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DETECTION OF HEPATITIS B VIRUS DNA IN HBsAg CHRONIC CARRIERS USING A LIQUID PHASE MOLECULAR HYBRIDIZATION (ABBOTT HBV DNA™) AND THE DNA POLYMERASE ASSAY: COMPARISON OF RESULTS

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Summary. - These data clearly show the sensitivity differences and the correlations between the HBV DNA and the DNA polymerase assay. The Abbott HBV DNA™ is a molecular hybridization test in a liquid phase using a ^{125}I-labelled probe and allowing the detection and the quantitation of HBV DNA in serum samples. This test was found to be 20-200 times more sensitive than the DNA polymerase assay and its sensitivity was evaluated to be about 0.3 pg HBV DNA. It is very easy to use and the results are obtained within 24 hours. In the patients with persistent HBV infection a close correlation between the positivity of the DNA polymerase activity and of the HBV DNA ($R = 0.94$) was observed. These two markers evolve quite parallel whatever the results obtained in the HBe system or whatever the sample time in a group of patients treated with recombinant interferon.

Key words: hepatitis B virus; liquid phase hybridization; DNA polymerase

Introduction

The detection of HBV DNA in serum by dot blot molecular hybridization generally uses a ^{32}P-labelled cloned HBV DNA probe predominantly of 3.2 Kb and the method essentially described by Scotto *et al.* (1983). The DNA samples are applied to a nitrocellulose filter and denatured by alkali, then the prehybridization and hybridization are carried out. The lowest level of HBV DNA detectable by autoradiography is in the range of 1-10 pg (Scotto *et al.*, 1983; Moestrup *et al.*, 1985; Morace *et al.*, 1985; Alberti *et al.*, 1986; Gowans, 1986). The optimum hybridization procedure with regard to its sensitivity and specificity was investigated by Walter *et al.* (1987). Other alternatives using a probe labelled with biotine or tritium have also been proposed. The sensitivity of the non-radioactive hybridization tests (1-10 pg) would be comparable to that using

^{32}P-labelled cloned HBV DNA probe with a short time exposure (Feinman *et al.*, 1984; Yokota *et al.*, 1986; Quibriac *et al.*, 1987; Saldanha *et al.*, 1987). Recently, a liquid phase molecular hybridization test using ^{125}I-labelled HBV DNA probe developed by Kuhns *et al.* (1988) and commercialised by Abbott North Chicago (Abbot HBV DNA™) has allowed the detection and the quantitation of HBV DNA in the serum. The sensitivity of this assay (0.15 pg) was found to be equivalent or slightly greater than that of the classical spot hybridization test (Zarski *et al.*, 1989).

The present paper describes our experience with this test, with emphasis on the comparison of the results with those of the DNA polymerase activity tests in serum samples of HBsAg chronic carriers.

Materials and Methods

Patients. HBV DNA has been retrospectively assayed in 118 serum samples from 66 HBsAg chronic carriers. All samples had been previously tested for the DNA polymerase activity and kept at -80 °C until HBV DNA assays. Each serum was tested once for DNA polymerase and for HBV DNA. Fifty-eight samples were taken from 21 HBsAg chronic carriers positive for HBeAg and who had been treated with a recombinant human interferon (IFN, Coppens *et al.*, 1989). A sample was available before the treatment from 19 patients and after the onset of treatment at 6 weeks from 16, at 12 weeks from 13, at 18 weeks from 5, and at 24 weeks from 5 patients. Sixty samples were taken from 45 untreated HBsAg chronic carriers, positive or negative for HBe markers and suffering from chronic liver diseases.

HBV DNA was detected in serum with a radiological molecular hybridization assay (Abbott HBV DNA™, North Chicago). According to the manufacturer, the single-stranded probe is complementary to the long strand of the HBsAg gene and contains no vector sequences. 0.1 ml of serum was pretreated with reagents to solubilize and to expose the HBV genome. Then the ^{125}I-labelled probe was added to the mixture. After an overnight incubation at 65 °C the hybridized and unhybridized forms were separated by chromatography on a pre-packed glass column and the hybridized radioactivity was read in a gamma counter (ANSR, Abbott). The presence or absence of HBV DNA was determined by comparing the net counts of the specimen to a cutoff value (the mean of negative control net counts plus 0.015 times the mean of positive control net counts). The results expressed in pg HBV DNA per ml were evaluated relative to the positive control used as standard. According to the manufacturer's information the positive control prepared from Dane particles contains about 103 pg HBV DNA per ml.

