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Monoclonal radioimmunoassays for hepatitis B surface antigen: Demonstration of hepatitis B virus DNA or related sequences in serum and viral epitopes in immune complexes*

(hybridoma/antibodies to hepatitis B surface and core antigens/molecular hybridization/recombinant DNA probe)

DAVID A. SHAFRITZ†, HARVEY M. LIEBERMAN†, KURT J. ISSELBACHER‡, AND JACK R. WANDS‡

†The Liver Center and the Departments of Medicine and Cell Biology, Albert Einstein College of Medicine; 1300 Morris Park Avenue, Bronx, New York 10461; and ‡Gastrointestinal Unit, Massachusetts General Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02114

Contributed by Kurt J. Isselbacher, June 14, 1982

ABSTRACT **High-affinity monoclonal IgM and IgG antibodies (anti-HBs) to hepatitis B surface antigen (HBsAg) have recently been developed and used by us in the construction of highly sensitive radioimmunoassays for the detection of HBsAg-associated determinants in serum. We now report that selected serum samples demonstrating monoclonal immunoreactive material also contain nucleic acid sequences complementary to hepatitis B virus (HBV) DNA by molecular hybridization analysis. In addition, monoclonal radioimmunoassays can detect viral determinants in HBsAg–anti-HBs immune complexes formed in anti-HBs excess; such determinants are undetectable by commercial radioimmunoassay. These and our previous studies suggest that there are HBV or "HBV-related" agents present in human serum that are detected by monoclonal antibodies but are not identified by conventional polyvalent antibodies.**

We have produced and characterized high-affinity monoclonal IgM and IgG antibodies to specific determinants or epitopes on hepatitis B virus (HBV) surface antigen (HBsAg) (1–5). These antibodies (anti-HBs) have been utilized to develop highly sensitive solid-phase radioimmunoassays (RIAs) for detection of HBsAg in human serum (5). Several such anti-HBs monoclonal antibodies, designated 5D3 (IgM), 5C3 (IgG2a), and 5C11 (IgG1), have been particularly useful in permitting detection of HBsAg at subnanogram levels.

During the course of these studies, it was discovered that sera from some patients with acute hepatitis and chronic active hepatitis, from certain blood donors incriminated in post-transfusion hepatitis, and from Australian aborigines in an isolated population with a high exposure rate to hepatitis B virus were reactive for HBsAg determinants with monoclonal anti-HBs RIAs but not with a commercial RIA that uses polyvalent anti-HBs (6, 7). In the present study, we report that monoclonal anti-HBs antibodies can detect HBsAg determinants in human serum when this antigen is present in immune complexes and that a significant proportion of selected sera that are positive by the monoclonal anti-HBs RIA but negative by polyvalent anti-HBs RIA contain HBV DNA sequences by molecular hybridization analysis. The presence of both immunoreactive material for HBsAg and nucleic acid sequences complementary to HBV DNA provides strong evidence that these individuals are infected with HBV or a "hepatitis B-like" virus that was not detected by conventional RIAs with polyvalent antisera.

MATERIALS AND METHODS

Serum Specimens. Serum specimens were selected from patients with acute hepatitis and chronic active hepatitis, normal blood donors, donors who had been incriminated in transmitting acute hepatitis, and Australian aborigines. These sera were tested for HBsAg by a standard commercial RIA and were negative (6, 7). All, however, were reactive by monoclonal RIA. The clinical, biochemical, and histologic features of these individuals have been reported (6, 7).

