

OCCULT HEPATITIS B VIRUS INFECTION IN PATIENTS WITH CHRONIC HEPATITIS C LIVER DISEASE

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ABSTRACT

Background Hepatitis B virus (HBV) infections in patients who lack detectable hepatitis B surface antigen (HBsAg) are called occult infections. Although such infections have been identified in patients with chronic hepatitis C liver disease, their prevalence and clinical significance are not known.

Methods With the polymerase chain reaction, we searched for HBV DNA in liver and serum samples from 200 HBsAg-negative patients with hepatitis C virus (HCV)-related liver disease (147 with chronic hepatitis, 48 with cirrhosis, and 5 with minimal histologic changes). One hundred of the patients had detectable antibodies to the HBV core antigen (anti-HBc); 100 were negative for all HBV markers. Eighty-three were treated with interferon alfa. We also studied 50 patients with liver disease who were negative both for HBsAg and for HCV markers. In six patients found to have occult HBV infection, we evaluated possible genomic rearrangements through cloning or direct sequencing procedures.

Results Sixty-six of the 200 patients with chronic hepatitis C liver disease (33 percent) had HBV sequences, as did 7 of the 50 patients with liver disease unrelated to hepatitis C (14 percent, $P=0.01$). Among the 66 patients, 46 were anti-HBc-positive and 20 were negative for all HBV markers ($P<0.001$). Twenty-two of these 66 patients (33 percent) had cirrhosis, as compared with 26 of the 134 patients with hepatitis C infection but no HBV sequences (19 percent, $P=0.04$). HBV sequences were detected in 26 of the 55 patients in whom interferon therapy was ineffective and 7 of the 28 patients in whom interferon therapy was effective ($P=0.06$). None of the sequenced HBV genomes had changes known to interfere with viral activity and gene expression.

Conclusions Occult hepatitis B infection occurs frequently in patients with chronic hepatitis C liver disease and may have clinical significance. (N Engl J Med 1999;341:22-6.)

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HEPATITIS B virus (HBV) and hepatitis C virus (HCV) infections account for a substantial proportion of liver disease worldwide. HCV is an RNA virus of the Flaviviridae family. HBV is a DNA virus of the Hepadnaviridae family. It contains four open reading frames: the *S* gene, coding for the envelope proteins; the core gene, coding for the core and "e" proteins; the *P* gene, coding for a DNA polymerase; and the *X* gene, coding for a transcriptional transactivator.

HBV and HCV are both transmitted through the blood and by sexual contact. Infection with both viruses is frequent, particularly in areas where the two viruses are endemic and among people at high risk for parenteral infections.¹⁻⁵ HCV infection is diagnosed by the detection of specific antibodies and viral RNA in the serum. HBV infection is usually diagnosed when circulating hepatitis B surface antigen (HBsAg) is identified.

Many studies have shown that HBV infection may occur in HBsAg-negative patients with or without serologic markers of previous infection (antibodies to HBsAg [anti-HBs] or to the hepatitis B core antigen [anti-HBc]).⁶⁻⁸ The reasons for the lack of circulating HBsAg in such patients are unknown. Recent observations have suggested that the lack of HBsAg may be due to rearrangements in the HBV genome that interfere with gene expression or lead to the production of an antigenically modified S protein.⁹⁻¹¹ Occult HBV infection has frequently been identified in patients with HCV-related chronic hepatitis. Considerable data suggest that this occult infection may contribute to chronic liver damage and the development of hepatocellular carcinoma.¹²⁻¹⁷ Despite its potential clinical importance, the prevalence of occult HBV infection in patients with hepatitis C is still undetermined.

We analyzed the prevalence of occult HBV infection in patients with chronic hepatitis C. Our goals were to determine whether the failure to detect HBsAg is related to viral genomic variability and to evaluate the possible relation between occult HBV infection and the clinical outcome of the liver disease.

METHODS

Patients

Between January 1991 and June 1997 at our institution, percutaneous needle biopsy of the liver was performed in 396 patients who had HCV-related chronic liver disease, had no detectable HBsAg, did not drink alcohol to excess or use intravenous drugs, and were not infected with the human immunodeficiency virus. HCV infection was defined by the presence of anti-HCV antibodies and HCV RNA in serum.¹⁸ One hundred forty of these patients had serum markers of previous HBV infection; all were anti-HBc-positive, and 112 were also anti-HBs-positive. The remaining 256 were negative for all HBV markers.

