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A Rapid and Sensitive Two-Site Sandwich Enzyme-Linked Immunosorbent Assay for Detection of α-Fetoprotein in Human Serum

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Abstract: A rapid and sensitive method has been developed to detect α-fetoprotein (AFP) in human serum by a two-site sandwich enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies (MAbs) for human AFP within 1 h. To obtain the most sensitive and reliable MAbs, 12 kinds of MAbs (HPJ1 to HPJ12) as a capture antibody and 4 kinds of horseradish peroxidase (HRP) conjugated MAbs as a tracer antibody were investigated. Among these, only HPJ 10-HRP conjugated HPJ 1 (HPJ 10-HPJ 1*) and HPJ 11-HRP conjugated HPJ 10 (HPJ 11-HPJ 10*) were chosen as candidates based on the linearity of the standard curve and the sensitivity of the assay. To further characterize these two pairs, MAbs against human AFP were purified from hybridoma cells, conjugated with HRP, and then characterized to optimize the two-site sandwich ELISA. The HPJ 10-HPJ 1* pair showed a sensitivity of 1 ng/ml and a better reproducibility than the HPJ 11-HPJ 10* pair when the human sera were incubated at 37℃ for 30 min. The results obtained for 480 randomly selected human sera showed 0~20 ng/ml of AFP values for the normal human sera. To test the utility of our kit, AFP concentrations were determined for 951 human sera (including 85 normal sera, 480 random blood sera, 213 HBsAg-positives, 50 anti-HCV antibody positives, and 47 malignant diseases) and compared with other commercially available AFP detecting kits. These results show that the present two-site sandwich ELISA method is a rapid, sensitive, and reliable procedure for detecting AFP in human serum.

Key words: α-fetoprotein, sensitivity, two-site sandwich enzyme-linked immunosorbent assay.

α-Fetoprotein (AFP) is a major plasma protein of the foetus (Bergstrand and Czar, 1956), where it is produced by the yolk sac and the liver (Gitlin and Perriceili, 1970). AFP has a molecular weight of 65,000~70,000 daltons, consists of a single polypeptide chain and contains about 4% carbohydrate (Smith and Kelleher, 1980). AFP concentration levels regularly increase during pregnancy up to a maximum level at the 14th week in fetal serum and at the 20th week in maternal serum, after which the levels begin to decrease (Brumfield *et al.*, 1990). In adults its concentration is extremely low (Ruoslahti and Seppala, 1972) except in patients with tumors such as primary hepatomas, teratomas (Abelev, 1974; Ruoslahti *et al.*, 1974). So AFP can be defined as a carcino-fetal protein because AFP levels are elevated in the foetus but not in adults. Patients affected by hepatic carcinoma or teratocarcinoma show a high incidence of elevated AFP values. We now report a rapid and reliable method of detecting AFP in human serum by a two-site sandwich enzyme-linked immunosorbent assay (ELISA) with MAbs directed against the human AFP.

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Materials and Methods

Materials

Human sera were obtained from Blood Bank (Korea), Catholic University Medical College, Yonsei University Medical College, and Chungnam University Medical College. Human AFP, used as working standard antigen in the development of two-site sandwich ELISA, was obtained from InPharm company (Russia); human AFP used as immunogen, horseradish peroxidase (HRP), pristane, and mouse MAb isotyping reagents from Sigma (St. Louis, USA); HRP-conjugated antimouse IgG goat antibody from Calbiochem; Balb/c mice for monoclonal antibody production, from Myung Jin Instrument Co. (Korea Laboratory Animal Center, Korea); polystyrene microtiter plate from Corning.

Production of monoclonal antibodies

For injection, the AFP (50 μg in a volume of 150 μl) was mixed with an equal volume of complete Freund's adjuvant by sonication for three 15 sec bursts at 30% maximum intensity. The antigen-adjuvant mixture was injected into a female Balb/c mouse (six weeks old) intraperitoneally. The first injection was followed by three booster injections at two weeks intervals. The final injection was given 3 or 4 days before the cell fusion without adjuvant. The feeder layer cells were prepared one day before fusion. A 12~18 weeks old mouse was killed by cervical dislocation, its abdominal skin was carefully removed and peritoneal cells were collected by centrifugation. The cells were suspended in 50 ml of HAT medium and 0.5 ml of the suspension was placed into each well of five 96-well plates. Prepared myeloma and SP2/0 Ag-14 cell suspensions were combined together and washed with RPMI 1640 by centrifugation for 10 min at 200×*g*. The cell pellet was mixed by tapping the tube with fingers and 1 ml of 50% PEG in RPMI 1640 was added slowly to the tube over a period of 1 min with constant swirling at 37℃. The fusion process was allowed to continue for another 90 sec at 37℃ (Goding, 1985). About two weeks after the fusion, culture supernatants were collected and screened by ELISA. Positive clones selected by the screening method were transferred to 24-well plates, or 6-well plates, and finally grown in tissue culture flasks (75 cm^2) and frozen in liquid nitrogen. All hybridomas showing positive reactions were frozen and cloned by limited dilution method (repeated three times) after thawing (Harlow and Lane, 1988). From these results, we finally selected 12 MAbs (HPJ 1 to HPJ 12) for further study.