RIAs. The immunization protocols, characteristics and purity of the immunization antigen (HBsAg), cell fusion technique, and the growth and cloning of hybridomas producing anti-HBs monoclonal antibodies have been reported (1). The 5D3 IgM anti-HBs used in the monoclonal RIA has been characterized with respect to specificity for HBsAg-associated determinants, affinity (Kasn) for a HBsAg viral determinant, and capability of agglutinating erythrocytes coated with HBsAg (subtypes adw and ayw) (1–5, 8, 9). The assay design, referred to as a "simultaneous sandwich" monoclonal RIA, has been described (5). This assay uses the 5D3 IgM anti-HBs coupled to a solid phase support (polystyrene beads). Serum (100 μl) was coincubated with 100 μl of ^{125}I-labeled 5D3 or 5C3 and 5C11 monoclonal antibodies (specific activity, 8–10 μCi/μg; 1 Ci = 3.7×10^{10} becquerels) for 5 hr at 45°C. The solid-phase support was then washed three times with distilled water, and radioactivity (cpm) bound to the beads was measured in a Packard gamma well counter. The results of the monoclonal RIAs were compared to those obtained with a standard commercial assay for HBsAg (AUSRIA II, Abbott). Additional serologic markers for HBV exposure, such as anti-HBs and antibodies (anti-HBc) to hepatitis B core antigen (HBcAg), were determined by Abbott commercial RIA kits (AUSAB and CORAB, respectively).

Because several individuals who were reactive only in the monoclonal RIAs had anti-HBs and anti-HBc antibodies in their serum, additional experiments were performed to ascertain the sensitivity of the monoclonal RIA for an HBsAg-associated determinant in HBsAg–anti-HBs immune complexes formed at various antigen/antibody ratios. In these investigations, several chronic carriers of HBsAg were selected, and serial dilutions were made of their serum (with HBsAg-negative serum). HBsAg binding activity was measured in each specimen by the monoclonal RIAs and was compared to that obtained with conventional polyvalent anti-HBs antibodies (AUSRIA II). Each dilution of HBsAg-positive serum (200 μl) was then incubated with 25 μl of serum from a multiply transfused hemophiliac patient (with an anti-HBs titer of 1–2.2×10^6 by passive hemagglutination) for 12 hr at 20°C. After this incubation, three

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; anti-HBs, antibodies to HBsAg; anti-HBc, antibodies to HBcAg; RIA, radioimmunoassay.

* This is paper no. 3 in a series. Papers 1 and 2 are refs. 6 and 7, respectively.

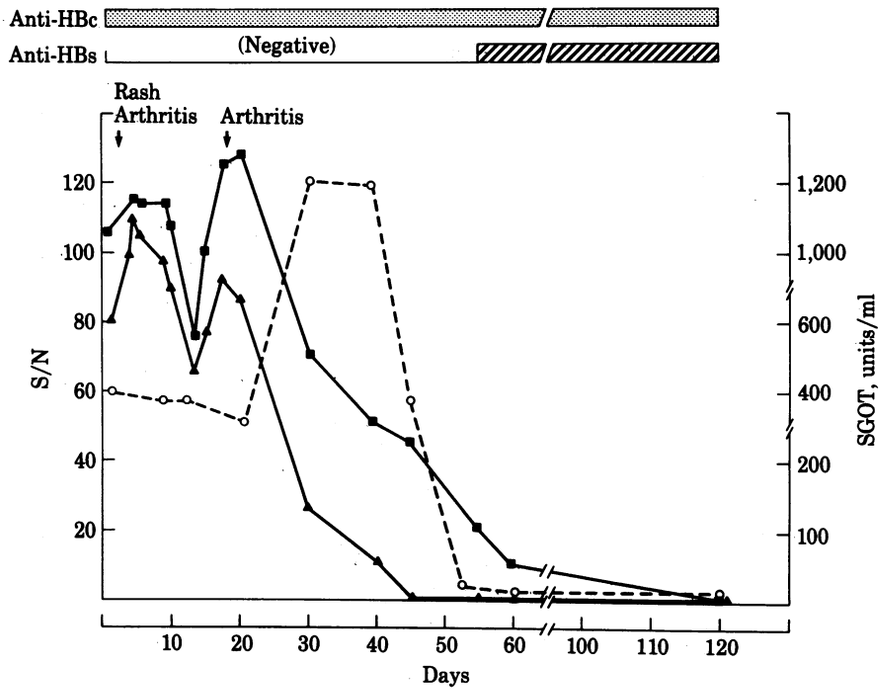

FIG. 1. Comparison of the IgM monoclonal RIA (■) to AUSRIA II RIA (▲) in a patient with acute hepatitis B and immune complex disease. ---, SGOT (serum aspartate aminotransferase, EC 2.6.1.1). S/N, signal-to-noise ratio defined as cpm bound in experimental samples/cpm bound in negative control.