During the same period, 50 patients with liver disease and no detectable HBsAg or HCV markers underwent liver biopsy (Table

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1). Part of each liver specimen was processed for histologic and possible immunohistochemical examination; the rest was immediately frozen and stored at -80°C for subsequent molecular analyses. Serum samples were collected from each patient and frozen for further studies.

We analyzed specimens from 200 HCV-infected patients (the HCV-positive group) and all 50 patients who were negative for HBsAg and HCV markers (the HCV-negative group) (Table 1). The HCV-positive group consisted of consecutively admitted patients, 100 of whom were anti-HBc-positive (the HCV-positive and anti-HBc-positive subgroup) and 100 of whom were negative for HBV markers (the HCV-positive and anti-HBc-negative subgroup). According to the histologic findings, the patients were divided into three categories: those with minimal or non-specific changes, those with mild-to-moderate chronic hepatitis, and those with severe chronic hepatitis with features of cirrhosis.

Three HCV-positive and anti-HBc-positive patients had a documented history of self-limited acute hepatitis B (one patient) or chronic hepatitis B (two patients). The liver specimens we analyzed were obtained from these patients five, four, and eight years, respectively, after the clearance of serum HBsAg. Retrospective analysis of their stored serum samples showed detectable anti-HCV antibodies from the beginning of the follow-up.

None of the patients we studied had been treated with antiviral or immunosuppressive drugs before undergoing liver biopsy. Subsequently, 83 patients with HCV infection (65 with chronic hepatitis and 18 with cirrhosis; 28 from the HCV-positive and anti-HBc-positive subgroup and 55 from the HCV-positive and anti-HBc-negative subgroup) were treated with 3 million to 6 million units of interferon alfa three times a week for four to six months, and if the results of liver-function tests became normal, with 3 million units three times a week for another six to eight months. In 28 of these patients (7 who were anti-HBc-positive and 21 who were anti-HBc-negative), the therapy was considered successful, since they had normal results on tests of liver function and had no detectable HCV RNA for at least six months after stopping therapy.

The study protocol was approved by the ethics committee of the University of Messina, and all the patients provided written informed consent.

HBV DNA Analyses

DNA was extracted from the frozen liver specimen of each patient by standard procedures.¹⁹ All liver DNA extracts were analyzed for HBV genomes with two different polymerase chain reaction (PCR) assays to detect the S and core genes, according to previously described methods.²⁰ Briefly, 100 µl of reaction mixture containing 10 µl of extracted DNA, 50 mM potassium chloride, 10 mM TRIS-hydrochloric acid (pH 8.3), 2 mM magnesium chloride, 200 µM deoxyribonucleosides, 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), and 20 pmol each of the oligonucleotide primers HBV1 and HBV2 for the S gene and primers HBV3 and HBV4 for the core gene was overlaid with 100 µl of mineral oil. Amplification was performed for 35 cycles, each consisting of denaturing for 30 seconds at 94°C, annealing for 45 seconds at 56°C, and extension for 1.5 minutes at 72°C. The amplification products were visualized on an ethidium bromide-stained 1 percent agarose gel. When no amplification product was seen on the gel, a second round of nested PCR amplification was performed with 10 µl from the product of the first reaction and the inner primers HBV5 and HBV6 for the S region and HBV7 and HBV8 for the core region.²¹

The patients who were found positive for only one of the two regions examined were retested by single-step or nested PCR procedures with the use of sets of oligonucleotide primers (HBV9 and HBV10, and HBV11 and HBV12) encompassing the viral X gene. They were considered to be definitely infected or uninfected with HBV according to the results of this additional test. The primers used were complementary to conserved regions of HBV genotype D at the following positions (from 5' to 3'): HBV1, 61-81; HBV2, 1004-985; HBV3, 1778-1800; HBV4,

TABLE 1. BASE-LINE CHARACTERISTICS OF THE PATIENTS.*

CHARACTERISTIC	HCV-POSITIVE		HCV-NEGATIVE (N=50)†
	ANTI-HBc-POSITIVE (N=100)	ANTI-HBc-NEGATIVE (N=100)	
Mean age (yr)	47±14	44±12	45±13
Sex (M/F)	67/33	61/39	32/18
Histologic diagnosis (no. of patients)			
Minimal changes	1	4	9
Chronic hepatitis	75	72	26
Cirrhosis	24	24	15

*Plus-minus values are means ±SD.

