

Identification and transmission of hepatitis B virus-related variants

(hepatitis B virus DNA/hepatitis B surface antigen/radioimmunoassay)

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ABSTRACT We have identified long-incubation viral agents that share epitopes with hepatitis B virus (HBV). During chimpanzee infectivity studies, these agents may be recognized in the liver since they possess complementary nucleic acid sequences with HBV DNA; the genomic size was found to be 3.2 kilobases, identical to that of HBV. Liver injury was produced and there was antigen expression in hepatocytes. Chimpanzees were not protected by prior immunization with hepatitis B surface antigen; conversely, they were still susceptible to HBV after recovery from infection with such agents. These findings suggest that these hepatitis B virus-related variants appear to be immunologically distinct from HBV.

Previous studies on sera using monoclonal anti-hepatitis B surface antigen antibody (anti-HBsAg) radioimmunoassays (M-RIAs) have established that there are individuals with acute and chronic hepatitis infection and hepatocellular carcinoma whose serum demonstrates positive binding activity only in these tests (1-8). Such patients were found to be negative for hepatitis B surface antigen (HBsAg) by polyclonal anti-HBsAg RIA (P-RIA) and some lacked evidence of recent or past exposure to hepatitis B virus (HBV) as shown by the absence of antibodies to the core (anti-HBcAg) and to surface antigens (anti-HBsAg) in the blood (4-8). HBV DNA hybridizable sequences were detected in the liver and serum of a significant number of such individuals and this finding correlated with the detection of antigen in serum by M-RIAs (4, 6). However, very little is known regarding the biologic properties and characteristics of the M-RIA HBV DNA-positive agents in serum. We report here the identification, transmission, and partial characterization of some viral agents during chimpanzee infectivity studies. These hepatitis viral variants appear to be antigenically related to and share nucleic acid homology with HBV.

METHODS

Sera from two individuals, designated G and M, were selected for inoculation into four chimpanzees. Patient G is a 44-year-old alcoholic with no clinical evidence of liver disease. HBsAg was undetectable by P-RIA but his serum was positive for anti-HBsAg and anti-HBcAg antibodies [AUSAB and CORAB, respectively (Abbott)]. The M-RIA measured binding activity at a S/N ratio of 3.51 (normal, <2.1), defined as the mean cpm of the patient G serum divided by the mean cpm of the negative control serum. Dot blot hybridization analysis with a ³²P-labeled HBV DNA probe revealed a weakly positive signal in the serum. Patient M was a 38-year-old with primary hepatocellular carcinoma.

His serum was negative for HBsAg, anti-HBsAg and anti-HBcAg antibodies by P-RIAs but was found to be reactive at a S/N ratio of 24.0 by M-RIA, and HBV DNA complementary sequences were detected by dot blot analysis.

Two chimpanzees (Dolf and Ianthe) were previously immunized with a yeast recombinant HBV DNA-derived HBsAg vaccine. These chimpanzees had been subsequently challenged with a standard National Institutes of Health HBV inoculum known to produce infection in unimmunized chimpanzees; both animals were found to be protected from HBV infection. The details of these experiments have been reported elsewhere (9). Two other chimpanzees (Sjef and Jimmie) had not been previously exposed to HBV or to the non-A, non-B hepatitis agents, and their serum was negative for HBsAg, anti-HBsAg, and anti-HBcAg antibodies. In contrast, Dolf and Ianthe had high titer anti-HBsAg antibodies as the result of past vaccination with HBsAg; anti-HBcAg antibodies, however, were not present. Ianthe and Sjef were inoculated intravenously with 3 ml of serum M, and Jimmie and Dolf were inoculated with 3 ml of serum G. After injection of serum, the chimpanzees were serially bled for measurement of alanine aminotransferase values, HBsAg, anti-HBsAg, and anti-HBcAg titers, and M-RIAs and HBV DNA hybridization studies. In addition, serial liver biopsies were performed for HBV DNA hybridization analysis as well as for immunoperoxidase staining of hepatocytes for antigen expression using a monoclonal anti-HBsAg antibody.