Purification of MAb

Six weeks old female Balb/c mice were treated with 0.5 ml of pristane per mouse. After 1 week, cultured hybridoma cells were introduced into the peritoneal cavities of the mice (10^5 cells/mouse). Then, ascitic fluids were collected after about 2 weeks. The collected ascitic fluids were precipitated by the addition of Na_2SO_4 solution and dialyzed against 10 mM sodium phosphate buffer (pH 8.0). The solution after dialysis was applied to a DEAE-Toyopearl 650 column (2×40 cm) and the MAb was eluted with the 10~160 mM sodium phosphate gradient. The purity of antibody was monitored by SDS-PAGE and biological activity was measured by two-site sandwich ELISA (Miles and Hales, 1968).

Affinity measurements using ELISA

The AFP at various concentrations (3.125×10^{-10} M to 100×10^{-10} M) was mixed with a constant amount of MAb (1×10^{-10} M) in PBS supplemented with 10 mg/ml bovine serum albumin (BSA). After overnight incubation at room temperature, each mixture was transferred and incubated for 1 h at room temperature into the wells of a microtiter plate previously coated with AFP (500 ng/ml). After washing with PBS supplemented with 0.05% Tween 80, the bound MAbs were detected by adding HRP conjugated anti-mouse IgG goat antibody. The dissociation constant (Kd) was obtained from the linear regression of a Scatchard plot (Friguet *et al.*, 1985).

Conjugation

The HRP conjugated MAb was prepared by the periodate method (Nakane and Kawaoi, 1974). Ten mg of MAb were dialyzed against 800 ml of 20 mM carbonate buffer (pH 9.6). Five mg of HRP, which was dissolved in 1.2 ml of deionized water, was oxidized by 0.3 ml of 0.1 M $NaIO_4$ (in 10 mM sodium phosphate buffer, pH 7.0). After incubation at room temperature for 20 min (in dark condition) with gentle shaking, the solution was dialyzed against 500 ml of 1mM sodium acetate buffer (pH 4.0) in dark condition overnight at 4℃. After mixing HRP-aldehyde with 10 mg of MAb solution, 0.25 g of Sephadex G-25 was added to reduce the reaction volume and incubated for 2 h at room temperature (in dark condition) with gentle shaking. Then 0.1 ml of $NaBH_4$ (4 mg/ml in deionized water) was added to the above solution, incubated for 2 h at 4℃ (in dark condition) with gentle shaking, and dialyzed against 1 l of PBS solution overnight at 4℃. After conjugation, the mixture was applied to a Sephadex G 200 column (2×80 cm), and the HRP conjugated MAb was eluted with PBS. The HRP conjugated MAb peak was identified by the RZ value (A_{403}/A_{280}) of about 0.3~0.5, 7.5% SDS-PAGE analysis, and direct antigen ELISA. The enzyme activity of HRP conjugate was determined as previously described by Catty (1989). Briefly, 20 μl of a diluted conjugate (1 μg/ml) was added to 3.0 ml of freshly prepared OPD substrate solution. After incubation at room temperature, 200 μl aliquots serially taken at 0, 5, 10, 15, 20, 25, 30 min were mixed with 50 μl 2 M H_2SO_4 solution and the absorbance read at 490 nm. In general, conjugates diluted to 1 μg/ml that achieve an absorbance of 1.0 in 10~15 min in the test, and a final absorbance of 1.5 or greater within 25~30 min have suitable enzyme activity for ELISA tests (Catty, 1989). From these results, HPJ 1*, 5*, 7*, and 10* conjugates were chosen as tracer antibodies among the 12 HRP-conjuagted MAbs.

Epitope cross-reactivity of HRP conjugated MAb

MAbs at various concentrations were mixed with a constant amount of HRP conjugated MAb. Each mixture was transferred and incubated for 1 h at room temperature into the wells of a microtiter plate previously coated with AFP (500 ng/ml). After washing with PBS supplemented with 0.05% Tween 80, the bound conjugate was detected by adding 100 μl of substrate solution containing 40 μl of 30% H_2O_2 and 40 mg *o*-phenylenediamine (OPD) per 100 ml of 0.1 M citrate buffer, pH 5.0. After incubation for 15 min at room temperature, the absorbance was read at 490 nm.

Two-site sandwich ELISA

Microtiter wells coated with MAbs (1 μg/well) were blocked with a BSA and then dried. For detection of AFP in human serum, the dried microtiter wells were incubated with 20 μl of human serum, 150 μl of HRP conjugated MAb, and incubated for 30 min at 37℃. The standard curve covered a range from 0 to 500 ng/ml of AFP. The standards are calibrated against the First International Standard of AFP (code 72/225) and show the following correlation: 1 ng=0.83 International Unit (IU). After washing, 200 μl of substrate solution was added and incubated for 15 min at room temperature. The reaction was stopped by addition of 50 μl 2 M H_2SO_4 and the color read on an automatic microtiter plate reader (THERMOmax™, Molecular Devices Co., USA) using the wavelength of 490 nm and an empty microtiter plate as a blank. The AFP concentrations in tested samples were obtained from the log-log standard curve using SOFTmax™ software (Molecular Devices Co., USA).