†Seven patients were positive and 43 were negative for HBV antibodies. The cause of the liver disease in these 50 patients was primary biliary cirrhosis in 5, hemochromatosis in 4, alcohol in 8, drugs in 5, and unknown in 28.

2483-2464; HBV5, 154-174; HBV6, 839-819; HBV7, 1928-1946; HBV8, 2391-2372; HBV9, 968-986; HBV10, 1951-1931; HBV11, 1266-1283; and HBV12, 1804-1784. The limits of sensitivity of our single-step and nested PCR methods were 1×10⁻³ pg and 1×10⁻⁶ pg of cloned HBV DNA, respectively. Direct sequencing of randomly chosen amplification products showed different sequences between patients, which confirmed the specificity of the reactions.²⁰

DNA was also extracted from serum samples from all the patients with intrahepatic HBV genomes and from 35 additional patients randomly selected from among those who were negative for intrahepatic viral DNA. The entire S gene of HBV isolated from three patients with occult infection who were randomly chosen from the HCV-positive group was amplified with the use of two hybrid oligonucleotide primers, cloned into a pUC19 vector, and sequenced by previously described procedures.^{22,23} Further studies in these patients included direct sequencing of the entire core gene of HBV isolates and testing for HBsAg and hepatitis B core antigen (HBcAg) in liver sections by immunohistochemical methods.¹⁹ The three patients who had been HBsAg-positive five to eight years before this study had occult HBV infection. The entire HBV genome from two of these patients was amplified and sequenced.²⁴ In the other, the amount of DNA was just sufficient for analysis of the entire S, core, and X viral genes. Liver sections from these three patients were also tested for HBsAg and HBcAg.

The HBV sequences we obtained were aligned and compared with those in the National Center for Biotechnology Information data bank.

HCV RNA Genotyping and Quantification

The HCV genotype was determined in 62 of the 83 subjects treated with interferon alfa and in 38 additional patients randomly selected from those with or without occult HBV infection (HCV Genotyping, Sorin-Biomedica, Saluggia, Italy). We also evaluated the levels of HCV virus in nine randomly chosen patients with occult HBV infection and nine without occult HBV infection (Amplicor-HCV-Monitor, Roche, Basel, Switzerland).

Statistical Analysis

Student's t-test and the Mann-Whitney test were used to analyze quantitative data. Fisher's exact test was used to analyze qualitative data and for comparing proportions.^{25,26} All P values are two-tailed; a P value <0.05 was considered to indicate statistical significance.

RESULTS

Intrahepatic HBV sequences were detected in 73 of the 250 specimens examined. In 64 patients both core and *S* genes were detected, whereas in 9 patients the *X* gene besides the *S* (4 patients) or core (5 patients) region was amplified. HBV genomes were found in the serum of 45 of the 73 patients with intrahepatic HBV and in none of the 35 patients without intrahepatic HBV. Liver HBV DNA was detected after single-step PCR amplification in 35 patients and after nested PCR in 38 patients. Serum HBV DNA was found in all patients by nested PCR. HBV sequences were found in liver tissue from 66 of the 200 HCV-infected patients (33 percent) and in 7 of the 50 HCV-negative patients (14 percent, $P=0.01$) (Table 2). Forty-six patients in the subgroup that was HCV-positive and anti-HBc-positive and 20 of those in the subgroup that was HCV-positive and anti-HBc-negative were HBV-positive ($P<0.001$) (Table 2). Six of the seven HCV-negative patients with occult HBV infection had cryptogenic liver disease, and one had hemochromatosis. The prevalence of occult HBV infection among HCV-infected patients did not differ with sex or age, except that among patients with cirrhosis, those with HBV tended to be younger than those without HBV (51 ± 10 vs. 56 ± 7 years, $P=0.06$). The prevalence of the different HCV genotypes was similar in patients with and those without occult HBV infection (Table 3). Quantification of serum HCV RNA showed very similar viral levels in patients with occult infection and those without such infection (Table 3).

