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Serum α-fetoprotein levels in human disease: Perspective from a highly specific monoclonal radioimmunoassay

(hepatitis B/hepatocellular carcinoma/cirrhosis)

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ABSTRACT **A rapid multisite radioimmunoassay for measurement of human α-fetoprotein (AFP) that uses two high-affinity monoclonal antibodies directed against distinct and separate determinants on the protein was developed and designated M-RIA. The sensitivity of the "simultaneous-sandwich" M-RIA is ≈0.5 ng/ml of serum after a 1-hr incubation period. Serum AFP levels have been measured in 1747 individuals with hepatocellular carcinoma (HCC), acute and chronic hepatitis B virus infection, chronic hepatitis B surface antigen (HBsAg)-carrier states, cirrhosis, other malignant tumors, and normal and disease controls to determine the specificity of the assay. Eighty percent (68/85) of patients with HBsAg-positive HCC had AFP levels of >200 ng/ml (range, 260 to >200,000 ng/ml). In contrast, all 450 normal subjects and 477 chronic HBsAg-positive carriers had levels of <20 ng/ml. More importantly, in acute and chronic hepatitis B, cirrhosis, and other malignant tumors and in the remaining disease controls, AFP levels were <20 ng/ml in 99.3% of the subjects, the great majority (>96%) being <5 ng/ml. Indeed only two of 1635 individuals, one with acute hepatitis and the other with carcinoma of the esophagus had AFP levels of >100 ng/ml. These observations are at variance with previous studies with conventional polyvalent RIAs of AFP levels of >20 ng/ml in ≈40% of acute and chronic hepatitis and in 30% of cirrhosis. This striking specificity of the M-RIA is probably due in part to recognition of epitopes unique to AFP and suggest that such an assay may be used in the detection, early identification, and monitoring of AFP-producing tumors in high-risk populations.**

α-Fetoprotein (AFP) is a major serum protein synthesized by fetal liver cells, by yolk sac cells, and in trace amounts by the fetal gastrointestinal tract (1, 2). Reappearance of AFP in adult serum often signals pathologic conditions, particularly the presence of hepatocellular carcinomas (HCC) and germ-cell tumors containing yolk sac cell elements (3, 4). Although existing assays may be successfully used for monitoring treatment of AFP-producing tumors and as an independent prognostic tool (5, 6), the finding of elevated serum AFP levels in some patients with nonmalignant liver diseases, particularly in acute and chronic viral hepatitis and cirrhosis (7, 8), has limited the value of such assays as an independent specific test to establish the diagnosis of cancer.

An important goal in current AFP research is to improve the cancer specificity of the test, particularly given the need for early detection of HCC in high-risk populations. Since some monoclonal antibodies have been reported to enhance either the sensitivity or the specificity of various immunoassays (9, 10), we began to evaluate a series of high-affinity monoclonal antibodies (MAbs) against AFP for use in the construction of multisite RIAs.

This work has led to our development of a rapid and simple one-step MAb-using RIA (M-RIA) for AFP that appears suitable for screening high-risk populations due to the extraordinary specificity of this assay for AFP-producing tumors.

MATERIALS AND METHODS

Production and Characterization of MAbs. Six-week-old BALB/c mice were injected with highly purified human AFP (Institut Behring, Marburg, Federal Republic of Germany) following several different immunization procedures, which varied the route and concentration of antigen and the interval between primary and secondary immunizations. MAbs used in this study (designated AF01 and AF03) were produced by two protocols. The first generated antibody AF01 and involved s.c. injection of 15 μg of AFP in Freund's complete adjuvant (FCA) as the initial dose, followed by i.p. injection of 15 μg in Freund's incomplete adjuvant 2 months later, followed 1 month later by injection of 100 μg i.p. in FCA, 100 μg i.p., and 100 μg i.v. 6, 5, and 4 days before cell fusion. MAb AF03 was produced from a second immunization in which 50 μg was given 1 year (s.c. in FCA), 3 months (i.p. in Freund's incomplete adjuvant), 23 days (i.p. in FCA), 22 days (i.p.), 21 days (i.v.), and 3 days (i.v.) prior to cell fusion.

