Immunodiagnosis of hepatitis B with high-affinity IgM monoclonal antibodies*

(radioimmunoassay/hepatitis B surface antigen/hybridomas)

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ABSTRACT High-affinity monoclonal IgG and IgM antibodies to hepatitis B surface antigen (HBsAg) have been prepared and their functional capabilities explored by means of solid-phase radioimmunoassays. ¹²⁵I-labeled HBsAg binding studies indicated that monoclonal IgM antibodies against HBsAg (anti-HBs) coupled to a solid-phase support quantitatively bound more HBsAg at a faster rate than conventionally prepared anti-HBs reagents or other high-affinity IgG monoclonal anti-HBs antibodies. Consequently, IgM anti-HBs was also radiolabeled, and an IgM-IgM radioimmunoassay was developed for the immunodiagnosis of hepatitis B. The lower limit of this assay was $\approx 100 \text{ pg} \pm 30 \text{ (SEM)}$ of HBsAg per ml of serum. Compared to available commercial radioassays, preliminary studies have shown the IgM-IgM assay to have increased sensitivity, which improved the detection of a HBsAg-associated determinant in acute hepatitis and post transfusion hepatitis. It is probable that the multivalent interaction between monoclonal IgM anti-HBs and the polydeterminant HBsAg is important in augmenting the performance of this monoclonal assay.

Acute hepatitis B causes significant mortality and morbidity, and chronic infection with the virus is associated with hepatocellular carcinoma, chronic active hepatitis, and cirrhosis (1). The specific viral marker for acute and chronic hepatitis B infection is the presence of hepatitis B surface antigen (HBsAg) in the blood (2). This high molecular weight complex viral protein is a surface component of the intact 42-nm hepatitis virus, but it is also present on a circulating 22-nm particle (3). HBsAg is distinct from other hepatitis B-associated antigens such as hepatitis B core antigen and hepatitis B e antigen (4, 5). In some patients, the amount of HBsAg in the blood reaches exceedingly high levels and may be measured by simple Ouchterlony gel diffusion (6). In most individuals, more sensitive techniques such as radioimmunoassays are required for detection of HBsAg (7). There may be others, however, in whom the virus or viral proteins are present but undetectable by the most sensitive currently available methods.

In the present investigation we have explored the use of high-affinity monoclonal antibodies directed towards epitopes on hepatitis B surface antigen (anti-HBs) in the immunodiagnosis of hepatitis B. A radioimmunoassay using IgM anti-HBs was constructed that demonstrated enhanced sensitivity compared to existing commercial assays. The multivalent nature of the IgM-HBsAg interaction probably played a role in improving assay performance.

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MATERIALS AND METHODS

Establishment of Somatic Cell Hybrids Producing Monoclonal Antibodies to HBsAg. The preparation of immunizing antigen, immunization schedule, cell fusion techniques, and culture of somatic cell hybrids have been described (8-12). Hybridomas were cloned twice at limiting dilutions (by use of Poisson statistics) on BALB/c 3T3 monolayers. After dilutional cloning, we selected three IgM- and two IgG-producing cell lines with high anti-HBs binding activity for intraperitoneal injection (4×10^6 cells) into pristane-primed BALB/c mice. Subsequently, the ascites fluid was collected and analyzed as described below.

Analysis and Properties of Anti-HBs Activity. Three separate assays were used for measurement of anti-HBs activity in cell culture supernatants and in ascites fluid. The first procedure involved a modification of the AUSAB (Abbott) solid-phase radioimmunoassay. Two hundred microliters of culture medium or ascites fluid was incubated with HBsAg-coated beads for 24 hr at room temperature followed by extensive washing with distilled water. Then ¹²⁵I-labeled HBsAg (¹²⁵I-HBsAg; 1.3×10^5 cpm) was added, the beads were again extensively washed with distilled water, and radioactivity was measured in a Packard gamma well counter. Binding of labeled antigen in excess of 2.5 times background was taken as an indication of the presence of specific antibody. The second solid-phase radioimmunoassay used ¹²⁵I-labeled goat anti-mouse F(ab')₂ and was identical to the one mentioned above except that ¹²⁵I-labeled goat anti-mouse F(ab')₂ (1×10^5 cpm) was added in place of ¹²⁵I-HBsAg (13). Finally, the capability of anti-HBs to agglutinate HBsAg-coated (ayw and adw subtypes) human O-negative erythrocytes in a microhemagglutination reaction was also evaluated (14).

