The Influence of Occult Infection With Hepatitis B Virus on Liver Histology and Response to Interferon Treatment in Chronic Hepatitis C Patients

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Occult hepatitis B virus (HBV) infections have been identified in patients with chronic hepatitis C virus (HCV) infection, although the clinical relevance of occult HBV infection remains controversial. We searched for serum HBV DNA in 106 HBsAg negative/anti-HBc positive patients with chronic HCV infection and in 150 blood donors HBsAg negative/anti-HBc positive/anti-HCV negative (control group) by nested-PCR. HCV genotyping was done in 98 patients and percutaneous needle liver biopsies were performed in 59 patients. Fifty-two patients were treated for HCV infection with interferon alone (n=4) or combined with ribavirin (n=48) during one year. At the end and 24 weeks after stopping therapy, they were tested for HCV-RNA to evaluate the sustained virological response (SVR). Among the 106 HCV-positive patients, 15 (14%) were HBV-DNA positive and among the 150 HCV-negative blood donors, 6 (4%) were HBV-DNA positive. Liver biopsy gave a diagnosis of liver cirrhosis in 2/10 (20%) of the HBV-DNA positive patients and in 6/49 (12%) of the HBV-DNA negative patients. The degree of liver fibrosis and portal inflammation was similar in HCV-infected patients HBV-DNA, irrespective of HBV-DNA status. SVR was obtained in 37.5% of the HBV-DNA positive patients and in 20.5% of the HBV-DNA negative patients; this difference was not significant. In conclusion, these data suggested that occult HBV infection, which occurs at a relatively high frequency among Brazilian HCV-infected patients, was not associated with more severe grades of inflammation, liver fibrosis or cirrhosis development and did not affect the SVR rates when the patients were treated with interferon or with interferon plus ribavirin.

Key Words: Occult HBV infection, chronic HCV infection, HBV-DNA, PCR, HCV genotype, interferon treatment.

Hepatitis B virus (HBV) is an important human pathogen that causes both acute disease and chronic infection. Studies based on serological assays for HBV have led to the concept that HBV is eliminated after the resolution of an acute infection [1], although a chronic carrier state [2-4], defined as being positive for hepatitis B surface antigen (HBsAg) for at least six months [5-7], has been reported to occur in about 5% of affected adults [8]. During acute and chronic HBV infections, antibodies against the core antigen of HBV (anti-HBc) are found together with HBsAg, whereas in resolved infections they are accompanied by antibodies against HBsAg (anti-HBs). About 10-20% of all individuals with hepatitis B have anti-HBc as the only marker of HBV infection [9]. In about 10% of these individuals HBV-DNA can be detected by a PCR assay [9,10]. HBV infection may also occur in HBsAg-negative patients who have serological markers of previous infection (anti-HBs) [11]. These HBV infections in patients who lack detectable HBsAg are called occult infections [11]; the serum HBV-DNA levels are usually less than 10^4 copies/mL [12].

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Recently, very low levels of HBV-DNA have also been identified in the sera of patients with chronic hepatitis C virus (HCV) infection, even though there is no HBsAg in the serum [11,13,14-18]. It appears that this occult infection contributes to chronic liver damage and to the development of hepatocellular carcinoma [11,15,19-22]. Occult HBV infection can also be associated with a lack of response to interferon treatment in patients with chronic hepatitis C [11,18]. Recently, we used PCR to study the prevalence of occult HBV infection in serum samples of 106 patients with chronic HCV infection who were both HBsAg negative and anti-HBc positive. HBV-DNA was detected in 15 (14%) of them [23].

We evaluated how occult HBV infection influences epidemiological, laboratorial, histological and therapeutic features of Brazilian HCV-infected patients.

Material and Methods

Between March 2000 and May 2002, we selected 106 patients who had been infected by HCV (anti-HCV and HCV-RNA positive) and who were being followed-up at the University Hospital of UNICAMP. None of the patients had detectable HBsAg or were infected by human immunodeficiency virus (HIV). All of them had serum markers of previous HBV infection: all were anti-HBc positive and 56 (53%) were also anti-HBs positive. In the first phase these selected patients were tested for HBV-DNA by an "in house" nested-polymerase chain reaction (nested-PCR) [23]. To estimate the viral load of the samples determined positive for HBV-DNA by "in house" PCR, a commercial test for HBV-DNA (HBV Monitor Roche, Roche Diagnostic Systems, Branchburg, NJ), with a lower detection limit of 1,000 copies/mL, was used.