Cell fusions were performed by incubating 5–10 × 10^6 SP2/0-Ag 14 mouse myeloma cells with 5–10 × 10^7 mouse spleen cells in 40% polyethylene glycol (M_r, 1000) as described (11). Hybridoma supernatants were tested for anti-AFP activity in a screening test with a polyethylene glycol precipitation test with ^{125}I-labeled AFP (^{125}I-AFP). Positive hybrids were cloned twice by limiting dilution, and ascites fluids were produced by i.p. inoculation of nude mice with 5 × 10^5 hybridoma cells. Anti-AFP MAb class and subclass were determined either by double-antibody RIA with ^{125}I-AFP and goat antiserum specific for mouse IgM, IgA, IgG, IgG2ab (Nordic, Tilburg, Netherlands) or by elution of the IgG bound to protein A-Sepharose column (Pharmacia) at different pH values as described by Ey *et al.* (12). Both were found to be of the IgG2a isotype. IgG2a was purified (2–4 mg/ml) from mouse ascites fluids by 50% ammonium sulfate precipitation, followed by dialysis against 0.2 M phosphate buffer (pH 8) for 18 hr at 4°C prior to protein A affinity chromatography (12). The affinities of the anti-AFP MAbs were determined with a double-antibody RIA technique by measuring the binding of ^{125}I-AFP in the presence of increasing amounts of AFP by Scatchard plot analysis (13). Anti-AFP MAbs AF01 and AF03 had measured affinity constants of 1.6 × 10^{10} and 5.0 × 10^8 liters/mol, respectively. Competitive inhibition studies established that each MAb recognized

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Abbreviations: AFP, α-fetoprotein; HCC, hepatocellular carcinoma; MAb, monoclonal antibody; M-RIA, MAb-using radioimmunoassay; C-RIA, conventional radioimmunoassay; HBsAg, hepatitis B surface antigen; FCA, Freund's complete adjuvant.

a distinct and separate AFP determinant (data not shown).

Development of Multisite M-RIA. Systemic testing of different combinations of anti-AFP MAbs demonstrated that the most sensitive assay for serum AFP determination was a "simultaneous-sandwich" M-RIA based on a mixture of AF01 and AF03 as capture antibodies on the solid-phase support and AF01 as the radiolabeled indicator antibody. This assay used polystyrene beads (outer diameter, 0.64 cm; Precision Plastic Ball, Chicago) coated with AF01 and AF03 antibodies. Serum samples or AFP-positive standards (200 μl) and 130,000 cpm of ^{125}I-labeled AF01 (specific activity, 10–16 μCi/μg; 1 Ci = 3.7×10^{10} becquerels) in 100 μl of buffer (50% fetal calf serum in phosphate-buffered saline, pH 7.2) were added simultaneously with the anti-AFP-coated beads. After an incubation at 45°C for 1 hr, the solid-phase support was then washed three times with distilled water, and the radioactivity bound (cpm) was measured in a Beckman gamma well counter. Assay standards consisted of either purified AFP diluted in pooled normal human serum or commercial AFP standard (Abbott). Details of the molecular interaction of anti-AFP MAbs to antigen, kinetics of antigen binding, and other characteristics of this multisite assay will be reported by us elsewhere.

Conventional Radioimmunoassay (C-RIA) for AFP. In selected samples we compared the results of a polyvalent C-RIA with the M-RIA for AFP. We used the RIA-GNOST, AFP-TACHISORB (Behring, Marburg) commercial system. This assay has a lower limit of sensitivity of 1.5 ng/ml and was performed according to the manufacturer's instructions.

Subjects. From our serum bank in the Gastrointestinal Unit at Massachusetts General Hospital, we chose sera from 450 normal subjects and 536 with miscellaneous liver and other disease controls, consisting of 27 with various tumors [colon (13), pancreas (7), breast (2), and lung (5)], 13 with hepatitis B surface antigen (HBsAg)-positive biopsy-proven cirrhosis, 10 with biopsy-proven HBsAg-negative chronic active hepatitis, and 17 with non-B acute viral hepatitis. The remaining 469 included other disease controls such as pneumonia, ulcerative colitis, coronary artery disease, hemodialysis patients, inflammatory bowel disease, etc. We also selected 477 chronic HBsAg carriers of the adw, ayw, adr, and ayr subtypes identified through routine screening of blood donors. We also studied sera from patients with HBsAg-positive and -negative HCC from the Far East and Africa, including chronic HBsAg carriers with and without liver disease from the same region of the world.

