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Spontaneous hepatitis B surface antigen clearance in a long-term follow-up study of patients with chronic type B hepatitis. Lack of correlation with hepatitis C and D virus superinfection

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Abstract: We investigated the frequency of HBsAg clearance and the possible role of viral superinfection in a long-term follow-up of 184 patients with chronic hepatitis B (CHB). Our subjects were 184 patients with chronic hepatitis B and the follow-up was 12–216 months (mean 66.2 ± 53.7 months). The investigative methods used were: immunoenzymatic assays for HBV, HCV, HDV, and HIV markers; polymerase chain reaction (PCR) for HBV DNA; and liver biopsy and immunoperoxidase. During the follow-up, 20 of the 184 patients cleared serum HBsAg. A comparison of patients with persistent HBsAg (group I) and of those who cleared this marker (group II) showed a significant difference in mortality ($P = 0.002$) between the two groups and a tendency to a more severe exacerbation (flare) in group II ($P = 0.07$). Antibodies to hepatitis C and D virus as well as antibodies to HIV were equally distributed in both groups. Thirteen patients (7.9%) from group I, but none from group II, subsequently developed hepatocellular carcinoma. These results suggest that the frequency of spontaneous clearance of HBsAg during chronic HBV infection is low. No determinant factor for the clearance was found, including the presence of liver cirrhosis. Serum HBV DNA was undetectable by PCR after clearance in 16 out of 17 patients.

Key words: hepatitis B, chronic hepatitis B, spontaneous HBsAg clearance

Introduction

During the natural history of chronic hepatitis B (CHB), seroconversion from HBeAg to anti-HBe is frequently observed at the same time as or preceded by a flare of the hepatitis.[1] After this seroconversion, HBV DNA may be absent or may be found in small amounts in serum, particularly by the polymerase chain reaction (PCR) technique. Despite the frequency of this seroconversion, the spontaneous clearance of HBsAg is considered to be an unusual event.[2] In a study of 204 blood samples with serum HBsAg in patients followed-up for 3–44 months, Sampliner et al.[3] found that the annual clearance rate of this antigen was 1.7%. A lower incidence (0.1%/year) of HBsAg clearance was described in Taiwan,[4] a hyperendemic area where HBV infection usually occurs perinatally or during early childhood.[2] In a series of 100 carriers from Britain, HBsAg was undetectable in one healthy carrier and one cirrhotic carrier (2%) after a follow-up of 14 and 60 months, respectively;[5] 77 of these carriers were male homosexuals. A higher frequency of HBsAg clearance (10.7%) was observed by Poralla et al.[6] in 65 patients with CHB during a 3-year period.

Several questions remain unanswered regarding the clearance of HBsAg in CHB, due to the wide variety in approach of the studies, particularly the incomplete characterization of the entire group of patients among whom clearance was detected, and the incomplete characterization of their morphological patterns.

According to some authors,[7,8] superinfection with hepatitis C virus (HCV) may suppress HBV infection and produce the clearance of serum HBsAg.

The Brazilian population includes individuals of different ethnic origins. As far as age, sex, race, and viral infections of the liver are concerned, these characteristics of our subjects permitted us to make a comparison between patients with CHB who cleared serum HBsAg and those who showed persistent serum

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HBsAg. The annual rate of clearance could also be determined.

The aim of the present study was to analyze clinical, biochemical, serological, and morphologic parameters of 184 chronic hepatitis B patients with a long follow-up, comparing 20 patients who cleared serum HBsAg with the remaining 164 patients with persistent HBV surface antigenemia.

Subjects and methods

This was a retrospective study of 184 patients with chronic hepatitis B consecutively followed at our institution. Criteria for inclusion were: (1) Diagnosis of CHB; persistence of HBsAg for at least 1 year and histological diagnosis of chronic hepatitis; (2) absence of hepatotoxic drug ingestion, chronic alcoholism, and other non-viral chronic liver diseases; (3) repeated detection of serum HBV markers. Of 214 patients with chronic hepatitis B followed at our institution from 1973 to 1991, 30 did not fulfill all these criteria and were excluded. The study population consisted of 184 HBsAg-positive patients with chronic hepatitis B. All were known to have been HBsAg-positive for at least 12 months when they entered the study; there were 159 men and 25 women, with a mean age of 40.2 years (±15.3 years).