Isotype analysis of anti-HBs was performed on the five double-cloned cell lines designated as 5D3, 2F11, 1F8, 1C7, and 4E8. In these experiments, 200 μ l of culture supernatant was incubated with HBsAg-coated beads for 24 hr followed by extensive washing with distilled water. The second probes, ¹²⁵I-labeled goat anti-mouse (1×10^5 cpm) IgG1, IgG2b, IgA, or IgM, were then added for an additional 36 hr. The antigen-coated beads were again extensively washed with distilled

Abbreviations: HBsAg, hepatitis B surface antigen; anti-HBs, antibodies against HBsAg; ¹²⁵I-HBsAg, ¹²⁵I-labeled HBsAg.

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water, and radioactivity was measured in a Packard gamma well counter.

Protein concentration in the ascites fluid derived from malignant growth of the above five double-cloned cell lines was measured by the method of Lowry *et al.* (15). Subsequently, 10 mg of ascites fluid protein was placed over a 1.5×30 cm Sephadex G-200 or Sepharose 6B column. Fractions were collected for assay of protein concentration, absorbance at 280 nm, and anti-HBs binding activity by the solid-phase radioimmunoassay with the ^{125}I -HBsAg probe.

Apparent association constants (K_a) between HBsAg and monoclonal antibodies were determined by binding purified ^{125}I -labeled anti-HBs of known specific activity to HBsAg coupled to a solid-phase support. Scatchard plot analysis was used to obtain values for K_a . Monoclonal IgM and IgG anti-HBs were purified from ascites fluid by gel-exclusion chromatography on Sepharose 6B and Sephadex G-200. Antibody used in these determinations was estimated to be 95–98% pure by NaDodSO₄/polyacrylamide gel electrophoresis. Iodination was performed with the Bolton–Hunter reagent (16) or by the lactoperoxidase method (17).

HBsAg Binding Studies. Monoclonal IgG and IgM anti-HBs were coated on 1/4-inch polystyrene beads. The beads were washed three times with distilled water. Subsequently, the beads were incubated with ^{125}I -HBsAg (1.2×10^5 cpm) at various times and washed three times with distilled water; radioactivity was then measured. Comparison of ^{125}I -HBsAg binding activity was made to commercial anti-HBs-coated beads (AUSRIA II, Abbott).

Anti-HBs Binding Studies. Monoclonal anti-HBs antibodies were radiolabeled with ^{125}I by either the lactoperoxidase method or the Hunter–Bolton reagent. Specific activity was 10–16 $\mu\text{Ci}/\mu\text{g}$ and 4–12 $\mu\text{Ci}/\mu\text{g}$, respectively (1 Ci = 3.7×10^{10} becquerels). ^{125}I -Labeled monoclonal anti-HBs were incubated with HBsAg-coated beads (AUSAB) at various times and washed three times with distilled water; radioactivity was then measured. We also studied the binding of ^{125}I -labeled anti-HBs prepared by conventional immunization (AUSRIA II) to HBsAg-coated beads in a similar fashion.

Construction of Radioimmunoassays for HBsAg. Several assays were investigated by using IgG and IgM monoclonal anti-HBs antibodies. In the first assay, 100 μl of monoclonal ^{125}I -labeled anti-HBs (1.5×10^5 cpm) was allowed to react with 100 μl of HBsAg-containing serum in a soluble phase for 1 hr at 45°C. Next, monoclonal IgG and IgM anti-HBs coated to the solid-phase support were added for an additional 1 hr at 45°C (“reverse sandwich” assay). The beads were washed three times

in distilled water and radioactivity was measured. In the second assay, beads coated with IgM monoclonal anti-HBs were first incubated with 100 μl of serum sample and 100 μl of 1% bovine serum albumin in phosphate-buffered saline for 1 hr at 45°C followed by washing three times with distilled water and the subsequent addition of 200 μl of monoclonal ^{125}I -labeled anti-HBs for 1 hr at 45°C (“forward sandwich” assay). Finally, an assay was constructed in which the monoclonal anti-HBs-coated beads, monoclonal ^{125}I -labeled anti-HBs probes (100 μl), and serum (100 μl) were added at the same time and incubated for 2 hr at 45°C (“simultaneous” assay). The beads were subsequently washed three times and radioactivity was measured.

To ensure the specificity of IgM and IgG anti-HBs for HBsAg and not other serum proteins, we developed a control radioimmunoassay. A monoclonal IgM with no anti-HBs activity was coupled to the same solid-phase support. The beads were incubated with dilutions of serum samples or HBsAg-positive standards and the monoclonal IgM ^{125}I -labeled anti-HBs probe. The beads were washed three times and radioactivity was measured.