In addition, serum samples from 60 patients were obtained from Institut Gustave Roussy. The diagnoses, confirmed by review of operative notes and pathology reports, were as follows: hepatocellular carcinoma, HBsAg status unknown (8); children with hepatoblastoma (3); placental tumors (18); testicular tumors (8); colorectal carcinoma (9); medullary thyroid carcinoma (2); lung metastases of unknown origin (2); and carcinomas of the esophagus (1), breast (6), uterus (2), and lung (1).

RESULTS

Multisite Monoclonal RIA. A sensitive simultaneous-sandwich assay was developed when a mixture of AF01 and AF03 was used on the solid-phase support and ^{125}I-labeled Mab AF01 was used as the indicator antibody. The sensitivity of the assay (1 hr incubation at 45°C) was ≈0.5 ng/ml, as determined by a signal-to-noise ratio (S/N) of >2.5 (defined as cpm measured in the experimental sample divided by the mean cpm of 10 negative controls). The standard curves from two different assays are shown in Fig. 1. The concentration range of the AFP standard varied between 1.5 and 200 ng/ml. The AFP standard curve is linear in the range of 1.5–40 ng/ml. Absolute serum AFP concentrations in experimental samples were determined from serial dilutions and by use of the AFP standard curve.

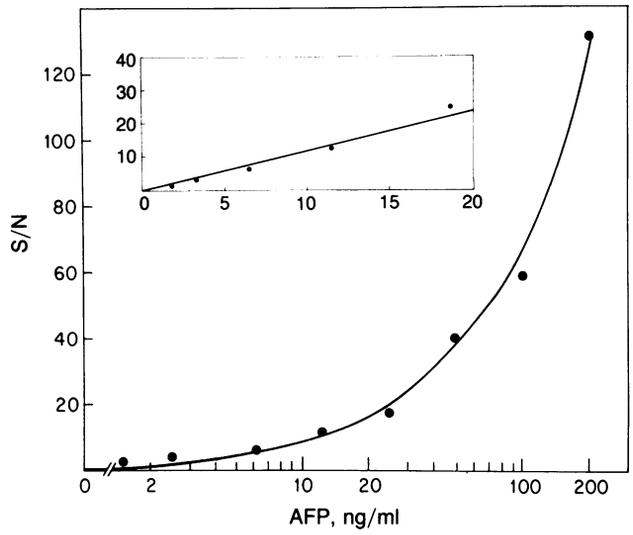

FIG. 1. M-RIA for AFP. There are two standard curves. (*Inset*) Emphasis on the linear portion of the curve between 1.5 and 20 ng/ml. The assay is a simultaneous-sandwich RIA and is performed at 45°C for 1 hr. S/N, signal-to-noise ratio defined as cpm bound in experimental samples divided by the mean of the negative controls.

Results of Serum AFP Values. Sera from 1747 individuals were studied. Each specimen was analyzed in duplicate on two separate occasions. The results are shown in Table 1 and may be summarized as follows. (*i*) All healthy subjects (450) had AFP values of <5 ng/ml. (*ii*) Of 564 HBsAg chronic carriers from three different continents, 98.2% had AFP levels of <5 ng/ml, and 100% had levels of <20 ng/ml. (*iii*) In 536 patients from the United States with nonmalignant disorders of the liver and other diseases (including carcinomas of gastrointestinal tract, breast, and lung), 96.4% had an AFP level of <5 ng/ml; 99.6%, <20 ng/ml; and 100%, <100 ng/ml. (*iv*) AFP levels were found to be <5 ng/ml in 23 of 24 patients (95.8%) from the Far East with acute or chronic hepatitis; only one had an AFP of >200 ng/ml (326 ng/ml). (*v*) In 20 cirrhotic patients from the Far East, 95% had an AFP level of <5 ng/ml, and only one had an AFP level of >40 ng/ml (57 ng/ml).