DNA polymerase activity was assayed according to the Kaplan's technique (1973). One ml of serum was centrifuged in the Beckman 70 Ti rotor at 55 000 rpm for 5 hr at 4 °C, the pellet was resuspended in 1/20 of the original volume in a phosphate buffer with 1 % NP-40 and 0.3 % mercaptoethanol, and added to 0.2 ml mixture containing 8 μmoles $MgCl_2$, 24 μmoles NH_4Cl, 56 pmoles ^{3}H-TTP (50 Ci/mmol) and dATP, dCTP, dGTP (0.1 μmole of each). The ^{3}H-thymidine incorporated into acid precipitable material was measured by scintillation counting. The activity was expressed as the ratio between the cpm in the sample after incubation for 4 hr at 37 °C and the cpm in the sample at zero time (T_4/T_0). A ratio higher than 3 was considered positive. A ratio ranging from 2.3 to 3 was considered borderline.

HBsAg, HBeAg, anti-HBe and anti-HBc were detected with reagents Ausria II, Abbott HBe, Corab, R. I. A. (Abbott North Chicago).

Results

Sensitivity of HBV DNA assay

From the plot of radioactive net counts versus the concentration of positive

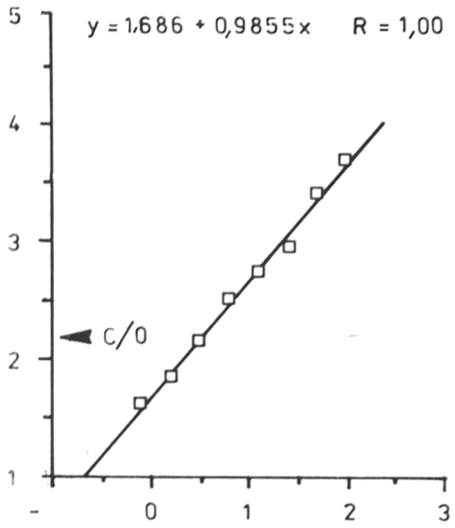

Fig. 1
Plot of radioactivity versus concentration of HBV DNA
Positive control HBV DNA (103 pg/ml) used. Ordinate: ^{125}I radioactivity, log of net c.p.m. Abscissa: DNA concentration, log of pg/ml.

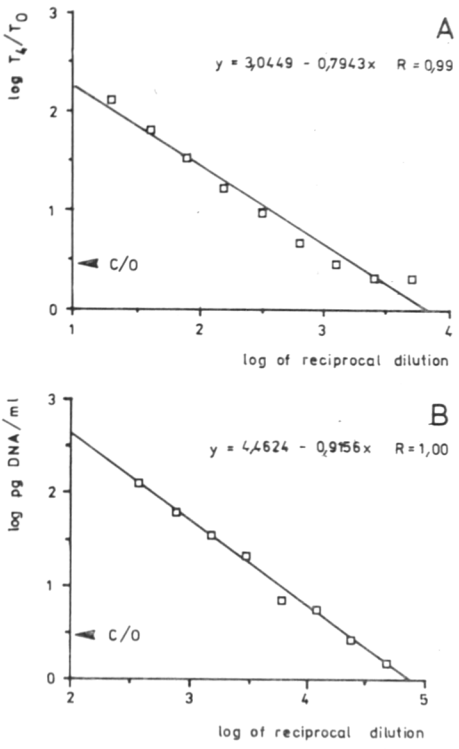

Fig. 2
The detection limit of the DNA polymerase (A) and the HBV DNA tests (B)

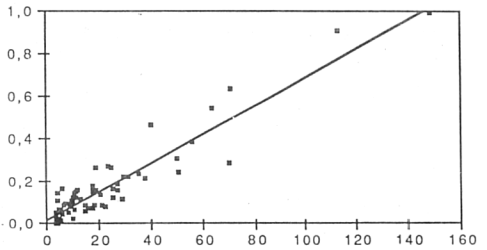

Fig. 3
Correlation between the DNA polymerase and the HBV DNA tests (R = 0.94)
Sixty-eight sera of HBsAg chronic carriers were tested. Ordinate: HBV DNA, pg/ml (x 10^3). Abscissa: DNA polymerase, T4/ T0.

control HBV DNA the cutoff value has been found to correspond to 0.3 pg HBV DNA (Fig. 1).