Based on the nested-PCR results the patients were divided into two groups: one group was HCV positive/HBV DNA positive (n=15) and the other was HCV positive/HBV DNA negative (n=91). The HCV genotype was determined in the serum of 98 patients, before initiating specific therapy for HVC infection.

Percutaneous needle biopsies of the liver were made in 59/106 patients, in order to evaluate the degree of histological lesion. All liver biopsy specimens were fixed, paraffin embedded, and stained with hematoxylin-eosin safranin and Masson trichrome for histological and immunohistochemical examination. A numerical score was calculated for each liver biopsy specimen, both for grading necroinflammatory activity and for determining the degree of fibrosis. The patients were divided into three categories according to the histological findings: those with minimal or non-specific changes, those with mild-to-moderate chronic hepatitis, and those with severe chronic hepatitis, with features of cirrhosis. The stage of fibrosis was determined according to METAVIR score and varied from 0 to 4 (F0 = no fibrosis; F1 = portal fibrosis without septa; F2 = portal fibrosis with few septa; F3 = septal fibrosis, without cirrhosis; F4 = cirrhosis) [11, 24, 25].

A control group to compare the epidemiological data was constituted of 150 blood donors who were HBsAg negative/ anti-HBc positive in blood bank screening. To identify occult HBV infection among these controls, we also tested these samples with "in house" nested-PCR [23], and we used a commercial test for HBV-DNA (HBV Monitor Roche, Roche Diagnostic Systems, Branchburg, NJ) with a lower detection limit of 1,000 copies/mL to estimate the viral load of these positive samples.

Based on PCR results the controls were subdivided into: one group that was HCV negative/HBV-DNA positive (n=6) and another group that was HCV negative/HBV-DNA negative (n=144).

All the HCV-infected and control individuals were interviewed with a questionnaire to investigate risk factors to acquire these viruses. This questionnaire included questions about age, sex, sexual contacts, transfusions of blood or derivatives, use of illicit drugs by intravenous routes, tattooing, and acupuncture.

At the end of the study, 52 patients had completed the treatment for HCV infection, and they were followed up for 24 weeks after ending therapy. These patients were treated with interferon a-2a 3 MU t.i.w, alone (n=4) or combined with ribavirin (1,000-1,200 mg/daily) (n=48) during one year. During the entire

follow-up period the ALT/AST levels were measured to evaluate the biochemical response. At the end of therapy, and 24 weeks later, the serum of these patients was tested for HVC-RNA. Patients who were HCV-RNA negative 24 weeks after the end of therapy, were considered to have a sustained virological response (SVR). Virological testing was done in accordance with the ethical guidelines of the authors´ institutions.

Serum markers for HBV, HIV and HCV infection

Serum HBsAg and anti-HBc were evaluated with commercial immunoenzymatic assays (Hepanostika HBsAg Uniform II and Hepanostika anti-HBc Uniform; Organon Teknika, Boxtel, Netherlands, respectively) and were tested for anti-HBs (AUSAB MEIA, Abbott Laboratories, North Chicago, IL), anti-HCV (anti-HCV assay version 3.0; Abbott Murex, Dartford, UK), and anti-HIV (anti-HIV1.2.O assay; Abbott Murex,Dartford, UK). HCV infection was defined as the presence of anti-HCV antibodies and HCV-RNA (HCV-AMPLICOR, Roche Diagnostic Systems, Branchburg, NJ) in the serum. The HCV genotype was determined by a commercial assay (INNO LIPA Innogenetics, Belgium) before starting interferon therapy. All the assays were performed according to manufacturers instructions.