RIAs were performed: monoclonal anti-HBs and AUSRIA II for HBsAg and AUSAB for anti-HBs levels.

HBV DNA Hybridization Studies. For molecular hybridization studies, 10-μl aliquots of serum were applied to nitrocellulose filter sheets and denatured and fixed to the filter with 0.5 M NaOH. The material was neutralized, digested with proteinase K (200 μg/μl) in 0.3 M NaCl/0.03 M Na citrate for 1–2 hr at 37°C, washed with 0.3 M NaCl/0.03 M Na citrate, air dried, and baked *in vacuo* at 80°C for 2 hr. The bound DNA was prehybridized and hybridized with HBV [^{32}P]DNA as reported (10, 11). For these experiments, recombinant cloned HBV DNA (≈3,250 base pairs) was repurified from plasmid pA01 HBV DNA by digestion of the plasmid with restriction endonuclease *Eco*RI, followed by agarose gel electrophoresis and electroelution of the purified HBV DNA band (12). HBV DNA was labeled with [^{32}P]dCTP and [^{32}P]dATP to a specific activity of 2–4 × 10^8 cpm/μg of DNA by nick-translation according to the method of Rigby *et al.* (13). Hybridization was performed in 0.75 M NaCl/0.075 M Na citrate/0.02% polyvinylpyrrolidone/0.02% Ficoll/0.2% bovine serum albumin containing denatured calf thymus DNA (150–200 μg/ml) and heat-denatured HBV [^{32}P]DNA (1 × 10^6 cpm/ml) at 65°C for 24–36 hr. After hybridization, the unreacted solution was discarded, and the nitrocellulose filter was washed, dried, and autoradiographed as reported (10–12). For some experiments, purified HBV DNA, DNA extracted from the PLC/PRF/5 cell line, which contains 5 or 6 copies of HBV DNA per genome equivalent (10, 14), or DNA isolated from serum Dane particles was utilized.

RESULTS

Fig. 1 depicts a serial study on a patient with acute hepatitis B and HBsAg–anti-HBs immune complex disease characterized by arthritis, rash, and arthralgias (15). In this figure, the signal/noise ratio for HBsAg (a measure of specific binding activity) is higher with monoclonal anti-HBs (IgM) than with polyvalent anti-HBs (AUSRIA II). More importantly, the monoclonal RIA for HBsAg remained positive for ≈3 wk after the polyvalent AUSRIA II RIA had become negative. During this period, anti-HBs was present in the serum, suggesting that the monoclonal RIA may detect HBsAg-associated determinant in HBsAg–anti-HBs immune complexes formed in anti-HBs excess and that such determinants are not detectable by polyvalent anti-HBs antisera.

To further explore this possibility, two additional studies were performed in which HBsAg–anti-HBs immune complexes were formed *in vitro* with serum from a chronic HBsAg carrier

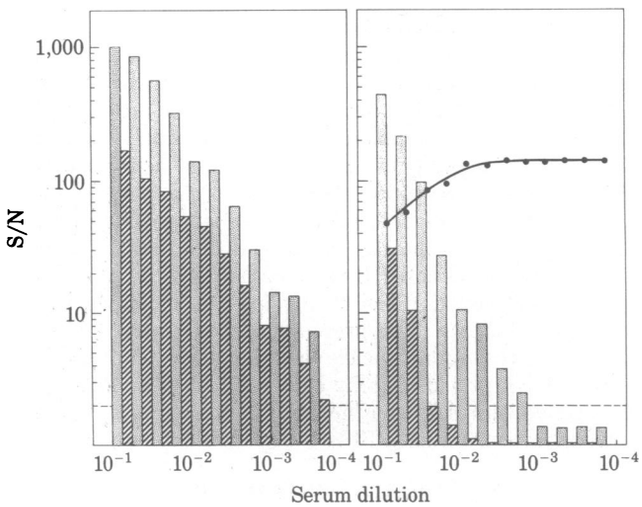

FIG. 2. Detection of a HBsAg-associated determinant by monoclonal IgM RIA (▨) and AUSRIA II RIA (▨) in HBsAg–anti-HBs immune complexes formed before (*Left*) and after (*Right*) addition of anti-HBs excess. ---, Cutoff for positive results; ●——●, concentration of anti-HBs at various antigen/antibody ratios. S/N, signal-to-noise ratio as defined in Fig. 1.