Results and Discussion

To obtain the most sensitive MAb and HRP conjugated MAb pair for detection of AFP in human serum, 12 kinds of MAb as a capture antibody (HPJ 1 to HPJ 12) and 4 kinds of MAb as a tracer antibody (HPJ 1*, 5*, 7*, and 10*, where asterisks denote the HRP conjugated MAb) were tested by two-site sandwich ELISA. All MAbs did not cross-react with human IgG and human serum albumin by direct ELISA (data not shown). Among these, only two pairs (HPJ 10-HPJ 1* and HPJ 11-HPJ 10*) were chosen as candidates based on the linearity and sensitivity of the standard curve. For the application of these pairs to detect AFP in human serum, HPJ 1, 10, and 11 were purified from the hybridoma cells. The doubling time for each hybridoma cell was about 20~24 h. The subtypes of MAbs from cell culture supernants were all IgG1.

Fig. 1 shows the results of the SDS-PAGE without (lane 2 to 4) and with (lane 5 to 7) β-mercaptoethanol.

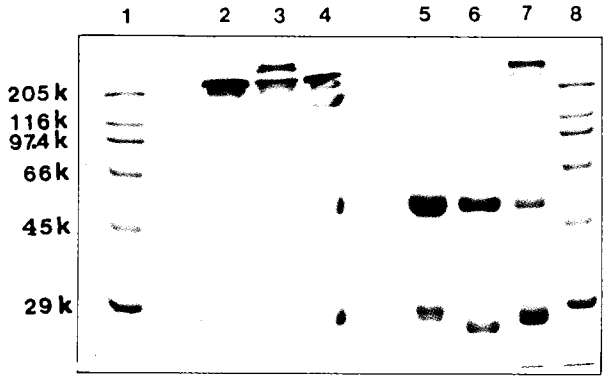

Fig. 1. SDS-PAGE of purified MAbs without (lane 2 to 4) and with (lane 5 to 7) β-mercaptoethanol. Lane 1: molecular mass standards (in kilodaltons); lane 2 and 5: HPJ 1; Lane 3 and 6: HPJ 10; lane 4 and 7: HPJ 11.

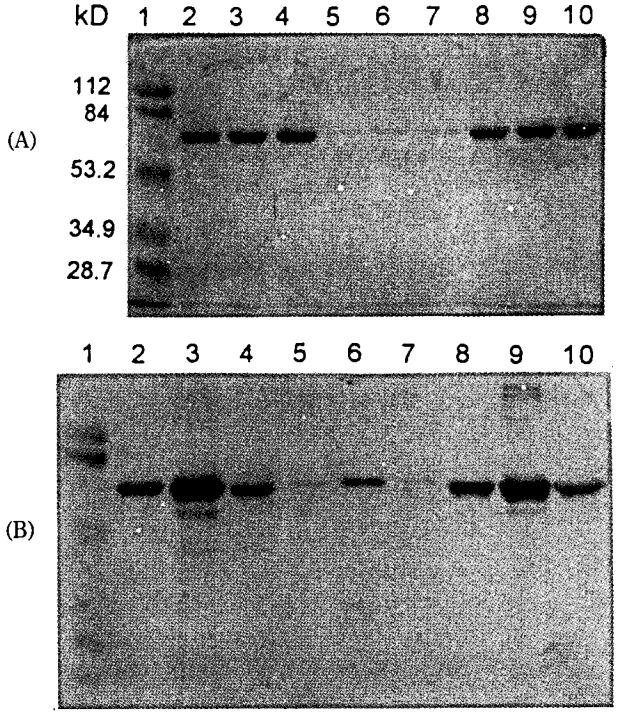

Fig. 2. SDS-PAGE of AFP and Western blot analysis. (A) SDS-PAGE analysis of AFP (3.3 μg/well). Lane 1: molecular mass standards (in kilodaltons); lane 2~4: human AFP used as working standard antigen; lane 5~7: human AFP used as immunogen; lane 8~10: an additional control human AFP from Russia. (B) Western blot analysis of the AFP by using purified HPJ 1 (lane 2, 5, and 8), HPJ 10 (lane 3, 6, and 9), and HPJ 11 (lane 4, 7, and 10) antibody (each 100 μg/ml).

