Rapid Identification of Low Level Hepatitis B-related Viral Genome in Serum

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Abstract

A sensitive and specific method has been developed to detect hepatitis B virus (HBV) in serum. The method involves two steps: the capture of viral genome from serum using a high affinity IgM monoclonal antibody directed against a common *a* domain epitope found on the envelope, and the amplification of viral DNA by the polymerase chain reaction (PCR). The amplification is initiated using "generic" primers derived from the core and pre-core sequences which are highly conserved amongst the hepadnaviruses. This rapid technique detects less than 10 infectious virions and may be useful in the study of individuals with acute and chronic liver disease of unknown etiology.

Introduction

We have developed a large library of MAbs directed against epitopes that reside on hepatitis B surface antigen (HBsAG).[1] These antibodies have been employed to identify genetic variations in hepatitis B virus (HBV) antigenic structure by a computerized technique called "signature analysis" (1, 2). With this approach, a number of high affinity MAbs that recognize common or *a* domain epitopes (present on all known HBV strains) have been defined. Using a combination of such antibodies, each of which binds to a separate and distinct epitope on HBsAG, a sensitive multisite monoclonal immunoradiometric assay (M-IRMA) was developed (3–5). This assay, in its most sensitive format, detects ~ 10–15 pg HBsAG-associated epitopes per ml of serum (5). Subsequent studies demonstrated that many individuals with acute and chronic liver disease as well as some "healthy" blood donors were reactive in this test only (3–5). Many of these subjects had no evidence of recent or past HBV exposure as determined by the absence of antibodies to HBV core and surface antigens (anti-HBcAG abd anti-HBsAG). Such monoclonal reactive blood has been shown to transmit a long incubation hepatitis infection to chimpanzees even if the animals had been successfully vaccinated against HBV (6). Thus, immunity against HBV does not afford protection against infection with some of these agents. Due to presumed low levels of viral replication, it has not been possible to identify and characterize the infectious virions in many of these subjects by dot blot and in situ hybridization of serum and liver with a full-length ^{32}P-HBV DNA probe (3–5).

With the development of the polymerase chain reaction (PCR) technique to amplify DNA, it became possible to detect low levels of viral DNA in serum, tissue and other biological fluids (7–9). However, the feasibility of PCR using serum containing extremely low levels of virus particles involves several considerations. The first is viral capture from serum and the second entails the selection of oligonucleotide primers and delineation of the region of viral DNA to be amplified. It has not heretofore been possible to perform PCR directly in serum containing viral particles without a complicated nucleic acid extraction step that may limit the sensitivity of the assay (10). To circumvent this problem, we have used a high affinity IgM (Kasn 4×10^{11} L/M) anti-HBsAG MAb directed against an *a* domain epitope of HBsAG and coupled this MAb to a solid phase in order to capture and thereby purify HBV particles from serum in a single step. All other serum components are removed by washing, leaving the viral particles tightly bound to the MAb linked to Sepharose beads. DNA is liberated from the viral particles by high temperature followed by PCR amplification.

Methods

Patients. In order to test the feasibility of the monoclonal antibody capture-PCR amplification technique to detect low level HBV related infection, we selected 13 individuals known to have very low levels of HBsAG-associated epitope serum as measured by a second generation M-IRMA (5). 2 of the 13 had HBV serologic markers (patient 12 with anti-HBsAG and patient 11 with anti-HBcAG alone, respectively): all were negative for HBsAG by a conventional assay (see below). Five individuals had persistent elevations of alanine aminotransferase (ALT) (patients 1–5) and four others had cirrhosis of uncertain etiology (patients 6–9). The final four had chronic active (patients 10 and 11) and chronic persistent hepatitis (patients 12 and 13), respectively. Their serum had been stored at −20°C before testing.

Monoclonal antibody capture. 5D3 IgM monoclonal antibody recognizing an *a* domain epitope of HBsAG was produced as described previously (1) and coupled to Sepharose beads using activated CNBr Sepharose at a protein concentration of 1 mg per 1 ml of Sepharose slurry (11). 25 μl of antibody-coupled Sepharose was added to 200 μl of serum and mixed overnight at 37°C. Serum was then decanted after a short centrifugation step. The beads were washed three times with PBS and once with H_2O to remove serum components from the antigen-

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1. *Abbreviations used in this paper:* ALT, alanine aminotransferase; DHBV, duck hepatitis B virus; GSHV, ground squirrel hepadnavirus; HBeAG, hepatitis B e antigen; HBsAG, hepatitis B surface antigen; M-IRMA, monoclonal immunoradiometric assay; PCR, polymerase chain reaction; WHV, woodchuck hepadnavirus.