by the addition of high-titer polyvalent anti-HBs antibodies. When polyvalent anti-HBs was added to serum from an HBsAg carrier, the monoclonal RIA (anti-HBs IgM) remained positive up to a 10-fold greater dilution than did the AUSRIA II RIA (Fig. 2). When, in place of IgM monoclonal anti-HBs, studies were carried out with IgG monoclonal anti-HBs 5C3 and 5C11, which recognize distinct and separate determinants on HBsAg (6), similar results were obtained (Fig. 3). These findings indicate that monoclonal anti-HBs RIAs can recognize their specific viral epitopes in the immune complexes when HBsAg is no longer detectable by polyvalent anti-HBs antibodies.

To determine whether HBV DNA sequences were present in serum samples that were positive for HBsAg by RIAs only with monoclonal anti-HBs antibodies, sera (10-μl aliquots) were applied as spots to a nitrocellulose filter sheet and denatured. The DNA material was fixed, hybridized with recombinant-cloned and repurified HBV [^{32}P]DNA, washed, and autoradiographed. All experiments were performed under code with two investigators independently interpreting the autoradiograms. A series of control samples either positive or negative for HBsAg by AUSRIA II were correspondingly positive or negative for HBV DNA by hybridization, respectively (Fig. 4). In several hundred random specimens from a clinical laboratory analyzed to date, there was no instance in which the HBV DNA hybridization test was positive when the AUSRIA II RIA was negative.

A select group of specimens that were positive for HBsAg by RIA with ^{125}I-labeled monoclonal anti-HBs IgM (5D3) but negative by RIA with ^{125}I-labeled polyvalent anti-HBs (AUSRIA II) were then analyzed by HBV [^{32}P]DNA hybridization. Three of seven samples were positive for HBV DNA by molecular hybridization (Fig. 5). Sample I was control serum, sample 2 was serum from a conventional HBsAg carrier (as determined by AUSRIA II), and samples 3–7 were sera positive for HBsAg by monoclonal anti-HBs IgM (5D3) but negative by AUSRIA II. Sample 2 (the positive control) and samples 5 and 7 (positive for HBsAg only with monoclonal anti-HBs) were clearly positive for HBV DNA sequences by molecular hybridization. These results were reproducible in three independent sets of experiments performed on separate days with different preparations of reagents. Hybridization analysis for HBV DNA sequences was then performed on additional sera positive for HBsAg by monoclonal anti-HBs RIAs but negative by AUSRIA II. As shown in Fig. 5*B*, five additional specimens positive for HBV DNA were identified.

To determine the frequency with which sera negative for HBsAg by AUSRIA II but positive for monoclonal anti-HBs were positive also for HBV DNA sequences, 36 selected specimens previously characterized by monoclonal RIAs (6, 7) plus additional samples were hybridized under code with HBV [^{32}P]DNA. Table 1 lists the results together with clinical information and data from other tests including various RIAs for HBV markers on those individuals whose serum was positive for HBV