Although the light chain of each MAb shows different mobility in the presence of β-mercaptoethanol, the SDS-PAGE patterns show the typical IgG pattern. These differences in the molecular weight might be explained by the variability of the light chain amino acid sequence. The purified MAbs have similar affinities for human AFP (Fig. 2). In addition, the AFP used

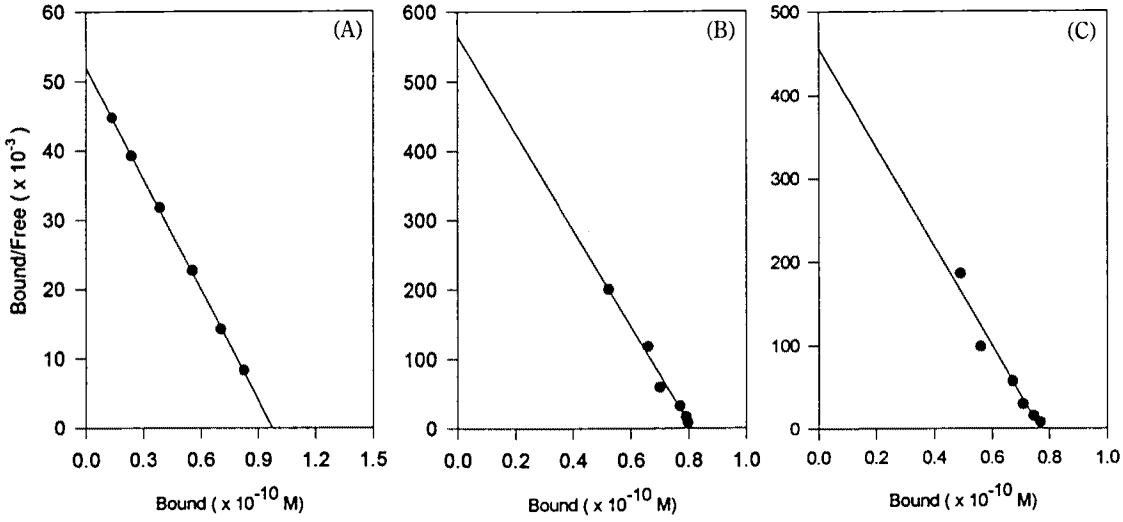

Fig. 3. Scatchard plots of binding of HPJ 1 (A), HPJ 10 (B), and HPJ 11 (C) MAb to human AFP measured by the ELISA. Bound is the fraction of bound antibody and free the concentration of free antigen at equilibrium. The dissociation constant can be deduced from the slope of the straight line.

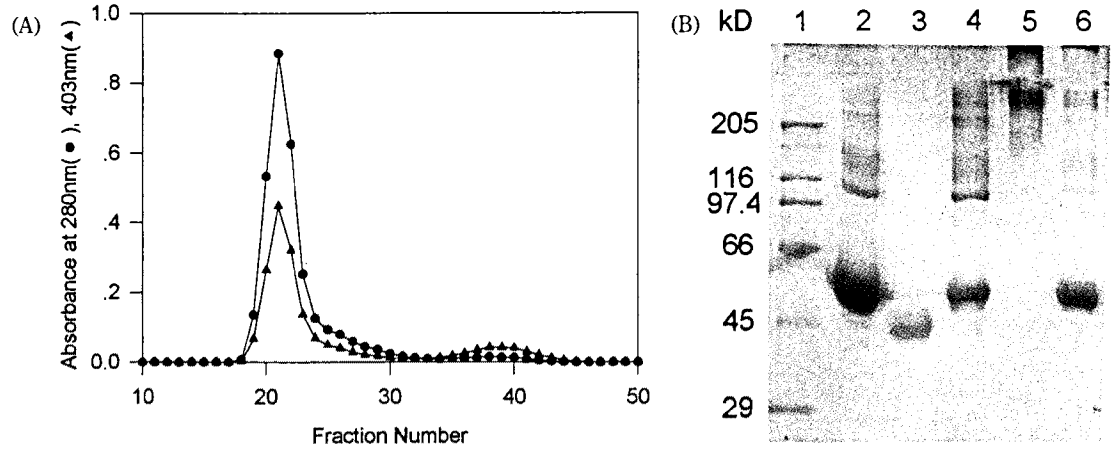

Fig. 4. The HRP-conjugated HPJ 1* antibody elution profile of Sephadex G 200 column chromatography (A) and SDS-PAGE analyses of conjugates (B). (A) Sephadex G-200 column chromatography. Flow rate, 12 ml/h; fraction size, 2 ml. (B) After purification, the fractions corresponding to fraction numbers from 19 to 28 were pooled and analyzed by SDS-PAGE. Lane 1: molecular mass standards (in kilodaltons); lane 2: HRP-conjugated anti-mouse IgG goat antibody as a control; lane 3: HRP only; lane 4: HPJ 1* conjugate used in the initial studies on the selection of the best MAb-MAb* pair; lane 5 and 6: prepared HPJ 1* conjugate before and after purification, respectively.

as the working standard has a molecular weight of about 70 kD similar to that reported by Smith and Kelleher (1980) (Fig. 2A). Although the AFP used in this experiment shows minor bands (lane 2 to 4 in Fig. 2A and B), these might be caused by the partial digestion of AFP and may have no side effect, judging from the following experiments.

