Detection and transmission in chimpanzees of hepatitis B virus-related agents formerly designated "non-A, non-B" hepatitis*

(monoclonal antibody to hepatitis B surface antigen/recombinant hepatitis B virus DNA probe/molecular hybridization)

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ABSTRACT Four chimpanzees have been infected with three different inocula containing "non-A, non-B" hepatitis virus(es). After inoculation, serial studies established the presence of antigenemia or viremia (or both) by radioimmunoassay with high-affinity monoclonal antibodies directed toward separate and distinct determinants on hepatitis B surface antigen (HBsAg) and by molecular hybridization analysis using a cloned hepatitis B virus (HBV) DNA probe. In contrast to results observed during HBV infection, elevations in serum alanine aminotransferase values indicate that liver injury preceded antigenemia by several weeks. Thus, the time between inoculation and development of antigenemia (incubation period) varied from 64 to 190 days and, in some cases, single or multiple episodes of antigenemia or viremia occurred in the absence of elevated aminotransferase levels. In this study, two chimpanzees were high-titer positive for antibodies to HBsAg (anti-HBs) from previous infection with HBV, suggesting that the antigenic composition of HBV-related virus(es) is substantially different from that of HBV, since naturally occurring anti-HBs antibodies were not protective. Demonstration of HBV-related virus(es) by the methods used in this study of experimental hepatitis infection in chimpanzees should now permit detection, isolation, and characterization of these previously elusive agents.

The development of sensitive diagnostic serologic techniques during the last several years has greatly facilitated understanding of the clinical, epidemiologic, antigenic, and molecular characteristics of hepatitis A and B viruses (1). However, acute and chronic viral hepatitis may occur without specific serologic markers for either the A or B agents. Thus, the term, "non-A, non-B" infectious hepatitis virus(es) has evolved (2-3). Indeed, these virus(es) are responsible for the majority of cases of acute and chronic posttransfusion hepatitis that represent a major problem and 25-30% of sporadic acute viral hepatitis may be due to such agents (4).

Epidemiologic studies suggest that non-A, non-B hepatitis shares many features with hepatitis B with respect to clinical severity, transmission by blood products or close personal contact, a chronic carrier state with or without evidence of ongoing liver injury, a long incubation period (>4 to 5 wk), and the tendency to progress to chronic liver diseases, cirrhosis, and death (5-10). Recent infectivity studies in chimpanzees show transmissibility of a hepatitis-like illness by such agents even in the presence of pre-existing antibodies to hepatitis B surface antigen (anti-HBs) (11-15). Despite intensive investigative efforts, however, the non-A, non-B hepatitis virus(es) have not yet been identified and characterized and this represents one of the greatest challenges in hepatitis research (16). During experimental viral hepatitis studies in chimpanzees, we have identi-

fied and partially characterized infectious hepatitis B virus (HBV)-related agents that, heretofore, would have been called non-A, non-B hepatitis virus(es).

MATERIALS AND METHODS

Radioimmunoassays (RIAs). The immunization protocols, characteristics and purity of the hepatitis B surface antigen (HBsAg), cell fusion technique, and growth and cloning of hybridomas producing anti-HBs antibodies have been reported (17). Such antibodies have been characterized with respect to specificity for determinants on HBsAg, ability to agglutinate erythrocytes coated with HBsAg (subtypes adw and ayw), antibody class and subclass, and affinity for HBsAg-associated epitopes (17-24). Four anti-HBs were selected from a library of monoclonal antibodies because they recognize distinct and separate determinants of the following subtypes of HBsAg: adw₂, adw₄, adr, ayw₁, ayw₂, ayw₃, ayw₄, ayr, and adyw (25). The monoclonal antibodies 5D3 and 3D4 (IgM), 5C3 (IgG2a), and 5C11 (IgG1) have affinity constants of 4×10^{11} , 8×10^{10} , 4×10^{10} , and 1×10^{10} liters/mol per molecule, respectively.

The 5D3 anti-HBs was coupled to a solid-phase support (18) and the other IgM and IgG antibodies were radiolabeled with ¹²⁵I to a specific activity of 8-10 μ Ci/ μ g (1 Ci = 3.7×10^{10} becquerels) by the technique of Bolton and Hunter (26). Chimpanzee serum samples were analyzed with "simultaneous sandwich" RIAs (18). In brief, 5D3-coated beads were incubated with 50-100 μ l of chimpanzee serum and 100 μ l (150,000 cpm) of ¹²⁵I-labeled IgM or IgG monoclonal antibody for 16 hr at 45°C. The solid-phase support was washed three times with distilled water and the radioactivity bound to the beads was measured with a Packard γ -well counter.