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antibody complexes. After the last wash, 25 μl of H_2O was added to the beads. HBV DNA from captured virions was released by heating at 95°C for 10 min. 12.5 μl of supernatant was removed for PCR in a total of 25 μl reaction volume containing 10 pmol of each oligonucleotide primer in a reaction buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% [wt/vol] gelatin, 200 μM of dATP, dGTP, dCTP, TTP and 0.625 U of Taq polymerase [Perkin Elmer Cetus, Norwalk, CT]). Reaction mixtures were overlaid with 30 μl of mineral oil to prevent evaporation. Samples were heated at 94°C for 30 s to denature the DNA, cooled to 45°C for 1 min to allow primers to anneal to the template, then heated to 72°C for 2 min for primer-directed DNA synthesis. Subsequent rounds consisted of repeated cycles of these three steps. After the PCR, 10 μl of reaction mixture was analyzed on a 1.2% agarose gel.

DNA extraction from serum. 200 μl of serum samples were treated with 1 mg/ml proteinase K in 10 mM EDTA and 0.5% SDS for 3 h at 55°C and extracted twice with phenol, once with $CHCl_3$ and once with ether. DNA was precipitated with ethanol and dissolved in 25 μl of H_2O. 12.5 μl of dissolved DNA was subjected to PCR amplification.

Hepatitis B antigen and antibodies. Monoclonal immunoradiometric assays (M-IRMAs) to detect HBsAG-associated epitopes were performed as described previously (3–5). HBsAG was also measured by Austria II; anti-HBcAG (IgM), HBeAG, anti-HBeAG, and anti-HBsAG were measured by Corab-M, Abbott HBe, and Ausab (Abbott Laboratories, North Chicago, IL), respectively.

Results

Since we suspected that there may be substantial genetic heterogeneity among HBV and related strains (2, 12, 13), a computer analysis of DNA sequences was done to search for regions of maximal homology representing functional and structural conservation. HBV strains from different parts of the world were shown to have significant sequence heterogeneity (12, 13). Thus, selection of primer sequences was considered essential for the detection of these viral genomes. A survey of the known DNA sequences of hepadnaviruses, including all HBV subtypes, ground squirrel (GSHV), woodchuck (WHV), and duck hepatitis B virus (DHBV) revealed several consensus sequences (14). Two stretches of nucleotides flanking the core gene were identified as remarkably conserved sequences. The oligonucleotides representing these two regions are shown: Oligo 1: 5′TTCAAGCCTCCAAGCTGTGCCTTGG3′; Oligo 2: 5′TCTGCGACGCGGCGATTGAGA3′.

The first oligonucleotide spans position 1865 to 1889 (adw subtype; see references 12, 13) and this sequence is 100% conserved in the pre-core region of all known hepadnaviruses. The second oligonucleotide from position 2410 to 2430 also contains sequences that are conserved except a C to T substitution at position 2418 in WHV and GSHV, and T to G at 2414 in adw2 subtype. When run in the PCR, these two oligonucleotide primers will direct and amplify the synthesis of a 566-bp HBV DNA fragment.

Fig. 1 demonstrates the sensitivity of the monoclonal antibody capture system followed by the PCR amplification step. In this example, serum from a patient who was initially reactive by both M-IRMA (concentration of HBsAG-associated particles ≈ 500 pg/ml) and DNA dot blot hybridization technique (HBV DNA concentration ≈ 500 pg/ml) was serially diluted. Binding activity of the M-IRMA was directly compared to viral capture with the IgM anti-HBsAG MAb linked to the solid-phase support followed by the PCR. At a serum dilution between 10^{-4} and 10^{-5} the immune reactivity of the M-IRMA became negative. However, a 566-bp band reflecting amplified virion DNA was easily detected at a dilution of 10^{-7}. Thus, the combination of MAb capture with PCR amplification is at least 100-fold more sensitive for detection of the hepatitis B related viral genome than the M-IRMA for viral antigen (10–15 pg/ml). This level of sensitivity corresponds to < 10 viral particles in 200 μl of serum. In Fig. 2, comparisons of the PCR have been made using the proteinase K/phenol extraction method of serum to the high affinity IgM anti-HBsAG MAb-capture technique. It is noteworthy that at