In a follow-up of 12–216 months (mean 66.2 ± 53.7 months) 20 patients (10.8%) showed permanent clearance of HBsAg. Two of the 20 patients (10%) admitted homosexual practices. These 20 patients form the basis of the present report.

Criteria for HBsAg clearance

Absence of serum HBsAg determined by both radioimmunoassay (RIA; polyclonal) and enzyme-linked immumosorbent assay (ELISA) (monoclonal antibodies) in at least three consecutive tests.

Serological studies

Routine follow-up studies included complete clinical and liver biochemical evaluation and serological tests performed at each visit at 3- to 6-month intervals. From 1973 to 1985, HBsAg was serially tested by RIA (Ausria II; Abbott Laboratories, Chicago, Il, USA). Testing for anti-HBs and anti-HBc was done by RIA (Ausab and Corab, respectively; Abbott Laboratories). From 1986 on, HBsAg was tested by ELISA using monoclonal antibodies (Abbott Laboratories). Sera were also tested for HBeAg, anti-HBe, anti-HBs, and anti-HBc IgM, using ELISA (Abbott Laboratories). Determination of viral polymerase activity and detection of HBV DNA in serum by molecular hybridization (dot-blot) were performed as previously described.[9] Anti-HCV was determined using second generation ELISA kits (Ortho, Raritan, NJ, USA or Diagnostics Pasteur, Marne-la-Coquette, France). Positive results were confirmed by second generation RIA (Ortho USA). Anti-HIV was detected using both viral lysate (Genetic Systems, Redmond, WA, USA) and recombinant (Wellcome, Kent, UK) ELISA kits, and results were confirmed by Western blot test (Dupont Biotech, Boston, MA, USA). Anti-HDV was detected using an Organon (Boxtel, Netherlands) ELISA kit. These techniques were performed on sera kept at −20°C or collected during an outpatient visit.

Polymerase chain reaction (PCR)

For the detection of HBV-DNA in serum by PCR, the technique developed by Kaneko et al.[10] was used, with minor modifications. To avoid carry-over between samples, the procedures described by Kwok and Higuchi[11] were strictly followed. Sample preparation: in a 0.5-ml Gene Amp reaction tube (Perkin Elmer, Branchburg NJ, USA), serum (10 µl) was mixed with 2.5 µl 0.5 M NaOH (Sigma, St. Louis, MO, USA), overlaid with 100 µl of mineral oil and incubated for 1 hour at 37°C. After this time, the solution was neutralized by adding 2.5 µl 0.5 M HCl (Merck, Darmstadt, Germany).

Oligonucleotides covering a well conserved sequence of the C gene were synthesized by Genomics (São Paulo, Brazil), according to the sequences proposed by Kaneko et al.,[10] as shown below:

1763: 5′ GCT TTG GGG CAT GGA CAT TGA CCC GTA TAA 3′ (30mer)

2032R: 5′ CTG ACT ACT AAT TCC CTG GAT GCT GGG TCT 3′ (30mer)

1778-E: 5′ GAC GAA TTC TGA CCC GTA TAA AGA ATT 3′ (30mer)

2017R-B: 5′ ATG GGA TCC CTG GAT GCT GGG TCT TCC AAA 3′(30mer)

For amplification, the first PCR employed the Gene Amp PCR kit (Perkin Elmer, USA). The final volume was 100 µl and contained 2.5 U Taq polymerase, 200 µM each deoxynucleotide, 1 µM primers 1763 and 2032R, 50 mM KCl, 50 mM TrisHCl pH 8.3, 1.5 mM $MgCl_2$, and 0.01% gelatin. The amplification was performed by placing the tubes in a DNA Thermal Cycler 480 (Perkin Elmer), programmed for 30 cycles (denaturation at 94°C for 1.5 min, annealing at 42°C for 1.5 min, and extension at 72°C for 1.5 min—total duration, 3 h). For the second PCR, after brief centrifugation, a 10-µl vol-

ume from the first amplification was transferred to another Gene Amp tube, and amplified with primers 1778-E and 2017R-B in the same way as described above. For PCR analysis, after brief centrifugation, 15-µl aliquots from the first and second PCR were analyzed on 1% agarose gel (Sigma, USA) in 0.5 × TBE and 0.5 µg/ml ethidium bromide (Sigma, USA). Samples were considered positive when a 258-bp band was visualized after the second PCR; sometimes, a 270-bp band was also visualized after the first PCR.