Sensitivity studies were performed with the above assays. HBsAg was purified from serum with a monoclonal IgM anti-HBs affinity column by coupling ascites fluid derived from cell lines 5D3 (28 mg) and 2F11 (65 mg) to cyanogen bromide-activated Sepharose 6B. Human serum (10 ml) derived from a high-titer, chronic HBsAg (adw subtype) carrier patient was placed over the columns, incubated for 24 hr at room temperature, and washed extensively with phosphate-buffered saline. Subsequently, 1-ml fractions were collected by elution with glycine-HCl buffer (pH 2.6). The pH of each fraction was adjusted to 7.4 with 0.1 M NaOH, and the protein concentration and HBsAg activity were determined. Thus, the sensitivity of each monoclonal assay configuration compared to a commercial assay was measured as the binding activity exhibited by a known concentration of purified HBsAg diluted in normal serum.

RESULTS

Properties of Monoclonal Anti-HBs. Ascites fluid derived from five double-cloned cell lines demonstrated high anti-HBs binding activity in two solid-phase assays (Table 1). Isotype analysis revealed that three cell lines produced anti-HBs of the IgM class and two others were IgG anti-HBs of the IgG₁ subclass. It is noteworthy that 5D3, an IgM anti-HBs, exhibited the highest binding activity for ^{125}I -HBsAg. Apparent association constants for the three IgM and two IgG anti-HBs antibodies were determined. Because, for IgM, both antigen and antibody were

Table 1. Properties of monoclonal anti-HBs antibodies

Clone	^{125}I -HBsAg,* cpm $\times 10^{-3}$	^{125}I - $\alpha\text{F}(\text{ab}')_2$,* cpm $\times 10^{-3}$	Immunoglobulin		Anti-HBs, [†] mg/ml	K_a , [‡]	Hemagglutination titer [§]	
			Class	Subclass			adw	ayw
5D3	96.3	12.4	IgM	—	6.3	4.0×10^{11}	1.22×10^{11}	1.27×10^{12}
1F8	54.4	9.2	IgM	—	24.7	1.4×10^{10}	1.17×10^{10}	1.26×10^4
2F11	60.8	10.6	IgM	—	20.1	4.8×10^9	1.13×10^{11}	1.10×10^6
1C7	46.4	9.6	IgG	IgG ₁	18.2	3.2×10^{10}	1.70×10^{10}	1.32×10^4
4E8	50.2	8.2	IgG	IgG ₁	15.2	9.1×10^9	1.43×10^9	1.21×10^6
Controls [¶]	0.2	0.1	—	—	—	—	<1:4	<1:4

* ^{125}I -HBsAg or ^{125}I -labeled goat anti-mouse $\text{F}(\text{ab}')_2$ bound in radioimmunoassays. Initial sample volume was 200 μl of ascites fluid.

[†] Expressed as mg of monoclonal anti-HBs per ml of ascites fluid.

[‡] Expressed as liters/mole per molecule of IgM or IgG.

[§] Hemagglutination titer in ascites fluid of HBsAg-coated O-negative erythrocytes.

[¶] Controls consist of ascites fluid containing IgG₁ monoclonal antibodies to cardiac myosin (18) reacting in the radioimmunoassay and with HBsAg-coated cells. Additional controls consist of the above five cloned cell lines reacting with human O-negative erythrocytes (HBsAg-negative) and mouse myeloma IgM (Bionetics, Kensington, MD), 1 mg/ml in phosphate-buffered saline, reacting with HBsAg-coated erythrocytes and control erythrocytes.

FIG. 1. Binding of ^{125}I -HBsAg to monoclonal anti-HBs on a solid-phase support. Cell lines 5D3, 1F8, and 2F11 secrete anti-HBs of the IgM class; 1C7 and 4E8 secrete anti-HBs of the IgG class. CR, commercial reagent.

potentially polyvalent, the values of K_a reported represent binding avidities between the antigen and antibody molecules. Values up to 4×10^{11} liters/mole per molecule of anti-HBs were observed.

The anti-HBs hemagglutination titers were exceedingly high. Indeed, 25 μl of ascites fluid derived from cell line 5D3 diluted at $1:2.2 \times 10^{12}$ still gave a positive agglutination reaction. This illustrates the apparent high avidity of this IgM anti-HBs for HBsAg coated on the indicator cells. In addition, 5D3 IgM anti-HBs recognized a common determinant on both adw and ayw HBsAg subtypes. In contrast, cell lines 1F8, 2F11, 1C7, and 4E8 appeared more specific for adw HBsAg- than for ayw HBsAg-coated cells. The variability of the hemagglutination titers exhibited by different monoclonal anti-HBs suggested that these antibodies may be directed towards different epitopes on HBsAg.