HBV DNA Hybridization Studies. For molecular hybridization studies, DNA was isolated from 250 μ l of serum by digestion with proteinase K at 200 μ g/ml for 2-4 hr at 37°C in the presence of 0.5% NaDodSO₄, extraction with phenol/chloroform/isoamyl alcohol, 50:48:2 (vol/vol), and ethanol precipitation. Precipitated material was suspended in 10 μ l of 10 mM Tris-HCl, pH 7.4/1 mM EDTA, spotted and fixed onto a nitrocellulose filter, and hybridized with recombinant cloned and repurified ³²P-labeled HBV DNA as reported (27-30).

Infectivity Studies. Two chimpanzees were inoculated with 1 ml of serum derived from an individual whose blood had been incriminated in transmitting non-A, non-B hepatitis. A third

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; RIA, radioimmunoassay; anti-HBs, antibodies to hepatitis B surface antigen; anti-HBc, antibodies to hepatitis B core antigen; AlaATase, alanine aminotransferase; P/N, cpm_{primate experimental}/mean cpm_{negative controls}.

*This is paper no. 4 in a series. Papers 1, 2, and 3 are refs. 20, 21, and 30, respectively.

FIG. 1. Clinical and virologic course of non-A, non-B hepatitis in chimpanzee 1. Note that elevations in AlaATase values (○) precede the appearance of antigen and HBV-related DNA sequences in the blood. RIAs: ●, monoclonal IgM; △, Ausria II; ▲, monoclonal IgG1 and IgG2a. ↓, Inoculation.

chimpanzee was injected with 40 ml of a clotting factor 8 concentrate previously shown to transmit non-A, non-B hepatitis to recipients. The fourth chimpanzee was inoculated with 1 ml of serum from another individual considered to harbor a non-A, non-B hepatitis agent. Serial measurements were carried out on sera, including immunoreactivity by four monoclonal anti-HBs RIAs, the presence of HBV DNA-related sequences by molecular hybridization analysis, HBsAg by the Ausria II RIA, and antibodies to hepatitis B core antigen (anti-HBc) and anti-HBs (Corab and Ausab, respectively; Abbott). On selected samples, IgM antibody to hepatitis A virus (Havab; Abbott) was also measured.

RESULTS

Observations in chimpanzee 1 (with pre-existing anti-HBs) after inoculation with the clotting factor concentrate are shown in Fig. 1. Evidence of liver injury was first apparent on day 40 with a rise in alanine aminotransferase (AlaATase) levels to 70 units/liter (normal, <38 units/liter); AlaATase elevations persisted for ≈ 35 days. Immunoreactive antigen appeared transiently at a low titer after inoculation of 40 ml of clotting factor concentrate and then disappeared from the circulation. On day 64, there was a striking rise in serum IgM anti-HBs binding activity, from 50 to 4,010 cpm [$\text{cpm}_{\text{primate experimental}}/\text{mean cpm}_{\text{negative controls}}$ (P/N) ≈ 80 , normal < 2.1]. Antigenemia continued for 56 days although titers fell with resolution of hepatitis. It is noteworthy that AlaATase levels returned to normal by day 78 but antigen was detected in the serum for an additional 42 days. Most importantly, the rise in serum AlaATase preceded development of antigenemia by approximately 30 days and thus provides a more accurate description of the incubation period (i.e., 64 days). We did not detect the 5C3 or 5C11 determinants in serum by RIAs, indicating that the virus transmitted to this chimpanzee is antigenically distinct from HBV. Finally, Ausria II was unreactive during the course of infection and both anti-HBc and anti-hepatitis A antibodies were undetectable.

Studies were then carried out to compare the appearance of antigen in the blood with the presence of HBV DNA-hybridiz-

able sequences. By molecular hybridization analysis, HBV-related nucleic acid sequences were not detected during the incubation period. However, as the antigen titer began to rise, nucleic acid material that hybridized to the HBV DNA probe was present in serum (Fig. 1), suggesting that virions were probably released into the circulation. The strengths of the hybridization signals with DNA extracted from the sera of three chimpanzees injected with separate non-A, non-B hepatitis inocula are shown in Fig. 2. Spots 1–3 (negative) are from a chimpanzee who developed monoclonal RIA immunoreactivity after inoculation with one non-A, non-B hepatitis serum; spots 4–6 (positive) are from chimpanzee 1, who had been inoculated with the factor 8 concentrate (immunological data shown in Fig. 1); spots 8 and 9 (positive) are from chimpanzee 2, who had been inoculated with a separate serum specimen known to transmit non-A, non-B hepatitis (see Fig. 3); and spots 7 and 10 are DNA extracts from negative control sera.