Twenty-two of the 66 patients with HCV infec-

tion and occult HBV infection (33 percent) had cirrhosis, as compared with 26 of the 134 patients with HCV infection and no occult HBV infection (19 percent, $P=0.04$) (Table 3). There was no significant association between occult HBV infection and chronic hepatitis ($P=0.13$). HBV sequences were detected in 26 of 55 patients in whom interferon therapy was unsuccessful and in 7 of 28 patients in whom the therapy was successful ($P=0.06$) (Table 4). By separately evaluating the 65 patients with chronic hepatitis and the 18 with cirrhosis, we found a trend toward an association between occult HBV infection and a lack of response to interferon therapy in the patients with chronic hepatitis ($P=0.07$) but not in those with cirrhosis (Table 4). The relation of occult HBV infection to both cirrhosis and a lack of response to interferon therapy was not significantly affected by sex ($P=0.8$ and $P=0.4$, respectively), age ($P=0.06$ and $P=0.7$, respectively), or infecting HCV genotype ($P=0.7$ and $P=0.6$, respectively).

The entire *S* gene of the HBV isolated from three patients with occult infection was amplified and cloned. The nucleotide-sequence analysis of five clones for each patient showed that the viral populations infecting each were genetically almost identical and were homologous with published genotype D prototypes. Consequently, we did not detect changes capable of preventing the synthesis or modifying the antigenic structure of HBsAg. Similarly, the direct sequencing of the core gene of HBV from these three patients showed very few irrelevant mutations in each (data not shown). HBsAg and HBcAg were not detected in liver tissue.

We considered it essential to sequence the entire HBV genome in order to verify whether specific mutations were associated with occult infection.²⁷ We extensively analyzed HBV genomes from three subjects with documented histories of acute or chronic hepatitis B. The sequencing analyses showed that two of them were infected by genotype D1 and one by genotype D5. No genomic changes known to be able to interfere with viral activity were detected. No HBsAg or HBcAg was immunohistochemically detected in their liver-biopsy specimens.

DISCUSSION

We investigated HBV infection in HBsAg-negative patients with chronic hepatitis C. We found that one third of the patients with HCV-related chronic hepatitis had detectable HBV genomes, despite the absence of circulating HBsAg. This prevalence was significantly higher than that among HCV-negative patients with chronic liver disease. The prevalence of occult HBV infection was particularly high among patients with anti-HBV antibodies. Occult HBV infection was also detected in patients who were negative for all HBV serum markers. The reasons for the disappearance of HBsAg and, in some cases, of all

TABLE 2. PREVALENCE OF OCCULT HBV INFECTION IN PATIENTS WITH AND WITHOUT CHRONIC HCV INFECTION AND WITH AND WITHOUT ANTIBODIES TO THE HBV CORE ANTIGEN (ANTI-HBc).

PATIENT STATUS	HBV-POSITIVE PATIENTS/TOTAL
HCV-positive	
Anti-HBc-positive	46/100*
Anti-HBc-negative	20/100
Total	66/200†
HCV-negative	
Anti-HBc-positive	2/7‡
Anti-HBc-negative	5/43
Total	7/50

* $P<0.001$ for the comparison with the subgroup of patients who were HCV-positive and anti-HBc-negative.

† $P=0.01$ for the comparison with the HCV-negative group. *P* values were calculated by Fisher's exact test.

‡ $P=0.25$ for the comparison with the subgroup of patients who were HCV-negative and anti-HBc-negative. *P* values were calculated by Fisher's exact test.

TABLE 3. CHARACTERISTICS OF THE PATIENTS WITH CHRONIC HCV INFECTION, ACCORDING TO THE PRESENCE OR ABSENCE OF OCCULT HBV INFECTION.

VARIABLE	HBV-POSITIVE (N=66)	HBV-NEGATIVE (N=134)
Sex — no. (%)		
Male	44 (33)	84 (67)
Female	22 (31)	50 (69)
Mean (±SD) age — yr	44±14	45±13
Histologic findings		
Minimal changes	0*	5
Chronic hepatitis	44*	103
Cirrhosis	22*	26
HCV RNA — copies/ml†	362±197	347±153
Genotype‡		
1a	5	1
1b	20	30
2a, b, c	11	18
3	9	6

*P=0.04 for the comparison with the HBV-negative group. P values were calculated by Fisher's exact test.

†HCV virus levels were evaluated in 18 samples: 9 from HBV-positive patients and 9 from HBV-negative patients.