In contrast, AFP levels from 112 patients with HCC are depicted in Fig. 2. The absolute value varied widely and ranged from 240 to >200,000 ng/ml. It is noteworthy that 82% of HBsAg-positive HCC from Africa and 79% from the Far East had AFP levels of >200 ng/ml. In contrast, only 6 of 16 (38%) of HBsAg-negative HCC from the Far East were reactive for AFP levels of >200 ng/ml ($P < 0.001$). Finally, a smaller group of 11 HCC from France, including 3 children with hepatoblastoma, were reactive for AFP levels of >200 ng/ml. Thus, the majority of HCC patients, particularly those who were HBsAg chronic carriers, had markedly high levels of AFP by the 1-hr simultaneous-sandwich RIA. These findings are in striking contrast to serum AFP levels in patients with other malignant diseases, which excludes tumors of the testis, where we found only 1 of 68 with a value greater than 100 ng/ml (Table 1). That patient developed an esophageal carcinoma and, at the time of study, his serum AFP level was 280 ng/ml. Thus, this M-RIA has a remarkable specificity for AFP-producing tumors, namely HCC from various regions of the world.

To determine if absolute serum AFP values measured by this M-RIA were comparable to those obtained by a polyvalent C-RIA, we studied 17 patients with various tumors. The C-RIAs and M-RIAs were quite comparable with respect to the absolute serum AFP concentration and over a wide range

Table 1. Percentage of positive AFP values measured in ng/ml by a 1-hr multisite M-RIA in healthy subjects and subjects with various malignant and nonmalignant diseases

Category	No. of subjects	Percentage of positive AFP values							
		0–5	5–10	10–20	20–40	40–60	60–100	100–200	>200
Healthy subjects	450	100	—	—	—	—	—	—	—
Miscellaneous*	536	96.4	2.4	0.93	—	0.18 (1)	0.18 (1)	—	—
$HBsAg^+$ chronic carriers (subtypes adw,ayw and ayr,adr)	477	98.1	0.84	1	—	—	—	—	—
$HBsAg^+$ chronic carriers (Africa)	77	98	2	—	—	—	—	—	—
$HBsAg^+$ chronic carriers (Far East)	10	100	—	—	—	—	—	—	—
AH, CAH $HBsAg^+$ (Far East)	24	95.8	—	—	—	—	—	—	4.1 (1)
Cirrhosis $HBsAg^+$ (Far East)	20	95	—	—	—	5 (1)	—	—	—
HCC $HBsAg^+$ (Africa)	28	10.7	—	3.6 (1)	3.6 (1)	—	—	—	82 (23/28)
HCC $HBsAg^+$ (Far East)	57	21	—	—	—	—	—	—	79 (45/57)
HCC (France)†	11	—	—	—	—	—	—	—	100
HCC $HBsAg^-$‡	16	62	—	—	—	—	—	—	38 (6)
Other tumors§	41	92.6	—	2.4 (1)	2.4 (1)	—	—	—	2 (1)
Total	1747								

Numbers in parentheses refer to numbers of patients. CAH, chronic active hepatitis; AH, acute hepatitis.
*See text.
†Three of 11 are children with hepatoblastoma.
‡$P < 0.001$ vs. HCC $HBsAg^+$.
§Includes tumors of placenta, colon, rectum, esophagus, breast, medullary thyroid, lung, uterus, and unknown origin (2).

of AFP levels (Table 2). We also studied patients with very high levels of AFP because this assay uses a simultaneous-sandwich mode, and we wished to examine the magnitude of the "hook effect" as shown by a representative example in Fig. 3. This patient has an AFP level of >1 mg/ml. Despite the exceedingly high level of AFP measured in some patients, we have not observed to date a false-negative value due to the hook effect (see ref. 14).