Detection of Hepatitis B virus DNA in the serum

The nested-PCR method for detection of HBV DNA was performed essentially as described previously by KANEKO et al [26,27]. A 10 mL aliquot of serum was pipetted into a 0.2 mL micro centrifuge tube. The serum was then incubated with 2.5mL of 0.5M NaOH at 37^{o}C for 60 minutes. After incubation, the solution was brought to a neutral pH with 0.5M HCl, and the sample was used for PCR amplification of HBV DNA sequences. The samples were amplified in a 100mL reaction volume containing 2.5 U of Taq polymerase (Bethesda Research Laboratories, Life Technologies Inc., Gaithersburg, Maryland), 200mM each of the four deoxyribonucleic triphosphates, 1mM of the primer pair (primers 1763 5'-GCTTTGGGGCATGGACATTGACCCGTATAA-3' and 2032R 5'-CTGACTACTAATTCCCTGGATGCTGGGTCT-3'), 50mM KCl, 10mM Tris-HCl (pH 8.3), and 1.5 mM $MgCl_2$. The amplification routine was: 25 cycles; 1.5 minutes at 94^{o}C, 1.5 minutes at 42^{o}C and 3 minutes at 72^{o}C, in a DNA Thermal Cycler (Perkin Elmer Cetus, Foster City, Ca, USA). For reamplification, a 10mL aliquot of the primary PCR reaction was amplified as described above, using primer pair (1778-E 5'-GACGAATTCCATTGACCCGTATAAAGAATT-3' and 2017R-B 5'-ATGGGATCCCTGGATGCTGGGTCTTCCAAA-3'). An aliquot of water was used as a negative control and serum from an HBV positive individual according to EIA quantified with the Amplicor HBV Monitor test (Roche Diagnostic Systems, Branchburg, NJ) (1.0 X 10^2 copies/mL) was used as a positive control. Serial dilutions were made in an HBsAg low-titer performance panel (PHA 105; Boston Biomedica, Inc., Boston, Mass), with which we determined the detection limit of our nested PCR as being 10^2 copies/mL. Carryover contamination was prevented as described by KWOK& HIGUCHI [28]. The final amplification product was mixed with bromphenol blue, eletrophoresed in a 2% agarose gel, visualized by UV fluorescence after staining with ethidium bromide, and photographed with Polaroid film. Samples were considered positive if they yielded at least two positive results from three amplifications. Samples were considered negative when there were two negative results in two different reactions.

Statistical analysis

The student's t-test and the Mann-Whitney test were used to analyze quantitative data. Fischer's exact test was used to analyze quantitative data and for comparing proportions [29]. All calculated p values were two-tailed; a p value < 0.05 was considered significant.

Results

The demographic characteristics and risk factors for HCV infection are shown in Table 1. Among our 106 HCV-infected patients, 88 (83%) were male and 18 (17%) females. In the HBV-DNA positive group 11/88 (12.5%) were men and 4/18 (22%) women ($p>0.05$). The median age was 47.2 ± 9.7 years among HCV positive/HBV-DNA positive patients and was 46.4 ± 9.3 years among HCV positive/HBV-DNA negative patients ($p>0.05$). Among the 150 HCV-negative blood donors, 101 (67%) were male and 49 (33%) were females. HBV-DNA was found in 4/101 (4%) men and in 2/49 (4%) women ($p>0.05$). The median age was 33.8 ± 11.8 years in the HCV negative/HBV-DNA positive blood donors and was 39.8 ± 10.8 years in the HCV negative/HBV-DNA negative blood donors ($p>0.05$). There was no significant association of sex or age and HBV-DNA in either group.

In the analysis of risk factors, among the 106 HCV-infected patients, 34 (32%) were intravenous drug users (IVDU); 23 (21.7%) had received blood transfusion, 20 (18.9%) had surgery, 2 (1.9%) reported unsafe sexual contact, 2 (1.9%) reported more than 1 risk factor (tattoo and acupuncture), and 25/106 (23.6%) denied risk factors. Among the 150 HCV-negative/anti-HBc positive blood donors 8 (5.3%) were IVDU; 17(11.3%) had received a blood transfusion, 1 (0.7%) had surgeries, 46 (30.7%) indicated sexual contact as a risk factor, 10 (6.7%) reported more than 1 risk factor and 68 (45.3%) denied any risk factors for HCV infection. No significant association was found between risk factors and HBV-DNA presence.