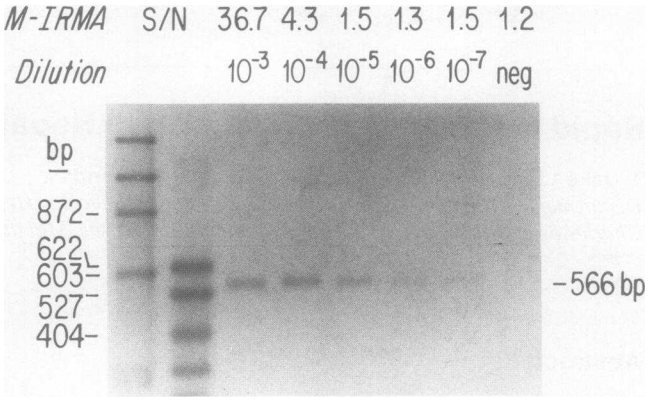

Figure 1. Sensitivity of IgM anti-HBsAG MAb capture-PCR amplification technique for HBV. Serum was serially diluted with negative control serum from 10^{-3} to 10^{-7}. Each dilution was analyzed separately for HBsAg-associated epitopes by M-IRMA and intact viral genome. The signal to noise (S/N) ratios (pos. S/N > 2.0) of the M-IRMA are indicated above each dilution. The corresponding IgM anti-HBsAG MAb capture-PCR amplified products are presented after 35 cycles. The 566-bp amplified fragments are shown on the agarose gel by ethidium bromide staining and on subsequent transfer to nylon membranes were shown to hybridize to full length ^{32}P-HBV DNA probe (data not shown). The last lane on the right is negative control serum.

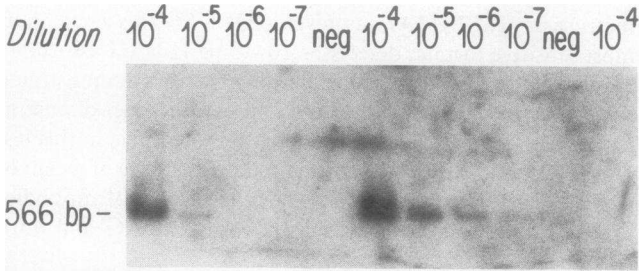

Figure 2. Comparison of the sensitivities of proteinase K/phenol extraction versus affinity capture on HBV detection. The HBV positive serum presented in Fig. 1 was serially diluted with negative control serum from 10^{-4} to 10^{-7}. Sample dilutions and negative control serum were extracted with proteinase K/phenol and subjected to 35 cycles of PCR amplification (lanes *1–5* autoradiogram, 8 h exposure). Additional 200 μl from each dilution are analyzed by affinity capture technique as depicted in Fig. 1. The PCR samples were then electrophoresed on agarose gel, transferred to nylon membrane (Biodyne A), hybridized with nick-translated ^{32}P-HBV DNA (3.2 kbp) probe. The autoradiogram was exposed with intensifying screens at −70°C. Duplicate samples were separately analyzed with affinity capture-PCR technique, (lanes *6–10*). Lane *11* represents the same 10^{-4} dilution of HBV positive serum reacted with a nonrelevant monoclonal antibody (anti-HCG) coupled to Sepharose.

higher concentrations of virus the hybridization signals observed by both techniques were comparable (10^{-4} dilution). As the viral concentrations decreased (10^{-5}), a low signal was observed for proteinase K/phenol extracted serum. Using the MAb anti-HBsAG-capture technique, however, it was possible to detect viral genome at a 10^{-7} dilution. We also found that extraction with proteinase K/phenol often gave widely variable signal intensities, particularly at low viral concentrations while the MAb anti-HBsAG affinity capture was more reproducible.

Using the sensitive M-IRMA, we identified HBsAG-associated epitopes in patients with chronic liver disease of unknown etiology who were serologically negative for HBV markers (anti-HBcAG, anti-HBsAG, and HBsAG) by commercial assays (3–5). Representative patients were analyzed by the IgM anti-HBsAG MAb-capture PCR amplification technique. We also included two patients serologically positive for previous exposure to HBV: patient 11 was reactive for anti-HBcAG only and patient 12 for anti-HBsAG only. By dot blot hybridization with a full length ^{32}P HBV DNA probe, sera from all 13 patients were negative at a sensitivity of 0.05–0.1 pg HBV DNA (data not shown). Fig. 3 demonstrates the results of these analyses. Patients 6 and 7, with M-IRMA S/N ratios of 1.8 and 1.9, respectively, are considered nonreactive (positive S/N > 2.0). All subjects were positive for HBV DNA sequences by the affinity capture-PCR amplification assay. The presence of anti-HBsAG (patient 12) suggests that the antibodies were either free or existed in soluble immune complexes. Such complexes were capable of binding to the IgM anti-HBsAG MAb linked to the solid phase and contained HBV or related virions.