Fig. 3 shows the affinity constants (Ka) of the purified MAbs. The Ka obtained from the slope of a Scatchard plot (-1/Kd) for HPJ 1, 10, and 11 were 5.3×10^8 M^{-1}, 7.0×10^9 M^{-1}, and 5.9×10^9 M^{-1}, respectively. These results imply that the HPJ 10 is a better capture antibody than the other two MAbs because of its higher affinity for AFP. Fig. 4 shows the purification profile of HRP conjugated MAb and SDS-PAGE analysis of the HPJ 1* conjugate. As shown in Fig. 4B, the HPJ 1* conjugate has a molecular weight of $\geqq$ about 200 kD similar as that of commercially available HRP-conjugated anti-mouse IgG goat antibody (lane 2 in Fig. 4B). The determination of the true molecular weight of the conjugate is difficult because of the oligomerization of HRP (Avrameas and Ternynck, 1971). From the RZ value of about 0.3~0.5, active fractions were collected and then used to determine the titer of HRP conjugated MAb (Fig. 5). As shown in Fig. 5A and B, each HPJ 1* and HPJ 10* showed a high titer

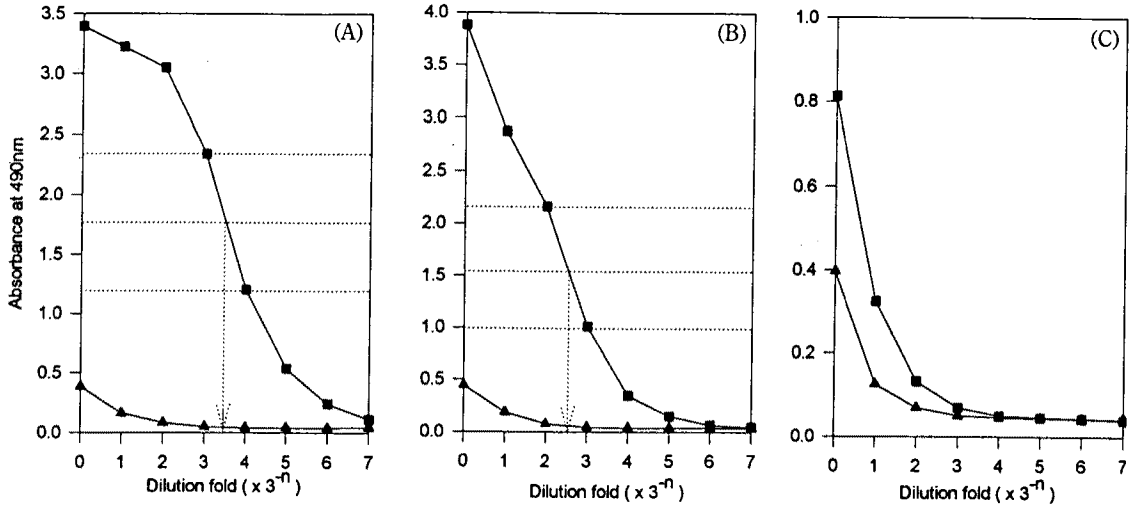

Fig. 5. Titration of HRP conjugate of HPJ 1 (A), HPJ 10 (B), and HPJ 11 (C). Specificities were tested in wells coated with AFP and background with BSA coated wells. The HRP conjugated MAbs were serially diluted starting from 1/100 dilution of the HRP conjugates. The arrows indicate the working dilution of HRP conjugate. AFP coated plate (■–■) and control plate (▲–▲).

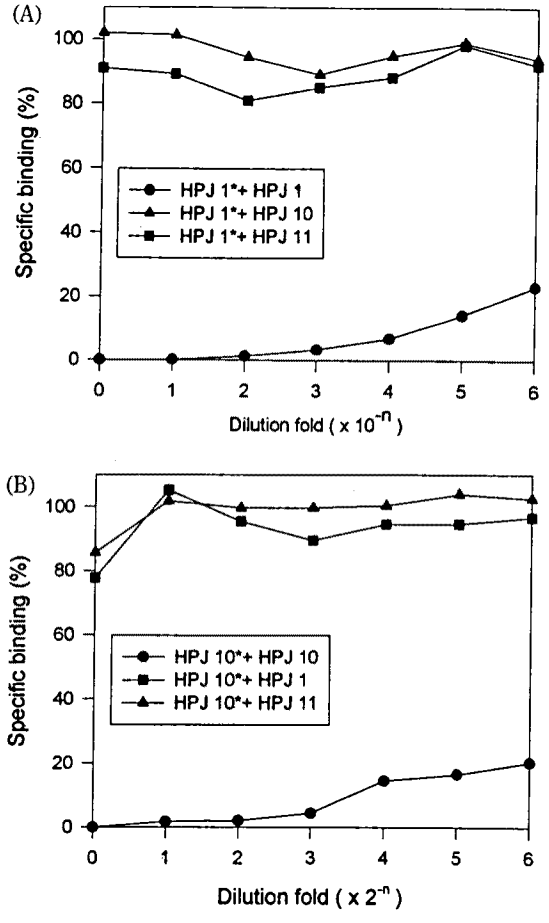

Fig. 6. Epitope cross-reactivies of HPJ 1* (A) and HPJ 10* (B). Specific bindings were expressed as percent binding compared with no addition of unlabeled antibody (100% binding).