Among the blood donors, 5.3% were IVDU, while 32% of the HCV-positive group were IVDU ($p<0.05$). Unsafe sexual practice was reported by 1.9% of the HCV-positive group and in 30.7% of the blood donors ($p<0.05$). When all sub-groups (HBV-DNA negative or positive) were compared, significant differences among risk groups were found for virus acquisition, mainly because there were more IVDU in the HCV-positive group, with or without occult HVB infection, when compared with the group of blood donors who were HBV-DNA negative (Table 1).

We were able to determine the HCV genotype in 98 HCV-positive patients. Genotype 1 was encountered in 62/98 (63%), genotype 2 in 1/98 (1%), genotype 3 in 32/98 (33%) and genotype 1 and 3 in 3/98 (3%). The frequencies of the different HCV genotypes were similar in HCV-positive patients with and without occult HBV infection ($p>0.05$, Table 2).

Liver biopsies were taken from 59 HCV-positive patients. Among them 2 (3%) presented minimal changes, 49 (83%) had chronic hepatitis and 8 (14%) had liver cirrhosis. Liver cirrhosis was diagnosed in 2/10 (20%) of the HBV-DNA positive patients and in 6/49 (12%) of the HBV-DNA negative patients ($p>0.05$). HBV-DNA presence was not significantly associated with more advanced histological lesions in the liver in HCV-positive patients ($p>0.05$, Table 2). Liver fibrosis and portal inflammation were found at similar rates in HCV-infected patients with or without HBV-DNA positive in serum ($p>0.05$, Table 3). Immunohistochemical staining was negative for HBsAg and HBcAg in all liver biopsies from HCV positive/HBV-DNA positive patients. Fifty-two HCV-positive patients were treated with interferon ($n = 4$) or with a combination of interferon plus ribavirin ($n = 48$) during 48 weeks. No significant differences were found in the rates of biochemical or virological responses in the HCV-infected patients with or without HBV-DNA in the sera ($p>0.05$, Table 4).

Discussion

Initially, we looked for occult HBV infection in HBsAg negative/anti-HBc positive individuals, with and without chronic HCV infection. This population of HCV-infected patients and blood donors is representative of the general population in our region. We found that 14% of the HCV-infected patients had detectable HBV genome in their serum. This prevalence was significantly higher than that found among HCV-negative individuals (4%) [23]. The reasons for the disappearance of HBsAg and, in some cases, of all HBV markers, despite the persistence of HBV infection, are not completely understood. Some researchers consider that HBV could be present at very low levels,

Table 1. Characteristics and risk factors in hepatitis C virus (HCV)-infected patients divided according to hepatitis B virus (HBV)-DNA positivity

Characteristic	**HCV (+) / anti-HBc (+)**						**HCV (-) / anti-HBc (+)**					
	HBV-DNA (+) n=15		HBV-DNA (-) n =91		Total n =106		HBV-DNA (+) n =6		HBV-DNA (-) n =144		Total n =150	
Mean age (yr)	47.2 ± 9.7		46.4 ± 9.3		46.8 ± 9.5		33.8 ± 11.8		39.8 ± 10.8		36.8 ± 11.3	
Sex (M/F)	11/4		77/14		88/18		4/2		97/47		101/49	
Risk Factors	**n**	**(%)**	**n**	**(%)**	**n**	**(%)**	**n**	**(%)**	**n**	**(%)**	**n**	**(%)**
IVDU	4/15	(26.7)	30/91	(33)	34/106	(32)	1/69	(16.7)	7/144	(4.9)	8/150	(5.3)
Transfusion	3/15	(20)	20/91	(22)	23/106	(21.7)	-	-	17/144	(11.8)	17/150	(11.3)
Surgery	4/15	(26.7)	16/91	(17.6)	20/106	(18.9)	-	-	1/144	(0.7)	1/150	(0.7)
Unsafe sexual practices	-	-	2/91	(2.2)	2/106	(1.9)	1/6	(16.7)	45/144	(31.2)	46/150	(30.7)
Not determined	4/15	(26.6)	21/91	(23)	25/106	(23.6)	4/6	(66.6)	64/144	(44.5)	68/150	(45.3)
Others*	-	-	2/91	(2.2)	2/106	(1.9)	-	-	10/144**	(6.9)	10/150	(6.7)

*Tattoo/acupuncture. **Transfusion/Sexual. IVDU: Intravenous drug use. p>0.05 for comparison of HBV-DNA(+)/HBV-DNA(-) patients in both groups. P<0.05 when all sub-groups (HBV-DNA negative or positive) are compared.