To test the biological relevance of these findings, a chimpanzee was inoculated with serum from patient 9. This individual was considered to have chronic hepatitis of uncertain cause since he had no serologic markers of recent or past exposure to hepatitis A or B virus. The chimpanzee was bled weekly and serum was analyzed for aminotransferase (ALT/AST) elevations, hepatitis B e antigen (HBeAG), antibody to HBeAG (anti-HBeAG), anti-HBsAG and anti-HBcAG and HBsAG-associated epitopes by M-IRMA. HBV DNA was measured by IgM anti-HBsAG MAb capture followed by PCR. As shown in Fig. 4, 7 wk after inoculation, HBsAG-associated epitopes were detectable in serum by M-IRMA. The antigenemic phase peaked at week 12 and cleared by week 16 followed by the appearance of anti-HBsAG. Acute viral hepatitis was evident by ALT/AST elevations and acute hepatic injury was confirmed by histology. HBeAG also developed during the peak of antigemia and later cleared with the appearance of anti-HBeAG. HBV DNA was first detected by the IgM affinity capture-PCR technique 5–6 wk after inoculation, earlier than the appearance of HBsAG as measured by M-IRMA. Even in the presence of anti-HBsAG at week 17, HBV DNA was still detectable in serum and suggests that HBV may remain in the blood for a longer period of time than has been previously recognized possibly in the form of immune complexes (15). HBV DNA was detected by standard dot blot hybridization only in serum from week 11 and 12 (data not shown), reflecting a significant difference in sensitivities between the two techniques. Similar to the observation in Fig. 3, the levels of HBV DNA measured by the affinity capture-PCR amplification method fluctuates significantly and appears not to correlate with the levels of HBsAG, a finding consistent with the observations of others (4, 16). Unlike classical HBV infection, anti-HBcAG of the IgM class did not develop (17). A weak IgG anti-HBcAG response was observed late in the course of infection, 3 mo after the onset of antigenemia. Of interest in this regard is that the inoculum derived from patient 9 also lacked anti-HBcAG antibodies. Finally, nucleic acid sequencing by the dideoxy method (18) of the amplified 566 bp fragment revealed the expected HBV core genomic sequences (data not shown).

Discussion

We have developed, therefore, a rapid, sensitive and specific method for detection of low level hepatitis B or related viral genomes in serum. This method offers high specificity since only virions that bear the epitope recognized by 5D3 IgM anti-HBsAG epitope are bound to a solid phase. Even though in serum there may be far more 22-nm "empty" particles present than intact virions (17), the presence of excess MAb anti-HBsAG bound to Sepharose allows enough virions to be captured for the detection of these agents by PCR. One advantage of "generic" hepadnavirus primers is that is permits amplification of all related viruses including different subtypes of HBV. As demonstrated here, amplification of several molecules of viral genome in serum is feasible.

With this technique, we now demonstrated the presence of intact and infectious HBV-like virions in serum of these patients with positive reactivity in the M-IRMA and no HBV serologic markers (3–5). Such patients would otherwise have been classified as chronic liver disease of unknown etiology. We do not know the prevalence of low level HBV or related agents in patients with acute and chronic liver disease and further studies will be required. Comparison of amplified viral sequences should allow us to define the extent and significance of genomic variations among these agents. In this regard, several other laboratories have reported preliminary molecular characteristics of HBV or related virons with sequence modifi-

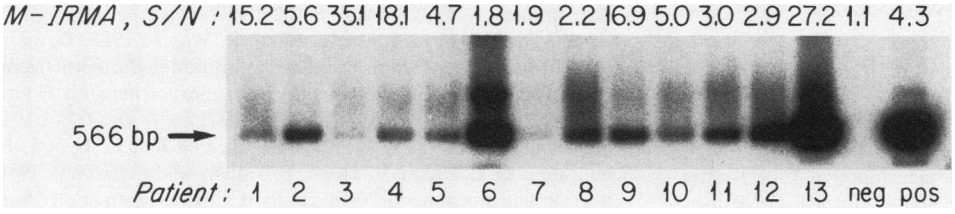

Figure 3. Analysis of 13 patients with chronic liver disease for the presence of HBsAG-associated epitopes and HBV-related virions. Serum was analyzed separately by M-IRMA and affinity capture-PCR amplification (35 cycles) and are displayed as patient 1 through 13 on the autoradiogram (8 h exposure). Corresponding S/N ratios for M-IRMA are listed above each lane (pos. S/N > 2.0). The last two lanes are negative and positive control serum.