The clinical and virologic course of chimpanzee 2 (with pre-existing anti-HB) after inoculation with 1 ml of serum carrying a non-A, non-B hepatitis agent is shown in Fig. 3. In contrast to chimpanzee 1, there was no rise in serum AlaATase levels during the observation period. A high level of antigenemia was observed ($P/N \approx 175$) with an incubation period of ≈ 190 days.

FIG. 2. Detection of HBV DNA sequences by molecular hybridization analysis in sera from chimpanzees 1 and 2 with non-A, non-B hepatitis. *, Positive results with recombinant cloned HBV DNA probe. Spots 1, 2, and 3 were negative for HBV DNA but positive for antigen by IgM anti-HBs RIA (see Figs. 4 and 5). Spots 4, 5, and 6 (chimpanzee 1) were positive for both antigen and HBV DNA sequences in serum on days 47, 58, and 64 (Fig. 1). Spots 8 and 9 (chimpanzee 2) were positive on days 190 and 204 (see Fig. 3). Spots 7 and 10 are negative controls.

FIG. 3. Clinical and virologic course of non-A, non-B hepatitis in chimpanzee 2. Despite infection, as shown by the presence of viral antigen and HBV DNA-related sequences, serum AlaATase values remained normal. Symbols are as in Fig. 1.

Antigenemia was prolonged (≈ 65 days) with a return to undetectable levels by day 260. Other HBV-related epitopes were absent as determined by the monoclonal RIAs and by HBsAg (Ausria II), as well as anti-HBc and anti-hepatitis A antibodies. Similar to chimpanzee 1 (Fig. 1), HBV DNA sequences in chimpanzee 2 were undetectable during the incubation period but were present at the peak of monoclonal IgM RIA binding activity.

The clinical and virologic courses of chimpanzees 3 and 4, each inoculated with 1 ml of serum from a separate individual who had been incriminated in transmitting non-A, non-B hepatitis, is shown in Figs. 4 and 5. For chimpanzee 3, evidence of liver injury as demonstrated by AlaATase elevation was apparent on day 50 and persisted with a relapsing pattern for 140 days. Antigenemia appeared on day 92 and the titers gradually fell by day 130 and became undetectable by day 180. The mag-

FIG. 4. Clinical and virologic course of non-A, non-B hepatitis in chimpanzee 3. Similar to Fig. 1, AlaATase elevations precede the appearance of antigen as detected by the monoclonal IgM anti-HBs RIA by ≈ 45 days. Symbols are as in Fig. 1.

FIG. 5. Clinical and virologic course of non-A, non-B hepatitis in chimpanzee 4. Note the presence of three well-defined peaks of antigenemia as measured by the monoclonal IgM anti-HBs RIA. Symbols are as in Fig. 1.

itude of peak binding activity by monoclonal RIA was less than that observed in previous animals ($P/N \approx 10$). It should be noted that anti-HBs was not present at the time of inoculation or during hepatitis infection and recovery nor were other HBV-associated epitopes, HBsAg, anti-HBc, and anti-hepatitis A antibodies. Similar to chimpanzee 1 (Fig. 1), the rise in serum AlaATase levels preceded the appearance of antigen in the blood by ≈ 50 days. HBV DNA-hybridizable sequences were not detected. For chimpanzee 4, inoculated with the same serum, however, AlaATase elevations did not occur. This was similar to the pattern observed for chimpanzee 2. There were three well-defined spikes of antigenemia with the highest value occurring on day 164. HBV-related DNA sequences were not detectable during any of the episodes of antigenemia.

DISCUSSION

We have used high-affinity IgG and IgM monoclonal anti-HBs antibodies to construct sensitive and specific RIAs to measure HBsAg-associated epitopes in serum (18, 20, 21, 30). During the course of clinical studies with these antibodies, many serum samples from patients with acute and chronic posttransfusion hepatitis and with acute and chronic sporadic hepatitis with or without anti-HBs and anti-HBc antibodies were reactive in monoclonal RIAs (18, 20, 21, 30). Although some of the positive results could be explained on the basis of enhanced sensitivity of the monoclonal assays for a HBsAg-associated determinant(s), others could not. It was also observed that monoclonal anti-HBs RIAs detected HBsAg-related epitopes in immune complexes formed in anti-HBs excess whereas polyvalent anti-HBs antibodies were unreactive. Thus, the monoclonal RIAs identified hidden viral antigens in immune complexes in clinically significant situations (30), consistent with recent *in vitro* observations (31). However, a substantial number of sera from patients with acute and chronic hepatitis were highly reactive in the monoclonal RIAs ($P/N > 20$) but lacked serologic evidence of hepatitis B or A. Therefore, we were not able to explain

the high binding activity in such sera either by increased sensitivity of the monoclonal anti-HBs RIAs or by the presence of immune complexes.