Antigen and Antibody Binding Studies. We then investigated the capability of monoclonal IgM and IgG anti-HBs to bind HBsAg when coupled to a solid-phase support. Fig. 1 illustrates that 5D3 IgM bound more ^{125}I -HBsAg and at a faster rate than

FIG. 2. Binding of ^{125}I -labeled anti-HBs to HBsAg on a solid-phase support. Note that a greater percent of monoclonal IgM anti-HBs from cell lines 5D3 and 2F11 binds to HBsAg than the commercial anti-HBs reagent (CR) and two other high-affinity IgG monoclonal anti-HBs (1C7 and 4E8).

the other high-affinity IgM and IgG monoclonal antibodies and a commercial anti-HBs reagent. Approximately the same concentration of monoclonal antibodies (100 ng) was coated on the beads. Therefore, the differences in HBsAg binding probably are a result of the different affinity constants of the antibodies rather than of the concentration effects alone. In support of this conclusion, it was found that 5D3 IgM still demonstrated a greater binding capacity for HBsAg when the reverse binding experiments were performed by incubating monoclonal ^{125}I -labeled anti-HBs (1.5×10^5 cpm) with HBsAg coupled to a solid-phase support (Fig. 2).

Monoclonal Radioimmunoassay for Epitopes on HBsAg. Several monoclonal assays were designed in order to determine and then compare the lower limits of sensitivity for detection of HBsAg in serum. As depicted in Table 2, the most sensitive assay was an IgM-IgM monoclonal system. This assay per-

Table 2. Sensitivity of various monoclonal radioimmunoassays for HBsAg compared to commercial radioimmunoassay

HBsAg, ng/ml	Commercial radio- immuno- assay, S/N	Assay design,* S/N							
		5D3R	5D3S	5D3F	1C7R	1C7F	4E8F	4E8R	1C7 + 4E8R
40	18.96	38.4	42.8	140.2	40.1	108.4	106.4	56.3	132.4
20	12.48	21.6	24.4	80.6	17.0	40.2	62.3	42.1	51.0
10	7.42	12.3	16.2	40.4	8.4	8.1	18.1	19.4	10.1
5	4.14	9.2	10.1	20.6	5.3	1.9	3.2	6.2	3.1
2.5	3.64	7.1	8.2	10.1	0.9	1.6	0.9	1.9	0.4
1.25	2.64	6.2	5.4	4.2	1.2	1.4	1.2	0.8	0.8
0.625	1.36	4.4	4.1	1.3	0.6	0.9	1.4	0.4	0.6
0.3125	1.22	3.9	3.5	0.8	—	—	—	—	—
0.156	1.04	3.1	2.9	0.4	—	—	—	—	—
0.078	1.04	2.1	2.0	—	—	—	—	—	—
0.039	0.95	0.9	1.2	—	—	—	—	—	—
0.020	0.62	0.8	0.6	—	—	—	—	—	—

S/N represents signal/noise calculated as mean cpm of ^{125}I -labeled anti-HBs bound divided by the mean cpm of negative control serum. Results were considered positive if S/N was greater than 2.0 (italicized). Commercial assay was AUSRIA II.

* Assays were run in triplicate. 5D3, a monoclonal IgM anti-HBs, was always bound to the solid-phase support. The probes were composed of ^{125}I -labeled 5D3 (IgM), 1C7 (IgG), and 4E8 (IgG). R denotes assay performed in reverse direction, F in forward direction, and S simultaneous (see *Materials and Methods*). Sample volume (HBsAg) was 100 μl .

formed equally well when run in the simultaneous or reverse configuration. The lower limit of detection was approximately $100 \text{ pg} \pm 30$ (SEM) of HBsAg per ml or $10 \text{ pg} \pm 3$ (SEM) of HBsAg (we used a volume of $100 \mu\text{l}$ of HBsAg standard in the assay). Similar differences in sensitivity were obtained with serial dilutions of HBsAg-positive sera (data not shown). A combination of IgM coupled to a solid-phase support preceded by or followed by monoclonal 1C7 and 4E8 ^{125}I -labeled anti-HBs (IgG) or both did not significantly augment the performance of the assay when compared to the commercial (AUSRIA II) radioimmunoassay.