Table 1. The reproducibility of two-site sandwich ELISA. The standard deviations (SD) and coefficients of variation (CV) are represented. The reproducibility of HPJ 11-HPJ 10* pair was also represented in bracket below the results obtained by HPJ 10-HPJ 1* pair

Intra-assay			
Control	Mean value (ng/ml)	SD	CV (%)
1	22.5(20.6)	1.66(1.51)	7.4(7.3)
2	97.1(87.5)	6.71(14.51)	6.9(16.6)
3	218.0(178.4)	17.68(26.03)	8.1(14.6)

Inter-assay						
	Singletons			Duplicates		
Control	Mean value (ng/ml)	SD	CV (%)	Mean value (ng/ml)	SD	CV (%)
1	20.7 (23.3)	1.93 (3.51)	9.3 (15.1)	22.1 (21.2)	1.52 (2.24)	6.9 (10.6)
2	99.3 (105.7)	8.75 (18.52)	8.8 (17.5)	92.1 (95.9)	6.21 (10.05)	6.7 (10.5)
3	215.1 (234.9)	19.65 (32.77)	9.1 (14.0)	209.8 (211.2)	13.61 (21.78)	6.5 (10.3)

(about 4,200 and 1,700, respectively), high enzyme activities (each conjugate showed an absorbance of 1.5~2.0 within 25~30 min), and high qualities (judging from the SDS-PAGE analysis, RZ value, and the enzyme activity). On the contrary the HPJ 11* showed a very low titer (Fig. 5C) and therefore this HPJ 11* was difficult for use as a conjugate. On the other hand, the AFP binding of HPJ 1* and 10* was inhibited only by the corresponding unlabeled HPJ 1 and 10, respectively (Fig. 6). None of the other MAbs showed any binding inhibition, even at high MAb excess. These

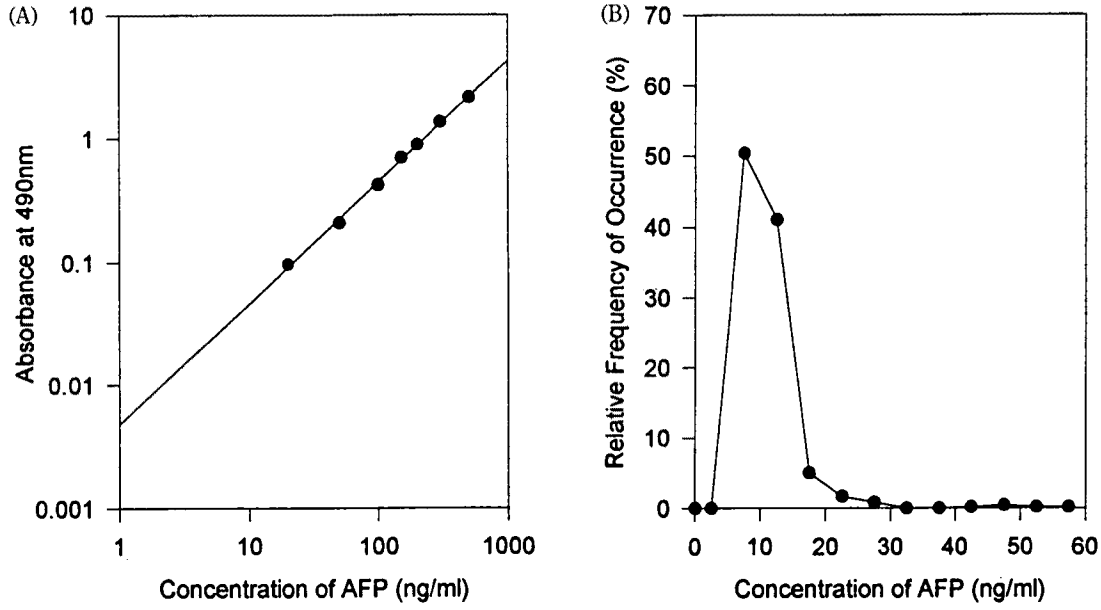

Fig. 7. Distribution of 480 determinations of AFP in unselected random populations. (A) The standard curve. (B) Distribution of AFP in random populations. The relative frequency of occurrence was expressed as percent occurrence compared with absolute occurrence (100% occurrence).