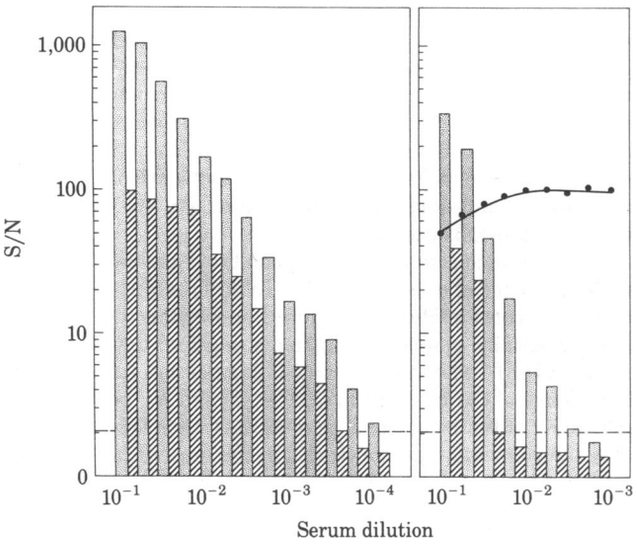

FIG. 3. Detection of HBsAg-associated determinants by monoclonal IgG RIA (▨) and AUSRIA II RIA (▨) in immune complexes formed before (*Left*) and after (*Right*) addition of anti-HBs excess. The 5D3 IgM anti-HBs was coupled to the solid-phase support, and the 5C3 and 5C11 monoclonal antibodies served as the radiolabeled indicator probes. Both the 5C3 and 5C11 antibodies, as compared with 5D3, recognized separate and distinct determinants on HBsAg. Thus, this multisite binding RIA demonstrates that other anti-HBs antibodies are effective in identifying their viral epitopes in immune complexes formed in polyvalent anti-HBs excess (see Fig. 2). S/N, signal-to-noise ratio as defined in Fig. 1.

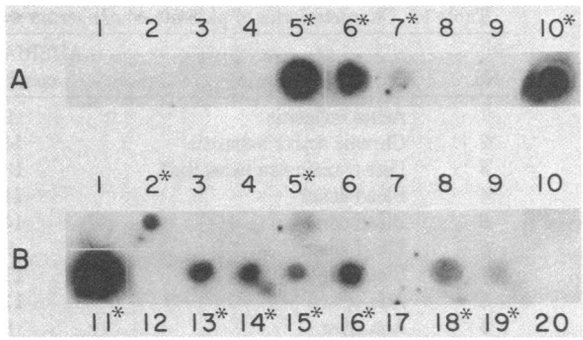

FIG. 4. Detection of HBV DNA sequences in human serum by spot hybridization test with recombinant cloned HBV DNA. *, Positive for HBV DNA by molecular hybridization. Human serum, Dane particle DNA, or DNA from a human hepatocellular carcinoma cell line, PLC/PRF/5, were applied to a nitrocellulose filter, fixed, digested with proteinase K, and hybridized with recombinant-cloned and repurified HBV [^{32}P]DNA (specific activity, 2–4 × 10^8 cpm/μg of DNA). (*A*) Control human serum negative for HBsAg by AUSRIA II RIA (spots 1–4, 8, and 9); 5.0, 0.5, and 0.05 μg of PLC/PRF/5 DNA (spots 5–7, respectively); and ≈0.5–1.0 ng of Dane particle DNA (spot 10). (*B*) Random serum specimens from the clinical laboratory. Spots 1, 3, 4, 6–10, 12, 17, and 20 were negative for HBsAg by AUSRIA II RIA.

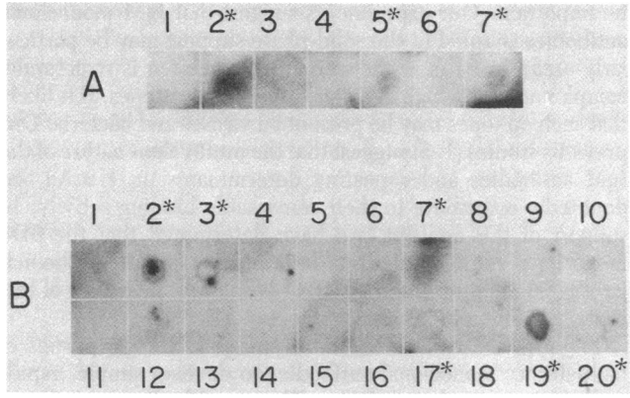

FIG. 5. Detection of HBV DNA sequences in human serum positive for HBsAg determinants only by RIA with monoclonal anti-HBs. *, Spots positive for HBV DNA sequences. (*A*) Negative control serum (spot 1), positive control serum (positive by AUSRIA II RIA) (spot 2), and serum positive for HBsAg determinants only by monoclonal anti-HBs RIA (spots 3–7). (*B*) Spots from 20 selected serum specimens, including one negative control (spot 1), one positive control (spot 7), and 18 samples from individuals positive for HBsAg by RIA with monoclonal anti-HBs (IgM) but negative with polyvalent anti-HBs.