Clinical Studies. We tested the hypothesis that a 5D3 IgM-IgM monoclonal radioimmunoassay may be of value in the immunodiagnosis of hepatitis B. Preliminary studies demonstrated that the sera from 8 of 22 patients with post-transfusion hepatitis, all of whom were negative for anti-HBs and HBsAg by two commercial and the control radioimmunoassays, were positive in the IgM-IgM monoclonal assay. Furthermore, five of these eight sera had high-titer anti-HBc antibodies, providing further serologic evidence that these patients had a prior hepatitis B infection (19). In 20 consecutive patients with presumed acute viral hepatitis, 10 were positive for HBsAg by both commercial and monoclonal assays; but in 5 others of this group, 3 of whom were positive for anti-HBc, a positive detection of HBsAg was obtained only by the IgM-IgM monoclonal system.

DISCUSSION

Five cloned hybridoma cell lines have been established which are stable and retain their capability to produce specific antibodies to antigenic components of HBsAg. Cloned cell lines 2F11, 1F8, and 5D3 produced IgM anti-HBs, and 1C7 and 4E8 produced IgG anti-HBs. Further, the specificity for HBsAg of the monoclonal IgG and IgM anti-HBs was explored by three independent measurements: two solid-phase radioimmunoassays and a microhemagglutination reaction. There is little doubt that the monoclonal IgG and IgM produced as the result of fusing NS1 myeloma cells with HBsAg-primed splenocytes possessed specificity for the yet to be defined antigenic components unique to HBsAg (20).

Ascites fluid derived from the five cell lines contained antibody that bound to HBsAg with extraordinary avidity (Table 1). The binding avidities of the IgM anti-HBs, especially the 5D3 antibody were particularly striking. Because most naturally occurring IgM antibodies have low avidity (21, 22), the multivalent nature of these antibodies and repeating epitopes on HBsAg undoubtedly contribute to their remarkable binding activity (23, 24).

In an attempt to develop radioimmunoassays using monoclonal anti-HBs, it was important to establish that the antibodies could be linked to a solid-phase support and still maintain their HBsAg-binding characteristics. Fig. 1 indicates that two IgM anti-HBs were superior to the other monoclonal antibodies and a commercial reagent in this regard. When the monoclonal anti-HBs antibodies were radiolabeled with ^{125}I and the binding to HBsAg on a solid-phase support was assayed, an IgM anti-HBs performed better than the other antibodies at all time points (Fig. 2).

Initially, we used the 5D3 IgM anti-HBs immobilized in a solid phase. After incubation of this solid phase with HBsAg-positive standards, the support was washed. Monoclonal IgG ^{125}I -labeled anti-HBs were selected for use as the indicating antibodies in this "forward sandwich assay" because it seemed likely that they were directed towards different epitopes on HBsAg. This assay procedure proved successful, but the lower limit of sensitivity was only 5 ng of HBsAg per ml. Moreover,

the performance of the assay was not enhanced by simultaneous addition of both high-affinity IgG monoclonal ^{125}I -labeled anti-HBs. Relatively higher concentrations of HBsAg ($>10 \text{ ng/ml}$) were easily detected by the assays. One possible explanation for these observations is that the IgG monoclonal antibodies were directed towards relatively low-density epitopes on HBsAg. As the concentration of HBsAg is progressively lowered by serial dilution, one would expect the binding to HBsAg by the highly specific IgG monoclonal antibodies to be limited by the number of available epitopes.

The most sensitive assay design for measurement of HBsAg was the use of monoclonal IgM-IgM. This assay was capable of detecting approximately $100 \text{ pg} \pm 30$ (SEM) of HBsAg per ml. It was somewhat surprising that the 5D3 IgM-IgM solid-phase radioimmunoassay could detect HBsAg, especially at low concentrations. In the reverse and simultaneous assays, the IgM on the solid phase and the IgM ^{125}I -labeled anti-HBs indicator probe are identical and compete for the same epitope. Consequently, one might have expected a blocking reaction which could restrict the performance of the assay at lower concentrations of HBsAg. However, a total 5D3 IgM monoclonal system proved to be the best design with respect to enhanced sensitivity. These experiments suggest that the monoclonal IgM derived from cell line 5D3 must be directed toward a highly represented epitope on HBsAg. Under these circumstances, the multivalent interaction of the IgM with a high-density repeating epitope was possible and presumably contributed to enhanced sensitivity in accord with theoretical determinations of Crothers and Metzger (22) and Ehrlich (24).

In some patients with acute hepatitis and posttransfusion hepatitis, the monoclonal assay detected an HBsAg-associated determinant not detected with a highly sensitive commercial assay (AUSRIA II). Thus, it would appear that one can take advantage of the special characteristics of this antibody (namely, multivalency, high affinity, and common epitope recognition) in the construction of a highly sensitive radioimmunoassay for hepatitis B. The efficacy of the monoclonal IgM-IgM assay reported here must now be demonstrated by more extensive trials.

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