SIGNIFICANCE OF MOLECULAR IDENTIFICATION OF HEPATITIS C VIRUS RNA IN DIAGNOSIS OF CRYPTOGENIC HEPATITIS IN CHILDREN

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Summary. – Viral etiology of hepatitis is routinely proved by standard immunological tests detecting specific antibodies. However, identification of specific antibodies cannot always be conclusive. Since specific hepatitis C virus (HCV) antibodies may appear after some months of the infection, identification of HCV RNA and/or hepatitis G virus (HGV) RNA should clarify the etiology of hepatitis. The aim of this study was to diagnose etiologically unknown hepatitis by a reverse transcription–polymerase chain reaction (RT-PCR) testing of the presence of HCV RNA and HGV RNA. The study involved 33 children with histologically proved hepatitis. The presence of HCV and any signs of autoimmune disease were not observed at the beginning of the follow-up study. During 2.5 years of the follow-up HCV-RNA was found in the blood and liver biopsies in 17 patients. Eight of them became HCV antibodies-positive during the follow-up. None of them eliminated the virus from the blood during the follow-up. In two other patients HCV-RNA was found only in the liver. HGV infection in all cryptogenic patients was excluded by PCR testing. Identification of HCV RNA RT-PCR allowed to diagnose 19 out of 33 (57.6%) patients with cryptogenic hepatitis. The etiology of the hepatitis in remaining 12 patients has to be established.

Key words: children; cryptogenic hepatitis; hepatitis C virus; RNA

Introduction

Among many factors causing hepatopathy viral etiology is most frequent (Alberti *et al.*, 1999; Chung *et al.*, 1997; Marcellin, 1999; Naoumov, 1999; Pawlotsky, 1999; Walewska-Zielecka *et al.*, 1996). Diagnostics of non-viral liver diseases is well established and includes genetic diseases causing hepatitis (Wilson disease, alpha 1-antitrypsin deficiency), toxic liver damage and autoimmune hepatitis. Diagnosis of viral hepatitis is not easy since immunological tests not always clarify the etiology of many cases. Hepatitis A virus (HAV), hepatitis B virus (HBV) and HCV are the most frequent causative agents of viral hepatitis (Januszkiewicz *et al.*, 1997; Maniva *et al.*, 1997; Pawlotsky 1999; Zancan *et al.*, 1996). Diagnosis of viral hepatitis is based on clinical picture, biochemical examination, and standard immunological tests detecting specific serum antigens or antibodies. However, identification of the etiologic agent based on the presence of specific antibodies is not always conclusive (Kocabas *et al.*, 1997a,b; Tada *et al.*, 1997). Especially in the case of hepatitis C, due to a long period of seroconversion, absence of specific antibodies cannot exclude the HCV infection (Lu *et al.*, 1998; Palomba *et al.*,

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Abbreviations: ALT = alanine transaminase, HCMV = human cytomegalovirus, EBV = Epstein-Barr virus, HAV = hepatitis A virus, HBc = hepatitis core antigen, HBV = hepatitis B virus, HCV = hepatitis C virus, HEV = hepatitis E virus, HGV = hepatitis G virus; HSVs = herpes simplex virus 1 and/or 2; RT-PCR = reverse transcription–polymerase chain reaction

1996; Thaler, 1997). In addition, lack of characteristic viral antigens relevant to HCV makes the diagnosis of hepatitis C more difficult as compared to hepatitis B. At present, the detection of HCV antibodies is still a standard method for diagnosis of HCV infection. Since the specific HCV antibodies production may occur after some months of the infection, the identification of HCV RNA by RT-PCR technique should clarify the involvement of HCV in cryptogenic hepatitis much earlier.

Exactly this idea was tested on a group of children with cryptogenic hepatitis in this study. In the present study molecular identification of HCV RNA allows us to diagnose the majority (57%) of children with cryptogenic hepatitis.