Details of the immunization protocols, cell fusion techniques, and growth and cloning of the hybridomas producing anti-HBsAg antibodies have been reported (10). Monoclonal antibodies designated 5D3, 5C3, and 5C11 (IgM, IgG_{2a}, and IgG₁, respectively) were selected from a library of monoclonal antibodies for use in the M-RIAs since they recognize all known subtypes or viral strains of HBV (11), distinguish separate and distinct determinants on HBsAg (3), and possess high-affinity constants for epitopes on the viral protein (10). A multiple-site "simultaneous sandwich" RIA using all three antibodies for measurement of HBsAg-associated epitopes in serum including the sensitivity and specificity of the assay has been reported (7, 8). In addition, we performed single HBsAg-associated determinant analysis with the M-RIAs where, for example, 5D3 was linked to the solid-phase support and also served as the ¹²⁵I-labeled tracer antibody, as well as using 5C11 and 5C3 separately as the radiolabeled indicator antibodies.

The spot test ³²P-labeled HBV DNA hybridization studies were performed on 200 μl of serum as described elsewhere (6, 12). Approximately 10-15 μg of cellular DNA was obtained from small-needle liver biopsies for Southern blot analysis.

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Abbreviations: HBsAg, hepatitis B surface antigen; M-RIA, monoclonal anti-HBsAg antibody RIA; P-RIA, polyclonal anti-HBsAg antibody RIA; HBcAg, hepatitis B core antigen; HBV, hepatitis B virus; kb, kilobase(s).

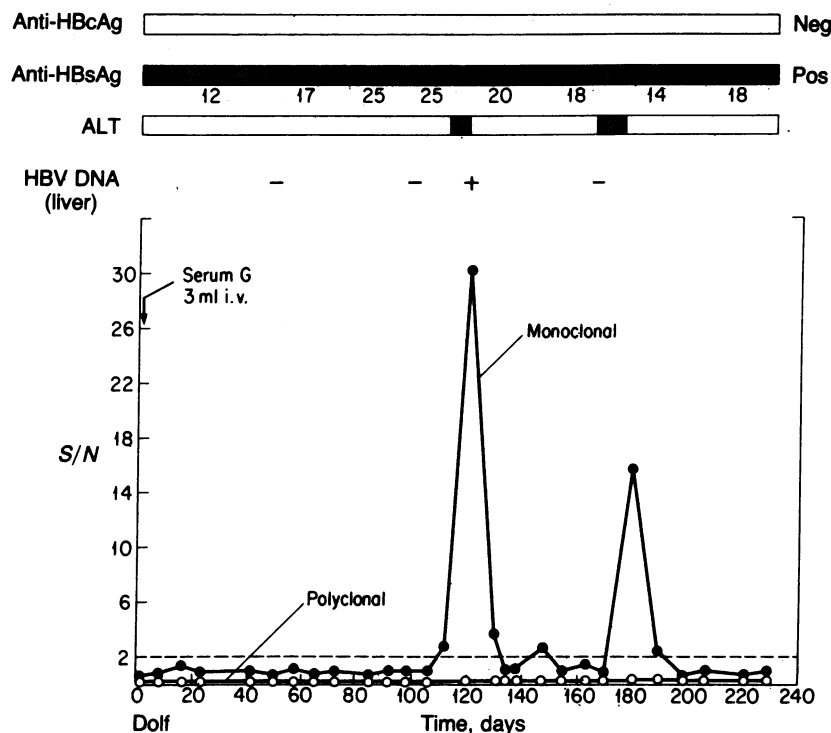


FIG. 1. Serologic course in a HBsAg-vaccinated chimpanzee (Dolf) after inoculation with serum G demonstrating the presence of a long incubation agent. The period of antigenemia is defined by the multisite M-RIA binding activities using antibodies 5D3, 5C3, and 5C11. The antigen was reactive to all three antibodies as shown by separate 5D3-5D3, 5D3-5C3, and 5D3-5C11 RIAs performed on day 120 (data not shown). The numbers below the anti-HBsAg bar are the S/N ratios, indicating the presence of high titer protective anti-HBsAg antibodies. There were two periods of alanine aminotransferase (ALT) elevations (>40 IU/ml). Light microscopy of liver tissue performed at the time of HBV DNA hybridization analysis showed that the tissue was normal.

Because of the very small amount of cellular DNA availability, it was only digested with *EcoRI* restriction enzyme prior to gel electrophoresis and subsequent hybridization with cloned ³²P-labeled HBV DNA under high-stringency conditions; the sensitivity and specificity of these techniques have been reported (6, 13).