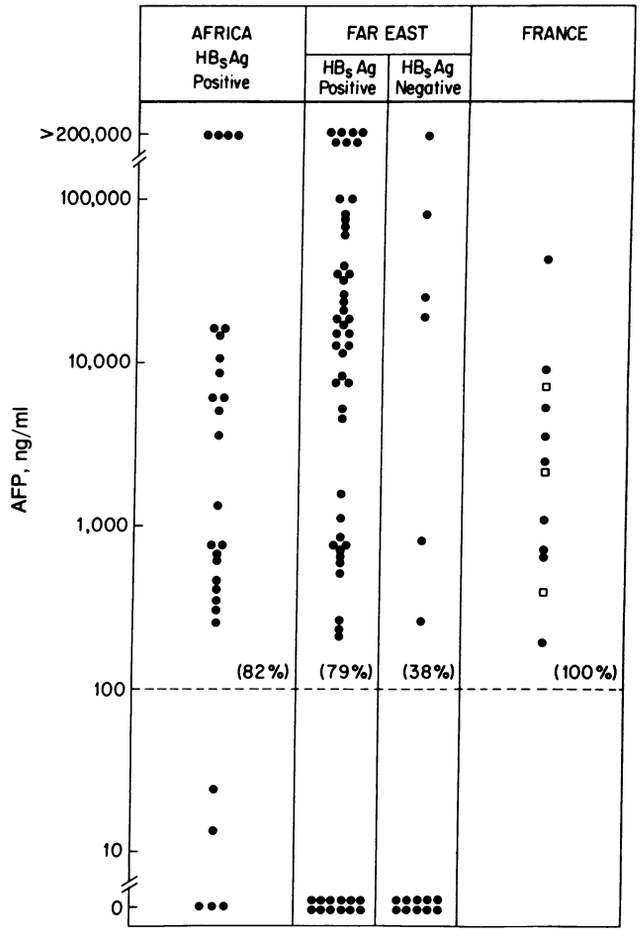

FIG. 2. Absolute AFP values in patients with HCC (●) or hepatoblastoma (□) from Africa, the Far East, and France. The numbers in parentheses represent the percent of individuals with AFP levels of >100 ng/ml.

DISCUSSION

We have developed a simultaneous-sandwich RIA by using a combination of two high-affinity MAbs directed against two separate epitopes of human AFP. This M-RIA is ≈4–10 times more sensitive than commercially available polyvalent C-RIAs; it has a lower level of sensitivity of ≈0.5 ng/ml. The assay is technically convenient and rapid, involving the simultaneous addition of all reagents and a 1-hr incubation at 45°C. The so-called hook effect previously described for "one-step" immunoassays (14) was observed also for high AFP values (i.e., >3,000 ng/ml). However, this phenomenon did not lead to false-negative AFP results but only required additional dilutions to accurately measure the abso-

Table 2. Comparison of serum AFP levels by M-RIA and C-RIA in 17 patients with various tumors

Tumor type	Serum AFP, ng/ml	
	M-RIA	C-RIA
Hepatocellular carcinoma	30,500	44,700
	9,000	6,700
	5,300	2,370
	2,400	2,670
	1,100	750
	600	443
Hepatoblastoma	6,900	5,800
	2,150	10,100
	380	410
Testicular tumors, embryonal carcinoma, or teratoma	58,000	18,300
	1,080	2,510
	190	107
	140	88
	60	48
Choriocarcinoma	<1.5	23
Seminoma	<1.5	<5
	<1.5	<5

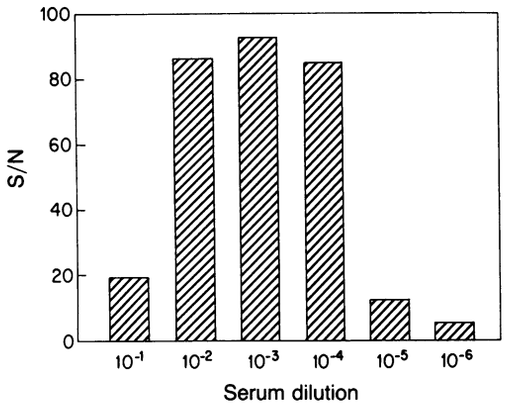

FIG. 3. Typical result of serial dilution of serum from a strongly AFP-positive patient with HCC. This shows the hook effect with the simultaneous-sandwich M-RIA.

lute AFP value from the standard curve. One of the major problems with current polyvalent AFP assays is their limitation in discriminating between AFP-producing carcinomas (especially HCC) and various benign liver diseases. Serum AFP levels have been reported to be >20 ng/ml in 67–82% of HCC (7, 15). However, given the limited specificity of the polyvalent assays, AFP increases to >20 ng/ml have been observed in 31–40% of patients with acute and chronic hepatitis and 8–33% of patients with cirrhosis (7, 8, 15). In one study serum AFP levels of >100 ng/ml were reported in 19% of patients with acute hepatitis and 5% with cirrhosis (8).