Criteria for exacerbation

Biochemical exacerbation of liver disease was defined as an abrupt elevation of serum alanine aminotransferase (ALT) level to 200 IU/l or more (normal 50 IU/l) or a greater than threefold increase in the previous abnormal ALT level.[12] Severe exacerbation was defined as an abrupt elevation of serum ALT of more than ten times the upper normal limit.

Histological and immunohistochemical studies

Liver biopsy and/or laparoscopy, performed with a Tru-cut needle (Baxter, Deerfield, IL, USA) were done in all but one patient. Repeat liver biopsies were obtained from three patients after HBsAg clearance. Besides the routine staining techniques, immunohistochemical methods[13] were used for the detection of HBsAg and HBcAg, with avidin biotin-peroxidase amplification.

Clinical features

The clinical features, studied in patients with persistent HBsAg (group I) and in those showing serum HBsAg clearance (group II), were: sex, age, ethnic group (Occidental and Oriental, all Orientals being of Japanese origin), clinical and/or morphological data, total time of follow-up, time after HBeAg clearance, and severity of biochemical exacerbation (Table 1). Informed consent was obtained from all patients and the study was approved by the Ethics Committee of the Department of Gastroenterology, University of São Paulo School of Medicine.

Statistics

Results were expressed as means and SD and analyzed using the following two-tailed tests: *t*-test or Wilcoxon's rank-sum test[14] and Fisher's exact test to compare proportions between groups. *P* values less than 0.05 were considered significant. Calculations were performed using Statistical Analysis System (SAS) (Cary, NC, USA) software.

Results

Twenty patients (10.8%) lost serum HBsAg. The annual clearance rate was 2.1%. In only one patient was reactivation of the disease observed, this occurring 4 years after clearance, in association with AIDS and multiple myeloma; the clinical details of this patient will

Table 1. Clinical and morphological data in patients showing persistent HBsAg (group I) and clearance of serum HBsAg (group II)

	Group I (n = 164)	Group II (n = 20)	P
Sex (% male)	85.4	95.0	0.319[e]
Age (years, mean ± SD)	38.5 ± 15.6	41.9 ± 15.1	0.354[c]
Race (percent Caucasians)	82.3	95.0	0.208[e]
Chronic active hepatitis (%)	42.7	65.0	0.094[e]
Liver cirrhosis (%)	42.1	30.0	0.344[e]
Hepatocellular carcinoma (%)	7.9	0	0.212[e]
Death (%)	18.2	0	0.022[e]
Time of follow-up, in years (mean ± SD)	5.1 ± 4.4	5.7 ± 4.0	0.290[d]
Time of follow-up after HBeAg clearance			
no. of patients[a]	29	7	
Months (mean ± SD)	41.0 ± 41.0	37.6 ± 25.1	0.731[c]
Severe exacerbation (%)[b]	35/53 (66)	17/19 (89)	0.072[e]

[a] Number of patients showing serum HBeAg upon admission
[b] Abrupt elevation of AST and ALT to more than ten times the upper normal limit
[c] *t*-test
[d] Wilcoxon's rank sum test
[e] Fisher's exact test