RESULTS

Figs. 1 and 2 demonstrate the course of infection after inoculation with serum G performed under two experimental conditions—namely, in the presence or absence of pre-existing protective anti-HBsAg antibodies. Fig. 1 illustrates

that Dolf developed two episodes of antigenemia with antigen titers reaching a maximum at approximately 120 and 180 days. Thus, the presence of high titer anti-HBsAg antibodies failed to prevent infection and the development of antigenemia. It is also noteworthy that this chimpanzee did not develop anti-HBcAg antibodies during infection or after recovery; HBsAg was not detected by P-RIA. Alanine aminotransferase levels were elevated [>40 international units (IU)/ml] at the time of antigen detection by the M-RIAs.

HBV DNA complementary sequences were identified in the liver but not in serum at the peak of antigenemia; such sequences were undetectable during the incubation period, as shown in Fig. 3. The genomic size was found to be 3.2

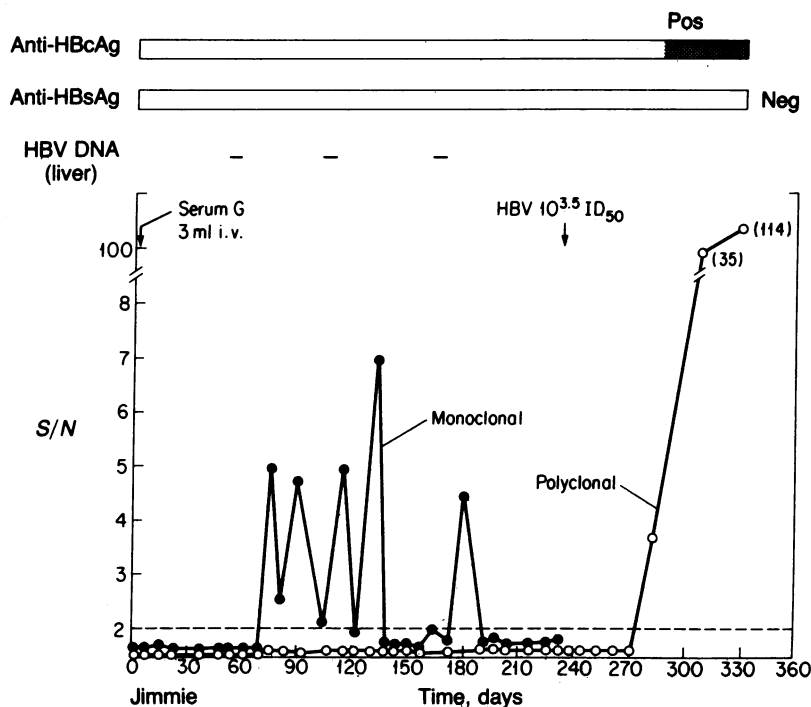


FIG. 2. Serologic course in a non-HBV-exposed chimpanzee (Jimmie) also inoculated with serum G, demonstrating an incubation period of 70 days and a prolonged period of antigenemia. After recovery from infection, the chimpanzee was still susceptible to a challenge with native HBV (arrow) and HBsAg and anti-HBcAg antibody were identified in serum. Histologic examination of the liver on day 100 revealed focal hepatocyte necrosis characterized by acidophilic degeneration of hepatocytes surrounded by mononuclear cells. In addition, there were lobular and sinusoidal inflammatory infiltrates, consistent with mild acute viral hepatitis. The liver histology was normal on day 40.