Table 2. Hepatitis C virus (HCV) genotypes and histological diagnosis in HCV-positive patients, divided according to HBV-DNA positivity in the serum

Variable	**HBV DNA (+) n=14**		**HBV DNA (-) n=84**	
Genotype	**n**	**(%)**	**n**	**(%)**
1	9	(64)	53	(63)
2	-	-	1	(1.2)
3	5	(36)	27	(32)
1+3	-	-	3	(3.6)
	HBV DNA (+) n=10		**HBV DNA (-) n=49**	
Histological diagnosis	**n**	**(%)**	**n**	**(%)**
Minimal changes	1	(10)	1	(2)
Chronic hepatitis (mild/severe)	7	(70)	42	(86)
Cirrhosis	2	(20)	6	(12.3)

P>0.05 for comparison of patients HBV-DNA(+)/HBV-DNA(-).

Table 3.Histopatological alterations observed in liver biopsies from 59 patients with chronic hepatitis C

Liver histology	**HBV-DNA (+) n=10**		**HBV-DNA (-) n=49**	
Fibrosis	**n**	**(%)**	**n**	**(%)**
No fibrosis (F0)	1	(10)	4	(8.2)
Portal fibrosis without septa (F1)	2	(20)	15	(31)
Portal fibrosis with few septa (F2)	3	(30)	15	(31)
Septal fibrosis without cirrhosis	2	(20)	9	(18)
Cirrhosis	2	(20)	6	(12)
Portal inflammation	**n**	**(%)**	**n**	**(%)**
None	1	(10)	-	-
Mild	5	(50)	27	(55)
Moderate	4	(40)	19	(39)
Severe	-	-	3	(6)

HBV: Hepatitis B virus. P>0.05 for comparison of patients HBV-DNA(+)/HBV-DNA(-).

Table 4. Response to interferon treatment in patients with chronic hepatitis C virus infection, with and without occult hepatitis B virus (HBV) infection

Response to treatment	**HBV-DNA (+) n=44**		**HBV-DNA (-)**	
	n	**%**	**n**	**%**
Sustained virological response	3	(37)	9	(21)
Biochemical response	1	(12)	11	(25)
Non-response	4	(50)	24	(55)

P>0.05 for comparison of patients HBV-DNA(+)/HBV-DNA(-).

undetectable by conventional serological assays. The HBsAg could elude detection by the polyclonal antibody used in conventional assays in the presence of HBsAg or HBsAg/anti-HBs immune complexes [30]. It is also possible that virions that are not transcriptionally active or replicating, quiescent or latent virions, are able to evade the surveillance of host immune cells [31]. Hepatocytes could be repeatedly re-infected by HBV virions, which could persist independently in peripheral blood mononuclear cells, or in other lymphoid tissues [10, 32]. We found that HBV-DNA persisted at low levels in patients with apparently resolved HBV infection. We utilized a highly sensitive in-house PCR test with a detection limit of 100 copies/mL [23]. The positive samples were tested afterwards with a commercial test that had a detection limit of 1,000 copies/mL, and all were negative. Thus, our HBV-DNA-positive patients, and the blood donors of the control group, had more than 100 and less than 1,000 copies/mL, which indicates that low levels of HBV-DNA are generally present in the sera of patients with occult HBV infection.

In co-infected patients, HBV and HCV may have a low rate of replication, due to mutual interference between these viruses [8,33-37). Some researchers believe that HCV has a strong suppressive effect on HVB replication [13,33,38-42], while others attribute to HBV the dominant role as an inhibitor of replication. [33 - 43]. It seems that inhibition of HBV replication by HCV is more common than the opposite [44].