Comparison of the HBV DNA and DNA polymerase assays

The difference of sensitivity between these assays was determined by comparing their detection limit obtained from graded dilutions of a serum sample previously known to be strongly positive for DNA polymerase activity and HBV DNA. The detection threshold has been evaluated to correspond to 1/1111 dilution in the DNA polymerase assay and to 1/24016 in the HBV DNA assay. As ten times more of the sample was used in the DNA polymerase assay, the HBV DNA assay was shown to be about 216 times more sensitive than the DNA polymerase assay for the complete virus particles detection in the serum (Fig. 2A, 2B).

The correlation between the two tests in HBsAg chronic carriers varies from 86 % (50/58) in treated patients (Table 1) to 75 % (45/60) in untreated patients (Table 2), with more samples positive for HBV DNA in the two groups. There are HBV DNA and DNA polymerase negative serum samples in the HBeAg

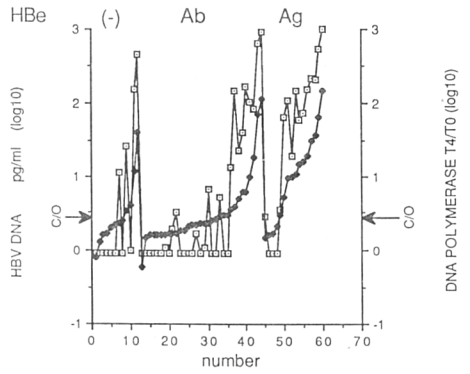

Fig. 4
A comparison between the DNA polymerase activity and the amount of HBV DNA in 60 sera from 45 HBsAg chronic carriers The carriers were subdivided into three categories related to the HBe markers. In each group the results have been sorted according to the DNA polymerase ascending values. The arrows indicate the threshold value.

positive group, and HBV DNA and DNA polymerase positive serum samples in the anti-HBe positive group (Table 3). Two samples weakly positive for DNA polymerase were found negative for HBV DNA. The coefficient of correlation between the positive results for the two tests is 0.94 (Fig. 3). There is a parallel evolution of the two markers whatever the results in the HBe system (R = 0.88, 0.81, 0.98, in HBe(-), HBeAb, HBeAg groups, respectively) or whatever the time of taking samples in patients treated with interferon (R = 0.82, 0.84, 0.94, 0.90 at 0, 6, 12, 18-24 weeks, respectively) (Fig. 4, 5).

Table 1. Results of the HBV DNA and DNA polymerase in 58 sera from 21 IFN-treated HBsAg chronic carriers

		DNA polymerase (T4/T0)		
	HBV DNA	>3 + (N = 46)	2.3 - 3 +/- (N = 5)	<2.3 - (N = 7)
> cutoff (N = 49)	+	45	2	2
< cutoff (N = 9)	-	1°	3	5

° T4/T0: 3.4

Table 2. Results of the HBV DNA and DNA polymerase in 60 sera from 45 untreated HBsAg chronic carriers

		DNA polymerase (T4/T0)		
	HBV DNA	>3 + (N = 24)	2.3 - 3 +/- (N = 5)	<2.3 - (N = 25)
> cutoff (N = 30)	+	23	4	3
< cutoff (N = 30)	-	1°	7	22

° T4/T0: 3.6

Table 3. Results of the HBV DNA and DNA polymerase in 60 sera from 45 untreated HBsAg chronic carriers related to the HBe markers

HBe	HBV DNA		DNA polymerase (T4/T0)		
	≥ cutoff +	< cutoff -	> 3 +	2.3 - 3 +/-	< 2.3 -
AG (N = 16)	13	3	11	1	4
AB (N = 32)	13*	19	9*	8	15
(N = 12)	4	8	4	2	6

* 7 sera were taken from 2 patients who were followed for 3 and 24 months.