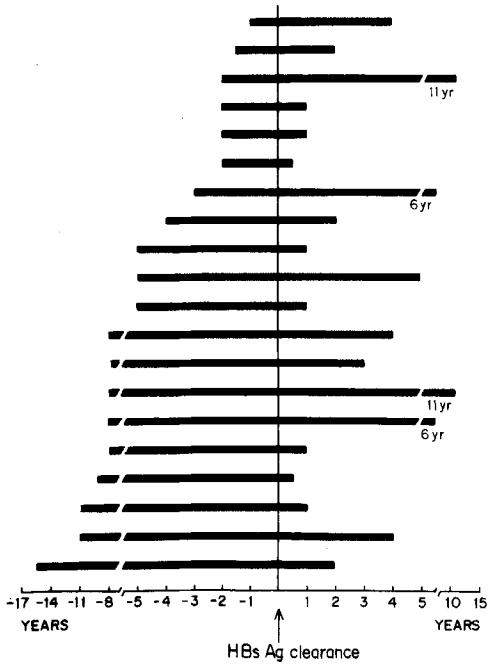

Fig. 1. Follow-up of patients with chronic hepatitis B and clearance of HBsAg

be reported elsewhere. Seroconversion to anti-HBs was observed in 14 of the 20 patients (70%). HBV DNA, determined by PCR was negative in 16 out of 17 patients and positive in the 1 patient with viral reactivation, as mentioned above. After clearance, liver biopsy, performed in 3 patients, showed a striking decrease in inflammatory activity. No HBV antigens were detected in their livers by the immunoperoxidase technique. The follow-up time of patients who cleared HBsAg is shown in Fig. 1. It is worth emphasizing that, despite the diagnosis of chronic active hepatitis and/or liver cirrhosis in all but two patients and the long-term follow-up of most, all but 2 are asymptomatic and show normal serum aminotransferase activity. Both exceptions are related to HCV superinfection. As shown in Table 1, only 7 patients from group II were HBeAg-positive upon admission.

When groups I and II were compared (Table 1), the only statistically significant difference was the number of deaths, which was lower in group II ($P = 0.022$). No other significant differences were found between groups in terms of the clinical, biochemical, or morphological parameters studied (including the presence of liver cirrhosis), except for a tendency to a more severe flare in group II ($P = 0.072$).

As shown in Table 2, the frequency of detection of antibodies to HCV, HDV, and HIV was similar in both groups.

Discussion

Despite the frequency of seroconversion of HBeAg to anti-HBe, spontaneous clearance of serum HBsAg during the course of untreated chronic hepatitis B is considered to be an unusual event,[5,15-20] which seems to be more frequent in Caucasian than in Oriental patients. The annual rate of spontaneous clearance in Caucasians has been reported to vary from 1% to 2%,[3,15] whereas in Orientals it seems to be between 0.5% and 0.8%/year.[2] The reported frequency of clearance varies widely, with figures being as low as 0.1% in Taiwan,[4] a hyperendemic area for HBV infection that usually occurs perinatally or during early childhood,[2] and as high as 10.7%.[6]

In our study, 20 of 184 patients (10.8%) cleared serum HBsAg, at an annual rate of 2.1%. Seroconversion to anti-HBs was observed in 14 of the 20 patients (70%). According to some authors,[21] the subsequent development of anti-HBs may be necessary to confirm that the infection has terminated; otherwise, the clearance may represent an HBsAg titer below the detection threshold. However, detection of anti-HBc alone for long periods after clearance has been observed by others[22] and may represent a protracted "window" period or an inability to develop anti-HBs[22] or, yet again, a low level of anti-HBs undetectable by ELISA.[2] Development of anti-HBs after vaccination in those individuals with isolated anti-HBc is strong evidence against a chronically infected, low-level HBsAg carrier state.[23] In our study, the importance of the clearance of HBsAg is emphasized by the absence of HBV DNA on PCR, even in those who did not develop detectable anti-HBs. Furthermore, in another series, we did not detect viral DNA by dot blot hybridization and PCR in 10 individuals with isolated serum anti-HBc determined by ELISA. It is worth mentioning that our criteria for HBsAg clearance were based on three consecutive negative results by RIA and ELISA, with polyclonal and monoclonal antibodies, respectively.

The low rate of detection of HBV DNA by PCR in our patients is in accordance with the results published by Baker et al.,[24] Koremman et al.,[25] and Hsu et al.[26] However, higher figures were reported by Liaw et al.[2] and Adachi et al.[27]

Table 2. Antibodies to HCV, HDV, and HIV in 164 patients with persistent serum HBsAg (group I) and in 20 patients with clearance of HBsAg (group II)

Antibodies	Group I *n* (%)	Group II *n* (%)	*P*[a]
Anti-HCV	8/87 (9.2)	2/16 (12.5)	0.652
Anti-HDV	1/77 (1.3)	0/16 (0)	1.000
Anti-HIV	7/79 (8.8)	2/17 (11.7)	0.658