Table 1. Characteristics of patients whose serum was reactive by both monoclonal RIAs and HBV DNA hybridization

No.	Diagnosis	AUSRIA II RIA, cpm bound	Monoclonal RIA, cpm bound	Anti-HBs	Anti-HBc
1	Acute hepatitis	93	2,641	+	−
2	Chronic active hepatitis	141	7,621	−	−
3	Post-transfusion hepatitis*	136	2,193	−	−
4	Blood donor	147	1,862	−	−
5	Blood donor	141	2,613	−	−
6	Blood donor†	96	1,562	−	−
7	Blood donor	114	684	−	−
8	Aus. abor.	119	1,281	−	−
9	Aus. abor.	88	691	+	+
10	Aus. abor.	94	663	+	+
11	Aus. abor.	110	934	+	−
12	Aus. abor.	113	2,600	+	+
13	Aus. abor.	138	1,084	+	+
	Controls (100)	136 ± 17	56 ± 9		

Aus. abor., Australian aborigine.
* Recipient of blood from patient 4.
† Incriminated in transmitting post-transfusion hepatitis. Serum was not available for analysis from recipient of blood from patient 6.

DNA sequences. A total of 13 of 36 samples (36%) of specimens from different individuals positive for HBsAg determinants with monoclonal anti-HBs but negative with polyvalent anti-HBs were positive for HBV DNA sequences by hybridization with recombinant-cloned and repurified HBV DNA. Amongst these individuals were three patients with acute or chronic hepatitis, four blood donors (two of whom have been implicated in transmission of hepatitis to recipients of their blood), and six Australian aborigines from an isolated population where HBV infection is endemic.

DISCUSSION

A potential major application of monoclonal antibodies (16) involves the immunodiagnosis of viral diseases (5). There are, however, certain critical properties that such antibodies must possess to permit the development of a sensitive assay. First, the monoclonal antibodies must be of high affinity. In the case of the 5D3 IgM anti-HBs monoclonal antibodies, the affinity (Kasn) is 4×10^{11} liters/mol per molecule for an HBsAg-associated determinant (1). Second, the isotype of the antibody may be important. Our experiments suggest that IgM monoclonal antibodies coupled to the solid-phase support may be particularly useful in RIAs, if the immunizing antigen is structurally complex and has a high density of repeating epitopes. It is likely that such epitopes may be present on viruses and bacteria. Our previous studies (1, 5) suggest that the multivalent nature of the IgM antibodies and repeating determinants on HBsAg undoubtedly contribute to their remarkable binding activity. In support of this concept, we have determined that the 5D3 monoclonal antibody binds to determinants located on the major 49,000-dalton protein of HBsAg (8); multiple copies of this protein are found on HBsAg particles (17).

Previously, we have taken advantage of the properties of high-affinity monoclonal antibodies to develop simple, rapid, and easily reproduced RIAs. These antibodies can detect HBsAg in serum over a concentration range of 100 pg to 2 mg. During the course of clinical investigation, it was observed that some serum samples derived from patients with acute or chronic hepatitis were reactive only in the monoclonal RIAs and not in commercial RIAs that used polyvalent anti-HBs antibodies (5). Partial characterization of the monoclonal immunoreactive material revealed that the binding activity had a $M_r > 2 \times 10^6$, shared several polypeptides in common with HBsAg, became reactive with polyvalent anti-HBs antibodies when concentrated ≈1,000-fold, and was particulate when examined by immune electron microscopy (7). Furthermore, detailed analysis of the binding activity by different IgG and IgM antibodies in RIAs has demonstrated additional HBsAg-associated determinants (6).