‡HCV genotypes were determined in 100 patients: 45 were HBV-positive, and 55 were HBV-negative.

TABLE 4. RESPONSE TO INTERFERON TREATMENT IN PATIENTS WITH CHRONIC HCV LIVER DISEASE WITH OR WITHOUT OCCULT HBV INFECTION.

CHARACTERISTIC	HBV-POSITIVE (N=33)	HBV-NEGATIVE (N=50)
Response to treatment	7*	21
Chronic hepatitis	6†	21
Cirrhosis	1	0
No response to treatment	26*	29
Chronic hepatitis	17†	21
Cirrhosis	9	8

*P=0.06 for the comparison with the HBV-negative group. P values were calculated by Fisher's exact test.

†P=0.07 for the comparison with HBV-negative patients with chronic hepatitis. P values were calculated by Fisher's exact test.

HBV markers despite the persistence of HBV infection are not known. Recent reports suggest that rearrangements of the viral genome, particularly in the S gene, may be responsible for the failure to detect HBsAg.⁹⁻¹¹ We cloned and sequenced the S region of three isolates and directly sequenced the entire genome from three more patients; no mutation known to be capable of interfering with viral activity or gene expression was detected. These findings support the view that variability of the virus is not a major reason that HBV infection can be occult.

In agreement with most reports,^{6-9,12,16,27} we found

very low levels of viremia in patients with occult HBV infection. Serum HBV DNA was detected with the very sensitive nested PCR technique in just 45 of the 73 subjects with intrahepatic viral genomes. Immunoperoxidase staining for HBV surface and core proteins was negative in all the liver-biopsy specimens examined, although molecular analyses showed that the corresponding viral genes in these specimens were normal. Together, these data suggest that occult HBV infection is usually due to a strong suppression of viral replication and gene expression. The mechanisms responsible for the inhibition of HBV activity are undefined. Much evidence suggests that the immune system may have a key role.²⁸⁻³³ In patients with HCV coinfection, it is also possible that this virus may suppress HBV activity.^{2,3,34-36}

HBV particles may persist for decades after self-limited acute hepatitis and clinical recovery.^{28,37,38} Thus, occult infection alone may not have clinical consequences and may become injurious only when the virus is reactivated after immunosuppression.²⁹⁻³³ Nevertheless, HBV genomes have also been found in patients with idiopathic liver disease.^{12,17} In our study six of the seven patients without HBsAg or markers of HCV and with occult HBV had a diagnosis of cryptogenic chronic hepatitis or cirrhosis. Such evidence might lead to speculation about a possible pathogenic role of HBV in liver injury, despite the suppression of its activity. However, other yet-to-be-identified factors (including unknown viruses) may be the main cause of liver damage in such patients.

Many epidemiologic and molecular studies indicate that persistent HBV infection may have a critical role in the development of hepatocellular carcinoma in HBsAg-negative patients.^{13-15,17,39-41} This hypothesis is supported by studies showing that both woodchucks and ground squirrels that have once been infected by woodchuck hepatitis virus and ground-squirrel hepatitis virus, respectively, are at high risk for hepatocellular carcinoma even after the apparent clearance of the virus.^{42,43} Occult HBV and its potential oncogenicity are traditionally considered a consequence of the capacity of the virus to be integrated into the host genome, although many observations show that free episomal HBV genomes may persist in the liver cells during occult infection.^{7,8}

Our study demonstrates that occult HBV infection is significantly correlated with cirrhosis among HCV-infected patients. This suggests that a masked HBV infection may interfere with the clinical outcome of chronic hepatitis C and favor or accelerate the evolution to cirrhosis. Cirrhosis is generally considered the most important risk factor for the development of hepatocellular carcinoma. Thus, in addition to its possible direct oncogenic properties, occult HBV infection may favor neoplastic transformation in HCV-infected patients through its contribution to cirrhosis.

A recent study showed that in HCV-infected patients with chronic active hepatitis and Child's class A cirrhosis, interferon therapy, as compared with no treatment, reduced the risk of hepatocellular carcinoma by a factor of more than six only in patients who were negative for all serum HBV markers.⁴⁴ Our results, by suggesting that occult HBV infection correlates with a lack of response to interferon treatment in patients with chronic hepatitis C, are in agreement with these findings⁴⁴ and with previous data obtained in a study of 14 patients.⁴⁵

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