[a] Fisher's exact test

The pathogenesis of serum HBsAg clearance is not well understood. Its appearance at different times after HBeAg clearance, and various clinical and laboratory observations, show that it frequently follows an acute exacerbation, characterized by clinical symptoms or laboratory changes quite similar to those found in acute hepatitis or in a sudden downhill course of liver cirrhosis. Severe spontaneous exacerbations, characterized by an abrupt elevation of serum aminotransferases (exeeding ten times the upper normal limit) were observed in 17 of 19 patients (89%). As shown in Table 1, this phenomenon tended to be more frequent (P = 0.072) in patients who had cleared HBsAg (group II). HBeAg was detected in 7 patients upon admission, whereas the others were anti-HBe-positive. Interestingly, in patients who did not show HBsAg clearance (group I), the time elapsed between the disappearance of HBeAg and the clearance of HBsAg varied widely and did not differ from the time of follow-up of group II patients. According to some authors,[15] HBsAg clearance tends to occur more often in females. This phenomenon was not observed in our subjects, where the male: female ratio was 19:1.

Although our virological findings stress the strong relationship of HBsAg clearance with the end of the infection, as assessed by the absence of viral DNA on detection by PCR, this phenomenon occurred late in the course of the illness, when severe histologicalchanges had already occurred in 90% of the patients, the vast majority of them being asymptomatic.

These findings reduced the level of enthusiasm about the higher probability of HBsAg clearance in asymptomatic carriers described by Liaw et al.,[2] since those authors did not perform biopsies, and therefore past necro-inflammatory changes could not be excluded.

Chronic active hepatitis occurring while a patient is HBsAg-positive usually indicates vigorous immune reactivity in an attempt to clear HBV-infected hepatocytes. Loss of both HBeAg and HBsAg, therefore, would reasonably be more likely to occur in this situation, particularly in patients who clear HBsAg shortly after HBeAg,[26] as was seen in some of our patients (Table 1).

The possibility that HCV superinfection is a cause of HBsAg clearance was mentioned by some authors.[7,8,28] In our patients, however, the detection of anti-HCV was similar in both patients who demonstrated clearance and those who did not (Table 2). Also, no difference was found with respect to anti-HDV and anti-HIV.

The clinical implications of HBsAg clearance in CHB are rather obvious. First, this phenomenon is always observed after HBeAg disappearance, which may represent a reduction or interruption of HBV replication. HBV DNA was not detected, even by PCR, in most patients who cleared HBsAg. Second, serum levels of aminotransferase were normalized, suggesting an interruption of the inflammatory process; our two exceptions had superinfection with HCV. Liver biopsies tended to show an inactive process with disappearance of HBcAg in the tissue. Third, the clearance of HBsAg is often followed by the appearance of anti-HBs, which is considered to be a protective antibody. Finally, a reduced tendency to evolve to hepatocellular carcinoma (HCC) was another important feature. Adachi et al.[27] reported evolution to HCC in 3 out of 7 patients with CHB, within 2–9 years after clearance of serum HBsAg. Serum HBV DNA, however, was detected by PCR in all 3. These results contrast sharply with our experience, based on 20 patients with a follow-up of 1–11 years after clearance; no evolution to HCC has been observed thus far, whereas this evolution was seen in 13 patients (7.9%) from group I. Furthermore, there was a fatal outcome in 30 of 164 patients (18.2%) from group I and in none from group II (P = 0.022), indicating a better prognosis for patients who clear their serum HBsAg.

The fate of HBV in CHB after sustained loss of HBsAg was recently investigated.[29–31] Fong et al.[29] and Kuhns et al.[30] concluded that HBV DNA may be detectable by PCR in liver tissue after the disappearance of HBsAg, even in the absence of detectable HBV DNA in serum. Some investigators.[30,32-34] have detected HBV DNA in peripheral blood mononuclear cells. This finding was more often observed in immunocompromised[35,36] than in immunocompetent patients.[31,37] Interestingly, serum anti-HIV was detected 2 years before death in the only patient in our series who presented with HBV reactivation, this being 4 years after HBsAg clearance.

Marcellin et al.[31] detected HBV DNA in liver tissue and serum but not in mononuclear blood cells collected after seroconversion from HBsAg to anti-HBs.

In conclusion, the annual rate of HBsAg clearance in patients with CHB is low in this series of Brazilian subjects. Despite a trend to more severe exacerbation of hepatitis activity observed in patients from this group during follow-up, no statistically significant difference was found between our two groups, except for a higher mortality in patients with persistent serum HBsAg (group I). HBV DNA was usually undetectable by PCR and seroconversion to anti-HBs was observed in most patients. The suggestion of possibly lower rates of development of HCC in these patients should be addressed in future studies.

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