Occult HBV infection in HCV-infected patients has been suggested as a possible co-factor, responsible for both impaired response to interferon therapy [13,18] and more severe liver disease [11,17,45,46].

In Italy, Cacciola et al., in a study of HCV-positive patients who were HBsAg negative/anti-HBc positive, were able to detect HBV-DNA in 46% of them. Liver cirrhosis was diagnosed in 33% of the HCV-infected patients who were HBV-DNA positive and in 19% of the HBV-DNA negative HCV-infected patients [11]. They hypothesized that occult HBV infection could favor or accelerate the evolution to cirrhosis in HCV-positive patients [11,40]. Since cirrhosis is the most important risk factor for the development of hepatocellular carcinoma, a relation between HBV occult infection and this complication should be considered.

We found liver cirrhosis in 20% of the HBV-DNA-positive patients and in 12% of the HBV-DNA negative patients. However, there was no significant difference between these two groups. Among our HCV-infected patients, the histopathological lesions in the liver were not more intense than in patients without occult HBV infection. Other researchers [35,46-48] also observed that occult HBV infection in HCV-infected patients does not seem to be associated with more severe liver disease. On the other hand, some researchers indicated that anti-HBc (previous HBV infection), rather than occult HBV infection, is associated with a worse outcome in HCV-infected patients [35,46].

Differences among the populations, different sensitivities of the assays used to detect HBV-DNA, different types of specimen used to detect the presence of HBV (serum or liver) and different prevalences of HBV infection in the general population of various countries, could explain the conflicting results. We were not able to detect liver tissue markers (HBsAg and HBcAg) in the HBV-DNA positive patients by immunoperoxidase staining. The same was observed in Italian patients [11]. We consider that this could be due to the very low HBV-DNA titers in the livers of the patients, the low intrinsic sensitivity of the immunohistochemical method, or because the HCV-infected patients with occult HBV infection had only residual replication outside of the liver.

Among oriental patients with occult HBV infection, it was observed that they are more frequently infected with HCV genotype 1b [13]. We did not find a significant difference in the frequencies of HCV genotypes in HCV-infected patients with or without HBV-DNA. The same was observed for similar HCV-positive patients in Italy [46].

A reduced response to interferon therapy has been described in individuals with chronic hepatitis C and serological markers of a resolved HBV infection (HBsAg negative/anti-HBc positive), perhaps due to an inapparent HBV co-infection in these patients [18]. More recently, some authors have observed that occult HBV infection correlates with a lack of response to interferon treatment in patients with chronic hepatitis C [11,18,45]. Cacciola et al. detected a virological response in 21% and 42% of HCV-positive patients who had an occult infection with HBV or not, respectively [11]. We did not find a significant difference in the virological response of HCV-positive patients, with or without occult HBV infection (37.5% and 20.5% respectively), when they were treated with interferon alone, or associated with ribavirin. The low levels of HBV-DNA in our patients did not interfere in the response to therapy. The same was observed in a recent study made in Taiwan with 110 patients who had chronic HCV infection. The prevalence of occult HBV infection did not parallel the severity of liver disease, and the sustained response to combination therapy against HCV was comparable between patients with or without occult HBV infection (38 versus 39%). These researchers concluded that occult HBV infection had no clinical significance in chronic hepatitis C [47].

Finally, we emphasize the high prevalence of occult HBV infection (14%) among our Brazilian patients with chronic hepatitis C who were HBsAg negative/ anti-

HBc positive when compared with the prevalence observed in HBsAg negative/anti-HBc positive/HCV negative blood donors (5.4%). We did not find an association among the risk factors to acquire both B and C viruses with higher rates of occult HBV infection. No significant association was found between more severe grades of liver fibrosis or cirrhosis in HCV-infected patients and HBV-DNA in sera. The SRV rates obtained with interferon therapy in HCV-positive patients apparently were not affected by occult HBV infection. New studies should be made to determine if occult HBV infection, with low levels of HBV-DNA, can accelerate the natural course of chronic hepatitis C and influence the response to therapy.

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