Additional experiments were carried out whereby the high binding activity was affinity purified from larger volumes of serum by using the monoclonal antibody of interest (21). After concentration, such material became reactive with polyvalent anti-HBs in the Ausria II RIA. This finding together with the observation that prior incubation of antigen with monoclonal anti-HBs blocked the binding of polyvalent anti-HBs suggested that a small amount of antibody in polyvalent antisera is like the monoclonal anti-HBs. It was apparent from other studies that these antigens were quite distinct from HBsAg with respect to epitope composition (21). Further evidence for this concept was provided by showing that the immunoreactive material detected only by monoclonal RIAs shared several common peptides with HBsAg but there were also other distinct peptides (21). In addition, immunoelectron microscopy of the monoclonal affinity-purified material revealed particles in the same size range as native HBsAg (22 nm). Furthermore, monoclonal anti-HBs was visible on the surface of such particles and the molecular weight exceeded 2×10^6 . Finally, detailed analysis of this binding activity in selected serum samples by different IgG and IgM monoclonal anti-HBs demonstrated additional HBsAg-related determinants as well (20).

Molecular hybridization experiments were then carried out with selected serum samples and a cloned HBV DNA probe (30). HBV DNA sequences related to or homologous with HBV were detected in 36% of these sera. This provided further evidence that HBV-like or related agents exist in man. Finally, since HBV DNA sequences and monoclonal anti-HBs-immunoreactive antigen were demonstrated both in blood donors and in recipients who subsequently developed non-A, non-B hepatitis (30), it seemed likely that the agent(s) identified by both techniques was infectious.

In the present study, we have been able to reproduce our previous human findings in chimpanzees—an accepted ex-

perimental animal model of non-A, non-B hepatitis. The major observations in the present investigation include (i) three different inocula injected into four animals were infectious; (ii) the incubation period, defined as the time from inoculation to the appearance of virus or viral protein in the blood, is longer than previously recognized; (iii) serum AlaATase elevations may precede the appearance of antigenemia by several weeks; (iv) antigenemia may occur in the absence of AlaATase elevations, a phenomenon identical to that observed in man (10); (v) the presence of antigen in the blood, as measured by the monoclonal IgM anti-HBs RIA, correlates well with the appearance of HBV DNA-like sequences by molecular hybridization analysis; (vi) the period of antigenemia or viremia may persist for weeks to months and usually disappears with recovery; (vii) antigenemia is still detectable in the resolution phase of illness when AlaATase levels are normal, a finding similar to HBV infection in man; (viii) several episodes of antigenemia may occur during the course of infection; (ix) pre-existing anti-HBs was not protective and thus the HBV-related virus is sufficiently different in antigenic composition from HBV. In support of this concept is the finding that polyvalent anti-HBs antibodies (Ausria II) and other monoclonal anti-HBs that recognize different HBsAg-associated epitopes were unreactive. Taken together, these and our previous studies (18, 20, 21, 30) provide strong evidence that non-A, non-B hepatitis agents in many circumstances may be related to but are distinct variant(s) of HBV.

Recent studies on viral antigenic structure using monoclonal antibodies suggest that there may be subtle but important antigenic differences among closely related viral strains. For example, the use of monoclonal antibodies has shown heterogeneity in the rabies virus; several rabies-related viral strains sharing common antigenic determinants have been identified (32). Similar important structural modifications in viral determinants have been found in the influenza virus by means of monoclonal antibody binding (33). Finally, there is a common antigenic relationship between herpes strains types 1, 2, and 3 and among the arenaviruses that can only be demonstrated by monoclonal antibodies (34–36). A similar phenomenon may be occurring with non-A, non-B hepatitis virus(es) in relationship to HBV.

Special comment is required regarding the finding that HBV-related nucleic acid sequences were not detected in two chimpanzees despite the presence of high titers of antigen. One possible explanation is that much more antigen was released into the circulation than intact virions, so that viral DNA was not detected when antigen was clearly present. Since 250 μ l of serum was used for each analysis and the lower limit of sensitivity of the hybridization method is less than 0.1 pg of HBV DNA sequence, this is unlikely. Most probably, more than one human hepatitis B-related virus exists but these agents are sufficiently different so that HBV DNA hybridization with the current probe may not occur. These and other questions can be addressed by isolating hepatitis B-related viruses made possible by the use of a combined immunological and DNA hybridization approach. Thus, we may now be able to define the properties of these elusive non-A, non-B agents, which produce significant human morbidity and mortality.

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