In the present study, the monoclonal RIA is able to bind to viral epitopes in HBsAg–anti-HBs immune complexes formed in the presence of anti-HBs excess. Possible explanations for this phenomenon are: (*i*) the high-affinity monoclonal anti-HBs may compete more effectively for their determinant(s) than do naturally occurring anti-HBs or (*ii*) the antibodies may have access to unoccupied determinants in the presence of polyvalent anti-HBs excess. Thus, polyvalent anti-HBs may contain only a small amount of antibody with immunologic properties of 5D3, 5C3, and 5C11 monoclonal antibodies, and, even though immunogenicity is directed against HBsAg-associated determinants, the region of immunologic reactivity with the monoclonal antibodies may extend beyond that present in polyvalent antisera. Such a phenomenon could permit detection of HBsAg in immune complexes by monoclonal RIAs, whereas conventional anti-HBs antibodies would demonstrate no binding activity under conditions of anti-HBs excess.

Although such activity could explain the detection of HBsAg in the presence of excess anti-HBs (Table 1, cases 1 and 9–13), additional consideration is required concerning the positive binding activity observed in patients negative for HBsAg by AUSRIA II RIA who were also anti-HBs negative (Table 1, cases 2–8). Some of these results may be explained by the increased sensitivity of the monoclonal immunoassays for HBsAg-associated determinants as demonstrated by the present and previous studies (5–7). In addition, HBsAg in some patients may be present in immune complexes circulating under conditions of anti-HBs equivalence or excess and, as reported here, would be detectable only by monoclonal RIAs. Such individuals would be comparable to those reported to have HBsAg in the cytoplasm of hepatocytes but no HBsAg or only anti-HBs and anti-HBc in the serum (18). Studies also have been reported in which HBsAg and HBcAg have been found in hepatocytes by immunohistochemical methods but with no positive serologic markers demonstrable in serum by conventional RIAs (19). It is quite possible, therefore, that the monoclonal RIAs could detect HBsAg-associated determinants in serum either in the presence or absence of anti-HBs and anti-HBc.

In terms of the HBV DNA reacting sequences present in 36% of serum specimens positive for HBsAg by monoclonal RIAs but negative by polyvalent RIAs (AUSRIA II), the present study was limited by the small amount of material available for hybridization analysis. However, the results indicate that DNA sequences related to or homologous with HBV DNA are present in these specimens. Aside from these selected cases, we thus far have not detected hybridization with human serum negative for HBsAg by the AUSRIA II RIA. Therefore, we are reasonably confident that the present findings do not represent biologically false-positive results. They also may explain the recent observations of Brechot *et al.* (20) that DNA sequences hybridizing with cloned HBV DNA were present in the liver of patients whose serum was negative for HBsAg. Because the serum from these individuals was negative for all known HBV markers by commercial RIA (including anti-HBc, hepatitis B e antigen, and its antibody, it is unlikely that the data can be explained simply by an equivalence of HBsAg and anti-HBs in serum.

At present, it is not clear whether the lack of HBsAg detection in serum by polyvalent anti-HBs reflects a sensitivity difference in the RIA or unique structural differences in HBsAg determinants expressed in these individuals. However, the presence of both immunoreactive material and hybridizable DNA sequences provides corroborative evidence for a "hepatitis B-like" virus in a significant proportion of these specimens. Whereas this is not surprising in those cases in which anti-HBs or anti-HBc (or both) was also present, such as in most of the Australian aborigines, we also found positive results for both tests under circumstances in which no HBV markers were detected by commercially available RIA. Although it is theoretically possible that these two tests might be detecting unrelated entities in serum, this is highly unlikely, especially in those cases in which transmissability of a hepatitis-like disease has been observed clinically (i.e., between a blood donor and transfusion recipient; Table 1, cases 3 and 4).

From the limited analyses performed, it is unclear whether the viral DNA detected by molecular hybridization is homologous to HBV DNA or represents a partially related but separate virus or group of viruses.

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