In our study, 86 of 116 (76.7%) HCC patients had AFP serum levels of >20 ng/ml, with 85 of 112 (75.8%) having levels of >100 ng/ml, a finding consistent with other reports (15). If, we consider those patients with HCC who were also HBsAg-positive carriers, 80% had AFP levels of >200 ng/ml. In patients with various types of malignancy, the AFP levels by both M-RIA and C-RIA were quite comparable. However, most significant was our finding that, compared to previous studies with polyvalent antibodies, the incidence of AFP increase when using our M-RIA was extremely low in normal individuals and in a number of different disease categories. Thus, no healthy subject had a level of >5 ng/ml, whereas other reports have shown 75% of normal human sera to have AFP levels of >5 ng/ml (16, 17). It is also noteworthy that 98.1% of HBsAg chronic carriers had a serum AFP level of <5 ng/ml and 100% had levels of <20 ng/ml. It was striking that 96.4% of patients with miscellaneous liver disorders and other disease controls had AFP values of <5 ng/ml, with 99.6% of these patients with levels of <20 ng/ml. More surprising was the finding that in cirrhotic patients (regardless of the etiology of the cirrhosis or their geographical origin), only 3% (1 of 33) had an AFP level of >20 ng/ml and none had levels of >100 ng/ml. In the acute or chronic active hepatitis patients studied, only 1.9% (1 of 51) had an AFP level of >20 ng/ml. Thus, the M-RIA demonstrates remarkable specificity for AFP-producing tumors; depending on the cut-off value for AFP-positive results, these patients show little overlap with nonmalignant liver disorders and other disease controls. Indeed, if one selects a cut-off value of 100 ng/ml, only 2 of 1185 disease controls (or 0.1%) exceeded that level.

Comment is required on the possible mechanisms that might account for the higher sensitivity and specificity of the multisite M-RIA. It seems possible that high AFP serum levels previously observed with conventional RIAs in benign liver diseases were due to the real production and secretion of AFP or "AFP-like" proteins. Nevertheless, it is also possible that these high AFP values reflected artefacts due in part to the presence of cross-reactive molecules of "AFP-like" material. For example, previous studies have shown that "specific" antisera raised against purified human AFP were not cross-reactive with native albumin, even though this protein shares a large sequence homology with human AFP; however, such anti-AFP antisera reacted strongly with derivatives of human albumin (18). Thus, conventional polyvalent RIAs may yield false positive AFP values because of cross-reactions of polyvalent antisera with "AFP-like" material, including various albumin degradation products, which may occur in patients with inflammatory disorders of the liver. In contrast, the multisite M-RIA may fail to detect such cross-reactive proteins.

There are several possible explanations for our findings. The first is the unique monospecificity of the antibodies directed toward a specific epitope on AFP. AFP is a molecule comprised of 590 amino acids. There are some limited amino acid sequences, particularly at the NH_2-terminal part of the chain (domain 1), that are AFP specific; otherwise human AFP shows a 39% overall amino acid sequence homology to human albumin (19). If one epitope, recognized by either AF01, AF03, or perhaps both, were localized to the AFP-specific region, we could readily explain the remarkable specificity of our assay. Furthermore, the assay design (multisite RIA) decreases the possibility of a cross-reaction with a nonrelevant protein because only one specific MAb is required for detection of the AFP-specific region—namely, AF01. Finally, the sensitivity of this assay is most likely due to the affinity of AF01 and AF03 MAbs for AFP-associated epitopes (K_a 1.6×10^{10} and 5.0×10^8 liters/mol, respectively).

Heretofore, it has not been possible to use serum AFP as a screening assay for HCC because most patients with acute and chronic active hepatitis (with and without cirrhosis) often showed AFP elevations up to 1000 ng/ml and sometimes >5000 ng/ml. Thus, the diagnosis of HCC could only be suspected in patients with AFP levels > 400–1000 ng/ml, and other methods were required to confirm the diagnosis. In addition, often at the time of diagnosis, the size of the tumor was already too large and/or the cirrhosis was too far advanced to warrant consideration of surgical resection (20). However, in a few patients, the discovery of an elevated AFP has led to curative resections of small tumors (21). Since with our M-RIA, the number of AFP-positive sera (>20 or 100 ng/ml) in chronic HBsAg carriers and in HBsAg-positive acute and chronic hepatitis (with and without cirrhosis) was extremely low (99.9% of patients have AFP levels of <100 ng/ml), it is quite possible that the M-RIA may be useful as a screening test for the early diagnosis of hepatocellular carcinoma in high-risk populations.

Clearly additional large-scale clinical studies are needed especially in HBsAg-positive carriers. However, in view of the data we have obtained, we are optimistic about the potential value of this rapid, sensitive, and highly specific M-RIA in the detection, early identification, and monitoring of patients with AFP-producing tumors.

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