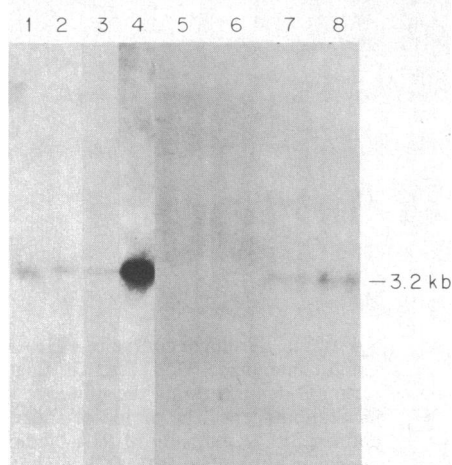


FIG. 3. HBV DNA hybridization pattern by Southern blot analysis of liver tissues obtained from chimpanzees during the course of infection with agents present in sera G and M. Lanes: 1 and 2, liver biopsies taken from Ianthe on days 90 and 138 and hybridized with ^{32}P -labeled HBV DNA (see Fig. 5); 3, liver biopsy from Dolf on day 120 (see Fig. 1); 4, a HBV DNA-positive control; 5 and 6, biopsies from Sjef taken during the incubation period on days 70 and 120, respectively; 7 and 8, results obtained from Sjef on days 180 and 210 during the period of antigenemia as measured by the M-RIAs (see Fig. 6). Exposure time was 72 hr. Note that the hybridization signals are weak compared to the HBV DNA-positive control. Each lane represents 12–15 μg of cellular DNA digested with *Eco*RI. The finding of a single 3.2-kb band in infected animals was consistent with either free monomeric HBV DNA complementary sequences or integration at multiple sites into the cellular DNA with a dimeric organization. No viral DNA replicative forms were identified and HBV DNA hybridization sequences were not detected in 200 μl of serum by a separate dot blot test.

kilobases (kb), a size identical to the HBV genome (14). In addition, there was low-level antigen expression on the plasma membrane and in the cytoplasm of hepatocytes at day 120 but not at day 50, as shown by the immunoperoxidase staining reaction with monoclonal antibody 5C3 (Fig. 4). The other chimpanzee (Jimmie) exhibited an incubation period of 70 days; the duration of antigenemia was far longer in this animal without prior exposure to HBV, as shown in Fig. 2. Similar to the results observed in Dolf (Fig. 1), anti-HBcAg and anti-HBsAg antibodies did not develop during active infection and after recovery. However, Jimmie was still susceptible to HBV and developed HBsAg and anti-HBcAg antibodies during infection as measured by P-RIAs (Fig. 2). Thus, there was no cross-protection induced by the agent present in serum G with HBV. HBV DNA-hybridizable sequences were not detected in liver biopsy specimens, and they also stained negative for antigen with monoclonal anti-HBsAg 5C3.

Figs. 5 and 6 depict the course of infection in chimpanzees Ianthe and Sjef after inoculation with serum M under similar experimental conditions. Infection as defined by the detection of antigen in the circulation occurred in the HBV immune chimpanzee (Ianthe) with pre-existing high titer anti-HBsAg antibodies as well as in Sjef, not previously exposed to HBV. Similar to the previous studies with serum G, anti-HBsAg and anti-HBcAg antibodies did not develop during antigenemia or after recovery. HBV DNA complementary sequences were identified in the liver of both animals at the time of antigen detection in serum but not during the incubation period (Fig. 3); the genomic size of the agents transmitted by serum M also was found to be 3.2 kb. Cytoplasmic and membranous antigen expression was demonstrated in hepatocytes by immunoperoxidase staining in Ianthe, but in Sjef, hepatocytes stained negative (data not shown).