Table 2. Predicted distribution of AFP values in human sera using HPJ 10-HPJ 1* pair. Total 951 human sera were tested by two-site sandwich ELISA. The AFP values obtained by HPJ 11-HPJ 10* pair were also represented in bracket below the AFP values obtained by HPJ 10-HPJ 1* pair

	Number of serums	AFP concentration (ng/ml)				
		0~20	20~50	50~100	100~500	>500
Normals	85	85(85)	0(0)	0(0)	0(0)	0(0)
HBsAg positives	213	208(205)	4(6)	1(2)	0(0)	0(0)
Anti-HCV positives	50	48(48)	2(2)	0(0)	0(0)	0(0)
Random blood donors	480	469(469)	11(10)	0(1)	0(0)	0(0)
Malignant diseases (malignant pregnancy, stomach cancer, breast cancer, primary liver cancer, rectal cancer, colon cancer, etc)	47	29(30)	6(5)	3(4)	5(4)	4(4)

results indicate that HPJ 1* and 10* can be used as a tracer antibody.

To obtain the better MAb-MAb* pair from HPJ 10-HPJ 1* and HPJ 11-HPJ 10*, the two-site sandwich ELISA method was optimized. In contrast to HPJ 10 as a capture antibody, HPJ 11 showed a relatively low stability when the AFP coated microtiter plate was dried after blocking with BSA. This might be caused by the relative low stability of the HPJ 11 antibody itself. The sensitivity of the HPJ 10-HPJ 1* pair is better than 1.0 ng/ml. This sensitivity was calculated as the smallest concentration that can be distinguished from the 0 standard (+3 standard deviation). The HPJ 11-HPJ 10* pair also shows the same sensitivity. Table 1 shows the reproducibility of the AFP assay determined with the HPJ 10-HPJ 1* and HPJ 11-HPJ 10* kit. Within-run precision (intra-assay) was determined by assaying 28 replicates of each of 3 serum pools. Between-run precision (inter-assay) was determined by assaying duplications of 3 serum pools in 60 separate runs. As shown in Table 1, the reproducibity obtained with the HPJ 10-HPJ 1* kit is better than that of the HPJ 11-HPJ 10* kit. Based on the reproducibity of the AFP assay, the stability of the MAb-coated plate, and the affinity for human AFP, we finally selected the HPJ 10-HPJ 1* kit as a candidate for the development of an ELISA kit for detection of AFP in human serum. To test the recovery of the AFP assay, the known quantities of AFP were added to a serum pool that contained a low concentration of endogenous AFP. When the assays were repeated three times, the average recovery was 92% to 108%. On the other hand, when the serum samples that have an AFP concentration greater than 500 ng/ml were serially diluted with the 0 ng/ml stand-