Discussion

The classical method for the detection of HBV DNA by molecular hybridization uses a solid support and a ^{32}P-labelled cloned HBV DNA probe. Recently, the firm Abbott (North Chicago) has commercialized a molecular hybridization test in a liquid phase with a ^{125}I-labelled HBV DNA probe (Abbott HBV DNA^{TM}). Using this test, the quantity of HBV DNA corresponding to the cutoff value has been evaluated to be about 0.3 pg. This value is practically comparable to that (<1 pg) of the hybridization test with a ^{32}P-labelled probe and an autoradiography after more than 3 days (Zarski *et al.*, 1989). Several studies have previously reported the higher sensitivity of molecular hybridization test in

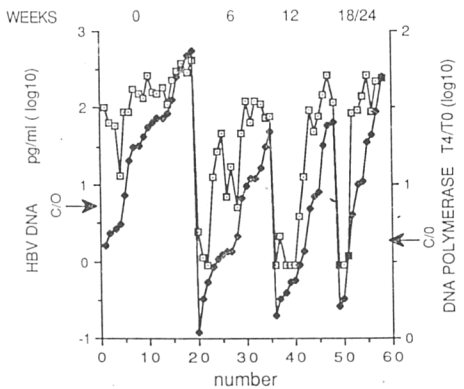

Fig. 5
A comparison between the DNA polymerase activity and the amount of HBV DNA in 58 sera from 21 HBsAg chronic carriers. The carriers were subdivided into four categories related to the sample time (before or after the onset of the treatment with a recombinant IFN). In each group the results have been sorted according to the DNA polymerase ascending values. The arrows indicate the threshold value.

detecting complete virus particles and the positive correlation between the results of molecular hybridization and DNA polymerase assays (Bonino *et al.*, 1981; Weller *et al.*, 1982; Fagan *et al.*, 1985; Quiroga *et al.*, 1987; Bartolomé *et al.*, 1988; Kuhns *et al.*, 1989). In this study the data clearly show the sensitivity differences and the correlation between the HBV DNA and the DNA polymerase measurements. The data obtained by comparing the detection limit of HBV DNA by the Abbott HBV DNA test and that of the DNA polymerase suggest that the HBV DNA test is about 200 times more sensitive than the DNA polymerase assay. Nevertheless, two weakly positive samples for DNA polymerase were found HBV DNA negative and some DNA polymerase positive samples showing a relatively low level of HBV DNA (5.1 - 27.6 pg/ml) were observed. This could be related to the difference in sample volume in the two assays, or it could perhaps reflect a stronger DNA synthesis in the polymerase assay than generally expected and perhaps the variations in the lenght of the incomplete strand of DNA. Another possibility is that some polymerase results could be false positive due to a bacterial contamination, but in this study, the sera and reagents were utilized so as to avoid the possibility of contamination during handling the samples. A close correlation between the positive results of DNA polymerase activity and the amount of HBV DNA detected with the Abbott HBV DNA is also observed. The linearity of the two assays has allowed to compare their quantitative evolution in HBsAg chronic carriers serum samples. These two markers of complete virus particles evolve quite parallel whatever the time of taking samples in a group of patients treated with IFN or whatever results obtained in the HBe system. In agreement with other authors (Lieberman *et al.*, 1983; Imazeki *et al.*, 1985; Berris *et al.*, 1987; Carloni *et al.*, 1987; Tassopoulos *et al.*, 1987), conflicting results such as the absence of HBV DNA in some HBeAg positive sera and its presence in some anti-HBe positive sera are also observed. It is interessing to note that in one anti-HBe positive patient the reactivation of HBV shown by the reappearance of HBV DNA and DNA polymerase in the serum was persisting during the follow-up period (24 months). In the group of untreated patients an important part of borderline DNA polymerase is related to positive HBe antibody results (8/11) and to negative HBV DNA results (7/11). This indicates that the Abbott HBV DNA test could improve the classification of cases with difficult interpretation. This was the case of the two groups of HBsAg carriers, where more samples positive for HBV DNA have been obtained.

In HBsAg carriers serum a close correlation exists between the positivity of the DNA polymerase activity and the amount of HBV DNA detected by molecular hybridization with the Abbott HBV DNA test. This test is about 200 times more sensitive than the DNA polymerase asay, it does not have the risks connected with the handling of ^{32}P used in the classical hybridization, and the detection time for a positive hybridization is strongly reduced since the results are already obtained after 24 hr. Besides the linearity of the assay also offers the possibility of quantitation that should be helpful for the evaluation of the clinical

significance of HBV DNA and the efficacity of treatment in the chronically infected patients. It is easy to use and could be applied to large scale clinical studies, however its high cost limits its utilisation.

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