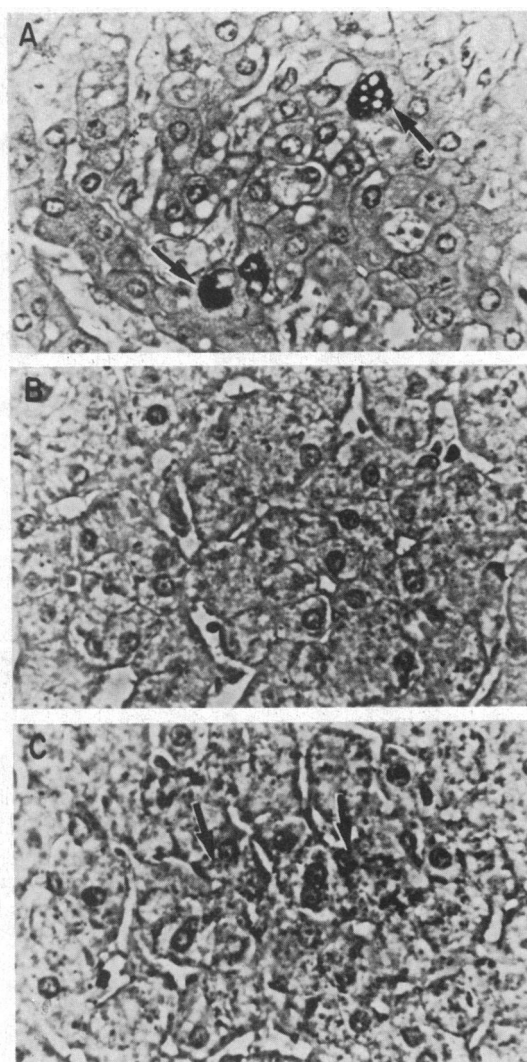


FIG. 4. Immunoperoxidase staining of liver tissue with monoclonal anti-HBsAg antibody 5C3. (A) Staining reaction of liver derived from a known human HBsAg-positive carrier, demonstrating the intensity of the immunoperoxidase staining reaction. (B) Liver biopsy specimens from Dolf (see Fig. 1) at day 40 during the incubation period; a positive staining reaction was not observed. (C) Positive staining reaction of hepatic tissue from Dolf (arrows) on day 120 at the time HBV DNA hybridizable sequences were also present in the liver.

DISCUSSION

We have demonstrated the presence of transmissible agents in M-RIA reactive HBV DNA positive serum derived from two individuals, one of whom had no recent or past exposure to HBV, as shown by the absence of HBsAg and anti-HBsAg and anti-HBcAg antibodies. The present results confirm and extend our previous observations—namely, that there are patients with acute and chronic hepatitis and hepatocellular carcinoma reactive for HBsAg-associated epitopes in serum and HBV DNA complementary sequences in liver and serum that are not detectable by conventional assays (6). The infectious potential of such serum has now been established and we are led to believe from our experiments that these long incubation agents share epitopes and nucleic acid homology with HBV. For example, the monoclonal antibodies used in the RIAs designated 5D3, 5C11, and 5C3 distinguish *a* domain epitopes on HBsAg and therefore have been found to identify all known strains of HBV (11). It is noteworthy that prior immunization with a HBV-derived recombinant HBsAg

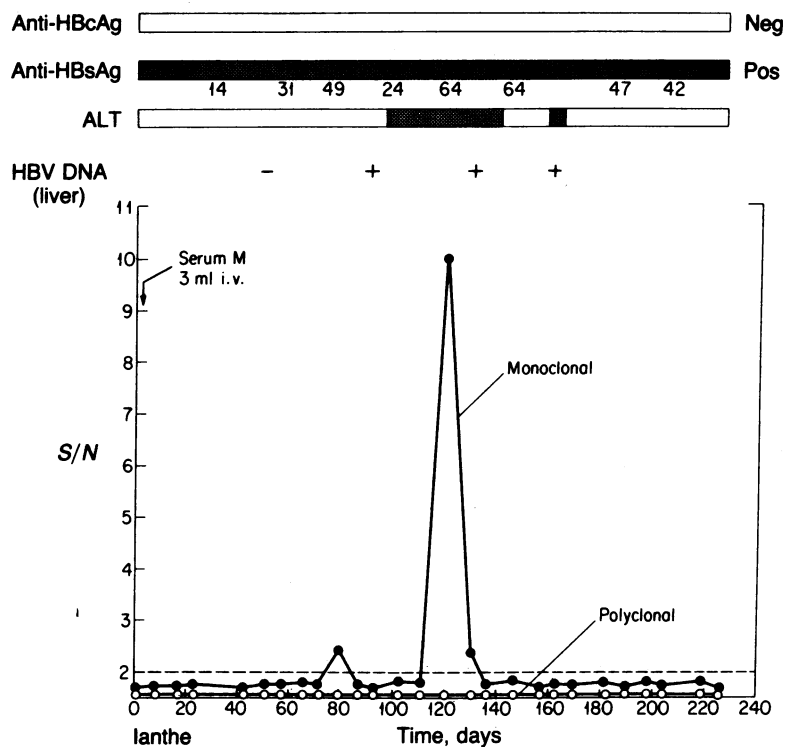


FIG. 5. Serologic course in a HBsAg-vaccinated chimpanzee (lanthe) inoculated with serum M. The incubation period was ≈110 days. Antigenemia as measured by the multisite M-RIA was associated with a prolonged elevation of alanine aminotransferase (ALT) values. Liver histology was normal during the entire course of infection, but on day 140 hepatocytes were positive for antigen expression by immunoperoxidase staining with monoclonal anti-HBsAg antibody 5C3 (data not shown).

