed that the length of infection ranged from 3 to 11 years, mean 7.2 years. (Table 1). Seven children had a history of malignant disease – acute lymphoblastic leukemia (ALL). The

Table 1. Baseline characteristics of patients.

Patient	Gender	Duration of infection (years)	Undergone malignant diseases	SVR
NK	f	11	ALL 1992	no
NP	f	10	ALL 1992	yes
BD	m	9	no	no
DB	f	9	ALL 1996	yes
MC	f	7	ALL 1991	no
CG	m	7	no	yes
EG	f	3	no	no
AB	f	7	ALL 1997	yes
KG	f	5	ALL 1999	yes
SJ	f	4	ALL 1998	no

ALL – acute lymphoblastic leukemia.

Table 2. Biochemical parameters of patients.

Patient	WBC ($\times 10^3$/μL)	RBC ($\times 10^6$/μL)	Hb (g/dL)	PLT ($\times 10^3$/μL)	Neut %	ALT (IU/L)	AST (IU/L)	Alb %	γ-glob %
NK	3.5	4.92	13.8	116	16	28	25	50.0	19.3
NP	7.7	5.13	13.8	230	65	40	23	52.9	19.89
BD	7.1	4.78	13.9	211	45	63	63	57.73	18.72
DB	7.7	4.48	12.9	344	57	17	32	47.91	24.38
MC	5.8	4.66	13.5	395	52	24	25	49.2	18.7
CG	11.5	5.52	14.9	241	60	38	44	51.24	15.0
EG	4.2	5.07	14.7	228	54	51	38	52.26	15.97
AB	4.9	4.53	12.6	243	48	58	42	58.48	15.74
KG	6.1	4.83	13.2	263	61	29	32	52.56	15.3
SJ	7.9	5.54	16.1	260	40	378	261	57.65	15.63

Table 3. RNA-HCV viral load (IU/mL) in serum, PBMCs, liver tissue, histopathological result and weight.

Patient	RNA-HCV in serum	RNA-HCV in PBMCs	RNA-HCV in liver	Liver biopsy	Weight kg
NK	2.68×10^5	bdl	2.31×10^4	S1 G1	55
NP	5.26×10^3	2.44×10^3	2.21×10^4	S1G2	71.5
BD	8.4×10^5	bdl	2.1×10^5	S1 G1	25
DB	2.45×10^3	6.03×10^2	8.31×10^4	S1 G1	38
MC	1.18×10^4	bdl	4.91×10^3	S1 G1	60
CG	5.63×10^7	3.32×10^6	2.71×10^4	S1 G1	21.5
EG	3.65×10^5	1.67×10^3	1.2×10^5	S3 G2	59.5
AB	2.66×10^5	bdl	1.04×10^6	S1 G1	37
KG	1.97×10^6	1.34×10^4	3.2×10^3	S1 G1	45
SJ	3.86×10^5	2.34×10^4	4.2×10^3	S1 G2	64

bdl – below detection limit.

remaining three children were frequently hospitalized (e.g., prematurity, surgical operations, injections, invasive diagnostic procedures). No family members of the children from the study group were infected with HCV. All children were infected with 1 genotype of HCV (Table 1). Basic laboratory parameters were correct, only the ALT activity in 3 children was slightly increased, in one girl nearly 10 times. None of children had clinical symptoms of the disease (Table 2).

Mean viral load in serum amounted to 117.087×10^5 IU/mL (children with SVR) and 3.7404×10^5 IU/mL (children without SVR). In 3 children without SVR, RNA-HCV was not detected in PBMCs, whereas in children with SVR only one was RNA-HCV free. Mean RNA-HCV viral load in liver in children with SVR amounted to 235.1×10^3 IU/mL and 72.442×10^3 IU/mL in children without SVR. Fibrosis has not exceeded 2 points in the group studied (Table 3).

Analyzing the relationship of HCV-RNA viral load between compartments (serum, PBMCs, liver) no significant correlation was found. It is worth to notice the positive correlation between serum and PBMCs ($r = 0.47$) viral load and negative correlation between PBMCs and liver viral load ($r = –0.47$). While analyzing the relationship between HCV-RNA viral load in serum, PBMCs or liver and the ALT, body weight, or the duration of infection no significant correlation has been found. However, attention should be paid to the negative correlation between body weight and HCV-RNA in liver ($r = –0.57$; $p = 0.08155$). The examined group consisted only of 10 children, therefore, it is hard to obtain statistically significant results. Some trends of dependence between various variables could however be observed.

DISCUSSION

HCV infection in adults and in children is determined by host and viral factors, but these precise and various mechanisms are not fully identified. Knowledge about the course of HCV infection in children is even more modest. Children with CHC are symptom free and do not show clinical signs of chronic liver disease (6, 11).

The group of children in the current study was homogeneous in terms of HCV genotype and parental infection route. All of them were symptom free. Laboratory parameters were within normal values, except of 4 children and especially a girl who moreover was obese (BMI 31). The infection's duration and oncological history have not influenced the level of development of pathomorphological changes. These observations were also noted by other researchers (7, 12, 13).

Any correlation between RNA-HCV viral load in serum or liver and obtained SVR has been observed. On the contrary, higher mean viral load in both these compartments in children with obtained SVR has been noted. It seems however, that this result should be treated with caution, due to the small number of the analyzed group. RNA-HCV viral load in three studied compartments had ambiguous value. Only HCV-RNA viral load in PBMC of every patient was lower than in serum, this tendency has not been observed in any other compartment. In 3 patients RNA-HCV viral load in liver was higher than in serum, and in 7 cases higher than in PBMCs. An interesting observation concerns high directly proportional correlation between viral load in serum and PBMCs, and inversely proportional between viral load in PBMCs and liver tissue. As it was mentioned before, higher viral load is not necessarily accompanied by the increase of aminotransferases activity or level of fibrosis, which suggests the presence of another factors, probably immunological or related to the virus biology, influencing the progress of disease.

The observations carried out already in the 90s seem to confirm this hypothesis (14, 15). There are still intense studies carried out on the factors directly responsible for both, the course of the disease and especially results of the treatment. The researchers focus on genetic studies concerning HCV replication, presence of certain, recurrent mutations in the genome, conditioning the escape from the host's immune system control or resistance to the given medications (16–20). The lack of animal models, constitutes a major difficulty in HCV research. Another critical study on HCV is in the field of acquired and innate immunity, which arises due to HCV infection (21, 22).

The biochemical and virological parameters have not been influenced by the duration of the disease. Only inverse correlation between body weight and HCV-RNA viral load in liver tissue has been observed. On the basis of previous own observations, the authors can make a suggestion that in children there are several clinical, biochemical, virological or histopathological factors that influence unambiguously the course of CHC or the results of treatment. However, the results are often conflicting, resulting probably from the small sample groups under assessment (23–25).

In conclusion, it can be said that HCV-RNA viral load in serum, PBMCs and liver in children

with CHC does not present statistically significant, mutual relations. Some trends of relationship of viral load between various compartments seem however, to exist. The duration of infection or oncological history apparently does not influence the viral load and the level of advancement of pathomorphological changes, irrespective of the viral load in the analyzed compartments. The laboratory values are within normal limits in most patients. Given the aforementioned results, it is clear that more data are needed, mostly more numerous groups of patients, especially those whose influence of RNA-HCV viral load had a major impact on the antiviral treatment.

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