Table 1. Presence of HCV antibodies and HCV RNA in the blood of 33 children with cryptogenic hepatitis during the 2.5 years follow-up study

Time after diagnosis of hepatitis (months)	HCV Ab- HCV RNA+	HCV Ab+ HCV RNA+	HCV Ab- HCV RNA-
6	16[a]	0	17
12	9	7	17
18	9	7	17[b]
24	11[c]	8	14
30	11	8	14

[a]All these patients had also HCV RNA in the liver biopsies.
[b]One out of 17 HCV-RNA negative children (in the blood) had HCV RNA in the liver biopsies.
[c]Another 2 patients had HCV RNA in the blood as well as in the liver biopsies.
HCV Ab = HCV antibodies.

Materials and Methods

Patients. The study involved 33 children selected from 366 chronic hepatitis patients. An informed consent was obtained from parents of all the 33 children. The duration of the disease prior to testing and biopsy was 3–6 months. Blood samples have been collected from the patients in 3–6 months intervals during the 2.5 years follow-up.

ELISA of HCV antibodies. A third generation ELISA kit from Organon Teknika (Maniva *et al.*, 1997) containing microplates precoated with a mixture of structural and non-structural proteins was employed.

RT-PCR for HCV RNA and HGV RNA were performed by use of oligonucleotide primers included in the HCV RNA or HGV RNA detection kit (Roche or Boehringer, respectively). The primers were derived from the highly conservative 5'-untranslated region sequences of HCV or HGV genome as described in detail elsewhere (Chen *et al.*, 1997; Chung *et al.*, 1997; Mondeli and Silini, 1999; Young *et al.*, 1993). The HCV primers KY78 and KY80 yielded a PCR product of 244 nucleotides. The conditions for HCV PCR consisted of 1 cycle at 95°C for 5 mins, 35 cycles of denaturation at 95°C for 2 mins, annealing at 65°C for 2 mins, and elongation at 72°C for 3 mins, and 1 cycle of final extension at 72°C for 7 mins. The conditions for HGV PCR consisted of 1 cycle at 94°C for 5 mins, 35 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min, and elongation at 72°C for 2 mins, and 1 cycle of final extension at 72°C for 7 mins. The Boehringer HGV test uses the primers define a sequence of 373 nucleotides within the highly conserved 5'-untranslated region of HGV genome (Linen *et al.*, 1996).

Agarose gel electrophoresis. PCR products were subjected to 2.2% agarose (Serva) gel electrophoresis and stained with ethidium bromide (Merck) (1 µl 1% solution of ethidium bromide/100 ml agarose gel). A DNA size marker, 50 bp DNA Ladder (MBI FERMENTAS GeneRuler™) was employed.

Results and Discussion

The 33 patients under study were selected out of 366 children with hepatitis of various etiology. In these 33 cases the absence of specific IgM antibodies to the viruses which may cause hepatitis (HAV, HBV, HCV, hepatitis E virus (HEV), herpes simplex virus 1 and/or 2 (HSVs), human cytomegalovirus (HCMV), and Epstein-Barr virus (EBV)) as well as IgG antibody to HCV was assessed in the beginning of the study. Autoimmune hepatitis was excluded by absence of anti-smooth muscle antibodies and anti-nuclear antibodies, and lack of elevated serum IgG. In the 33 patients followed no signs of genetic diseases causing hepatitis (Wilson disease, alpha 1-antitrypsin deficiency) were found. No possible source of infection was found. All the patients did not have history of blood transfusion, hospitalization and surgical interventions. Also maternal status of HCV of the patients was negative. All the patients had fluctuating alanine transaminase (ALT) level, and only in some of them physical examination revealed hepatomegaly or hepatosplenomegaly.