vaccine protects against infection with native virus but does not afford protection to chimpanzees inoculated with these HBV-related variants. More importantly, after recovery from this infection, one animal was still susceptible to HBV as shown by the appearance of HBsAg and anti-HBsAg

antibody in the blood. There appears, therefore, to be little if any cross-protection, a finding consistent with an interpretation that these agents are immunologically distinct from HBV. Furthermore, anti-HBcAg and anti-HBsAg antibodies were not measurable after infection with such HBV-related

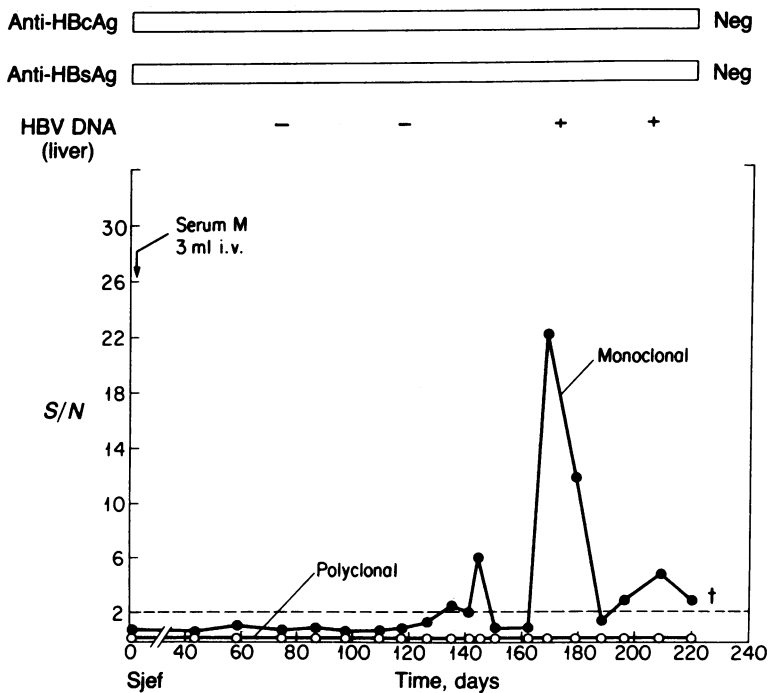


FIG. 6. Serologic course in a non-HBV-exposed chimpanzee (Sjef) inoculated with serum M. There was a long inoculation agent in the serum as demonstrated by two antigen peaks at approximately 140 and 170 days, as measured by multisite M-RIA. The 5D3, 5C3, and 5C11 epitopes were present on the antigen by the individual single determinant assays (see text). Liver histology was normal. †, Death unrelated to infection.

variants, and this finding may reflect in part the antigenic differences between the virus(es) or a lack of a host immune response to low levels of virus.

HBV infection and these variants seem to have similar biologic properties. Indeed, we found that there was an incubation period of >50 days. At the genomic level, HBV DNA complementary sequences were detected in the liver during antigenemia as defined by the multisite M-RIA binding results but not during the incubation period. The size of the genome is identical to that of HBV. It should be noted that the ³²P-labeled HBV DNA hybridization signal was weak; this observation may be due in part to a low concentration of virus in the liver, and/or there may be only a partial nucleic acid homology with HBV. Similarly, the level of antigen present in serum is probably quite low. If there is any parallel between the present findings and our previous studies with HBsAg, as measured by the same multisite M-RIA, one would predict that the level of binding activity exhibited in chimpanzee sera would represent HBsAg equivalent concentrations ranging between 30 and 150 pg/ml; such levels would not be detectable by a P-RIA (7). These results may reflect either low antigen expression by the viral agents, or a substantially different antigenic composition compared to HBsAg, or both. It is noteworthy, however, that antigen expression was detected in hepatocytes derived from two chimpanzees during active infection with monoclonal antibody 5C3, the same antibody used in the M-RIAs.

The present findings are of interest, especially in view of the recent observations on the considerable antigenic heterogeneity of HBsAg reflecting the existence of several new HBV strains; these subtypes were only identified by M-RIAs (11, 15). In addition, viral replication may occur without an anti-HBsAg and anti-HBcAg immunologic response. The genomic size of 3.2 kb suggests that there have not been major nucleic acid deletions compared to HBV. It will be of interest to clone and sequence the genome of these presumed variants and to compare directly their nucleic acid composition to the known sequences of HBV (16).

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