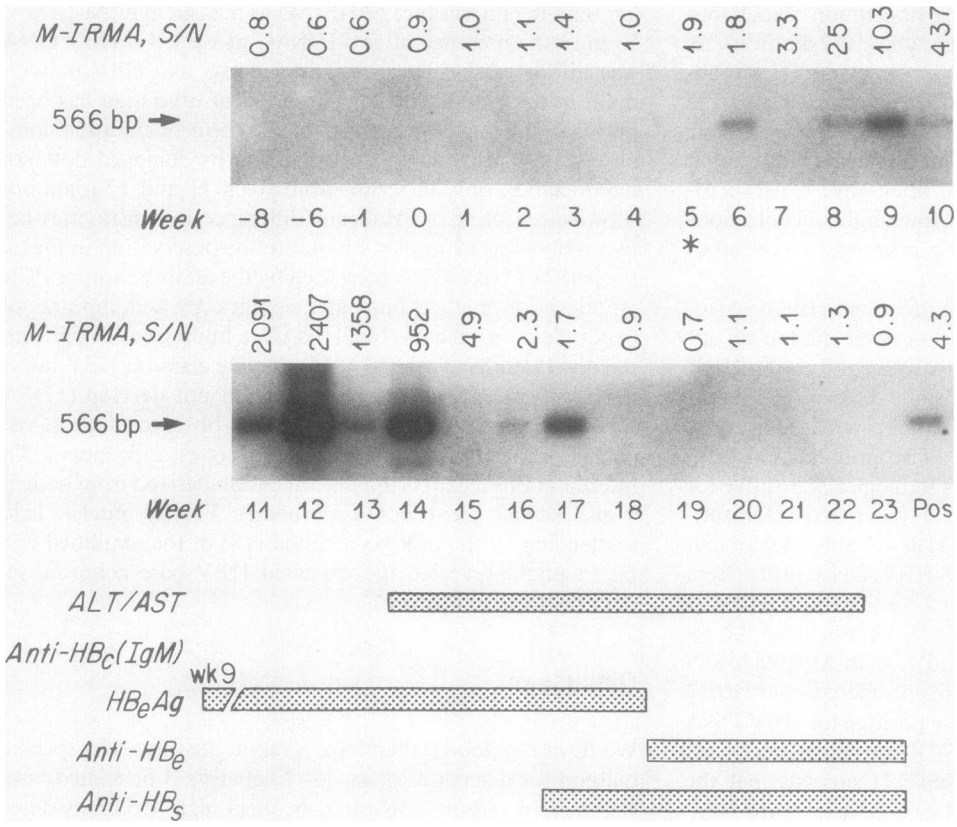

Figure 4. The serological course and detection of HBV DNA during experimental infection of a chimpanzee. 3 ml of serum from patient 9 (Fig. 3) was inoculated at week 0. Serum samples were also obtained several weeks before inoculation. The chimpanzee was bled weekly and serum analyzed for HBV DNA by affinity capture-PCR (35 cycles; autoradiogram represents a 3-h exposure), transaminases and other HBV serologic markers. *A longer exposure and repeat assay demonstrated a weak signal in serum at week 5.

cations in S and C regions that may lead or contribute to antigenic variations (19, 20). Indeed, a recent report demonstrated that 50% of HBsAG negative patients with anti-HBsAG and anti-HBcAG and chronic liver disease have HBV sequences in serum following phenol extraction and PCR amplification using S region primers (21).

The use of high affinity MAb capture followed by PCR amplification should provide a rapid and sensitive assay for the detection of any encapsulated viral genome assuming that the appropriate MAbs are available and well characterized. For example, one would only need to know the genomic sequence of the virus and have a specific antibody with high affinity directed against an envelope antigen(s). The MAb capture technique serves as the major purification step of the virion from complex biologic fluids such as serum, urine, cerebral spinal fluid, saliva, or even stool, and also helps to assure the specificity of the assay. Appropriate selection of oligonucleotide primers and probes will further enhance specificity. The use of type-specific MAb and oligonucleotide probes may identify the presence of various viral subtypes. The immunoassay described here is simple and rapid and provides a degree of specificity and sensitivity that far exceeds conventional viral diagnostics and serologic antibody tests.

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