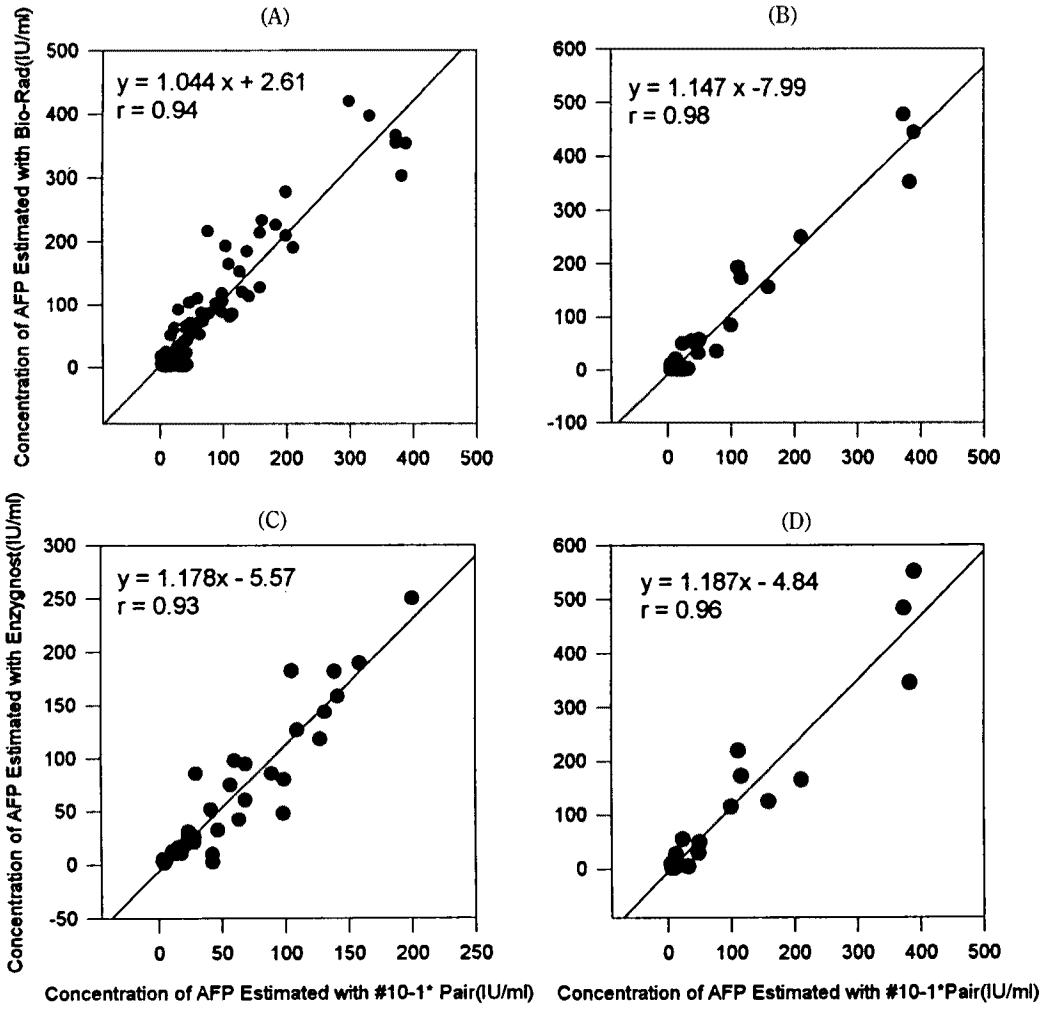

Fig. 8. The AFP correlation data from patient sera between Bio-Rad NovaPath™ AFP (A), Radim IEMA AFP (B), Behring Enzygnost AFP (C) and Abbott AFP-EIA kit (D). The AFP concentrations obtained with four kits were compared with that of HPJ 10-HPJ 1* kit. The correlation equation and correlation coefficient (r) are shown in each set of figure.

ard in a linearity study, the average recovery was about 83% to 107%. These results show that the present two-site sandwich ELISA method for detection of AFP in human serum is very sensitive and reproducible.

The range of normal serum was determined from data obtained from 480 various randomly selected samples (Fig. 7). The relative frequency of occurrence was expressed as a per cent compared with absolute occurrence (100% occurrence). As shown in Fig. 7B, 91.5% of the tested serum samples had AFP values of less than 15 ng/ml and 96.5% had values of less than 20 ng/ml. From this result, the range of normal serum was determined to be about 0~20 ng/ml, similar to that reported by others (Ruoslahti *et al.*, 1972; Ruoslahti and Seppala, 1972; Crandall, 1981). For the test of utility, AFP concentrations of 951 human sera including 47 malignant diseases, 213 HBsAg-positives, 50 anti-HCV antibody positives, 85 normal sera, and 480 random blood sera were investigated with the HPJ 11-HPJ 10* kit as well as the HPJ 10-HPJ 1* kit. Table 2 shows the results. Normal sera used in this study were found to be negative for the HBsAg, anti-HCV antibody, and anti-HIV antibody. No relationship between seropositivity for viral hepatitis (HBsAg and anti-HCV antibody) and AFP was noted in 263 patients (see Table 2). These observations are in keeping with those obtained by others (Ruoslahti *et al.*, 1973; Silver *et al.*, 1974). On the contrary in the case of malignant diseases, a direct relationship has been observed between the incidence of elevated AFP levels and the stages of the diseases. Elevated AFP levels have been observed in patients with malignant pregnancy and primary liver cancer but have not been observed in patients with other cancers (including stomach cancer, breast cancer, rectal cancer, and colon cancer). In the malignant patients which showed no such relationship, however, it cannot be ruled out that AFP could not show a high level. Previously Sarcione *et al.* (1983) have

shown that markedly increased amounts of previously undetected nonimmunoreactive AFP, which became immunoreactive after treatment with 0.4 M KCl, was detected and measured in primary human breast cancer tissue cytosol, and subsequently biosynthesis of free and bound forms of endogenous AFP were demonstrated in MCF-7 human breast cancer cells (Sarcione and Hart, 1985). In this respect, the results of the present investigation may correlate with those described above. Although the results obtained with the HPJ 10-HPJ 1* kit were similar to those of the HPJ 11-HPJ 10* kit, there are some differences in the AFP values between the HPJ 10-HPJ 1* kit and the HPJ 11-HPJ 10* kit. These differences in the AFP values might be caused by the different affinities of MAbs for human AFP and different enzyme activities of the conjugates.

The AFP values obtained for patient sera using the HPJ 10-HPJ 1* pair, shown in Fig. 8, were similar to the results obtained for the four commercially available AFP detecting kits (including Bio-Rad NovaPath™ AFP, Radim IEMA AFP, Behring Enzygnost AFP, and Abbott AFP-EIA testing kits). The extent of differences in AFP concentrations between the HPJ 10-HPJ 1* kit and four AFP detecting kits, fall within a range of 10~50% for several sera and these ranges would be general in determining the correlation between kits (Fleisher *et al.*, 1984; Zucchelli *et al.*, 1986). However, judging from the slope of the correlation equation showing a high degree of confidence (r), the overall degree of correlation between our kit and other commercial kits was high (84.2~95.8%). The results obtained with the Bio-Rad and Radim kits better agree with our HPJ 10-HPJ 1* kit compared with the Enzygnost or Abbott kit. These results demonstrate the reliability of our kit for detecting AFP in human serum.

In conclusion, the present two-site sandwich ELISA method using the HPJ 10-HPJ 1* pair is a rapid, sensitive, and reliable procedure for detecting AFP in human serum and may be the method of choice for the determination of the MAb-HRP conjugated MAb pair among a large series of MAbs.

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