None of the 33 patients had signs of primary or secondary immunodeficiency. It should be stressed that they all did not have HCV antibodies at the time of diagnosis and during next 6 months of observation. Test for HCV RNA was performed after 6 months of observation. Histological examination of liver biopsies documented different degree of chronic persistent hepatitis without signs of cirrhosis. All the children were well compensated. After 6 months from the diagnosis of cryptogenic hepatitis HCV-RNA was tested in all studied patients and has been detected in 16 children both in the blood and liver biopsies as well (Table 1). Specific HCV antibodies appeared during first 3 months in 1 patient and during subsequent 12 months in another 7 out of 16 HCV-RNA positive patients. During remaining 18 months HCV RNA was found only in 1 patient in the blood and liver biopsies, followed by seroconversion. In another 2 patients HCV-RNA was found only in the liver. It is worthwhile to mention that in 2.5 years these 2 patients did

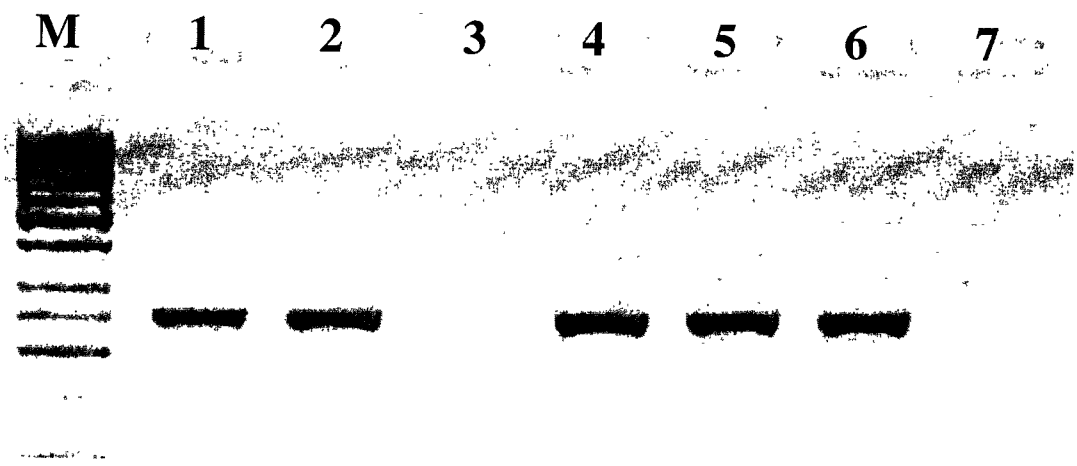

Fig. 1
Agarose gel electrophoresis of a RT-PCR product of HCV sequence from the serum of a child with cryptogenic hepatitis
DNA size marker, 50 bp ladder (lane M), serum from a child with cryptogenic hepatitis (lanes 1, 2, 4, 5), positive control (lane 6), negative control (lane 7).

not developed HCV antibodies. None of the 17 HCV RNA-positive children eliminated the virus from the blood during 2.5-years.

On the basis of the obtained results it may be concluded that the immune response to HCV as measured by specific antibodies production varied greatly. Among the patients under study seroconversion appeared after 3 to 18 months of observation. A delayed appearance of seroconversion can be explained by different immune response to HCV. It cannot be excluded that in remaining 11 HCV RNA-positive children (either in the blood or liver biopsies) seroconversion may take even longer time then 24 months due to various genetically determined immune response to HCV infection. It should be mentioned that a persistent or fluctuating appearance of HCV RNA or HCV antibodies is typical for hepatitis C (Alberti *et al.*, 1999; Fujisawa *et al.*, 1997).

An interesting finding concerning the age of the patients appeared after completing the study. The group of HCV RNA-positive patients was of significantly higher age than that of HCV RNA-negative patients (11.6 and 7.8 years, respectively; $P = 0.003$). Another observation revealed that HCV RNA-negative patients had a significantly higher ALT level as compared to the HCV RNA-positive patients (144.5 and 98.1 IU/l, respectively; $P = 0.0027$)

In order to assess the presence of another hepatotropic virus, HGV (Chen *et al.*, 1997; Chung *et al.*, 1997), molecular identification of HGV RNA in the blood was performed. However, the result was negative for all the patients under study.

It can be concluded that molecular identification of HCV RNA in the blood or liver biopsies allowed to diagnose hepatitis C in 19 out of 33 children with cryptogenic hepatitis. Our results demonstrated high rate of hepatitis C positivity in children with idiopathic chronic liver disease, especially when the viral RNA is looked for directly by using PCR technology. It should be added that the etiology of hepatitis in remaining 14 patients still awaits clarification.

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