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Development of a One-step Two-site Enzyme Immunoassay for Measuring Human Alpha-fetoprotein by Eliminating Hook-effect

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A one-step, two-site enzyme immunoassay was developed for measuring human alpha-fetoprotein (AFP) in serum and amniotic fluid using monoclonal antibodies (McAb) by eliminating the high-dose hook effect. Three McAbs that recognize different epitopes were selected among 16 different clones on the basis of epitope mapping, two for immobilization and one for horseradish peroxidase conjugation. This one-step immunoassay system is more convenient and rapid compared to a conventional two-step sandwich immunoassay system. It did not exhibit the hook effect to around 2.7 mg/ml of AFP, which is probably one of the highest concentrations of AFP in the serum. The dose-response curve of the system was linear to 500 ng/ml of AFP and the system could differentiate as low as 1 ng/ml of AFP. The intra- and inter-assay variations were in an acceptable range; 95~104% and 97~105% respectively. Its correlation with other commercial systems was around 95%.

Keywords: AFP, Epitope mapping, Hook-effect, Immunoassay, Monoclonal antibody

Introduction

Immunoassay technologies are being used extensively in clinical evaluations and biological research. Also, the advent of monoclonal antibody technology (Köhler and Milstein, 1975) revolutionized the development of immunoassay technologies. Generally two-site or sandwich immunometric assays are being performed in a two-step format, in which the test sample first reacts with the solid phase (capture) antibody and then, after incubation and the washing step, with the labeled antibody. However, the two-step format has the disadvantage of an additional step and a longer incubation period. Although the one-step sandwich immunoassay format is more rapid and convenient than the two-step format, the major limitation of the one-step sandwich immunoassay format has been the high-dose hook effect, in which there is a paradoxical fall in the response of the assay at high sample concentrations (Ryall *et al.*, 1982; Khosravi, 1990; Charrie *et al.*, 1995; Devine 1996).

AFP determination in serum and amniotic fluid represents a useful marker for monitoring the therapeutic response and the clinical evolution of neoplastic disease, as well as fetal abnormalities such as open neural tube defects (Abelev, 1971; Glitin, 1975; Masseyeff *et al.*, 1984; Seregni *et al.*, 1995; Abelev & Eraiser, 1999; Chalasani *et al.*, 1999; Khalifa *et al.*, 1999). The frequency and level of elevated serum AFP are highest in hepatocellular carcinoma (HCC) and yolk sac tumors (YST). Most levels of serum AFP in HCC are greater than 500 ng per mL, whereas the serum AFP in most of the benign liver diseases is only moderately elevated and is transient in nature (Wu, 1990; Johnson, 1999).

Production of AFP-specific McAbs, development of immunodiagnostic kits and mapping of antigenic epitopes of AFP using McAbs were performed (Chung *et al.*, 1984; Kimm *et al.*, 1984; Noh *et al.*, 1986; Kim *et al.*, 1990). In this report, a one-step sandwich enzyme immunoassay was developed, which is more convenient and rapid than the two-step sandwich enzyme immunoassay. This system exhibited reliable measurements. The main progress is that the high-dose hook effect was eliminated by employing three McAbs that recognize different epitopes from each other; two for immobilization and one for enzyme conjugation.

Materials and Methods

Production of McAbs McAbs to AFP were produced by immunizing Balb/c mice with purified AFP and fusion of spleen cells with mouse myelloma cells SP2/0 as described (Kimm *et al.*, 1984). In the end 16 different clones were produced.

Coupling of McAb with horseradish peroxidase Each McAb was purified by protein A-agarose gel (Sigma, St. Louis, USA) from ascites and coupled with horseradish peroxidase (HRP,

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Sigma) by the two-step glutaraldehyde method as described (Avrameas and Ternynck, 1971).

Determination of McAb pair for ELISA Each McAb was diluted to 10 μg/ml in a carbonate buffer, pH 9.6, and a 200 μl solution were added to immunotubes (NUNC, Maxisorp, Roskilde, Denmark) and incubated overnight at 4°C. After removing the antibody solution from the tubes, 1 ml of 1% BSA-PBS was added and incubated for 1 hr at room temperature. After washing the tubes with PBS-T (phosphate-buffered saline pH 7.4, containing 0.05% Tween 20), 20 μl of 500 ng/ml AFP and 200 μl McAb-HRP conjugate, which was diluted in 1% BSA-PBS, was added to the tubes and incubated for 1 hr at 37°C. After washing the tubes with PBS-T again, 200 μl of o-phenylenediamine (OPD) substrate (Sigma) were added and incubated for 30 min at room temperature. The reaction was stopped by the addition of 1 ml of 1 N H_2SO_4 and the O.D was measured at 492 nm.

ELISA for AFP measurement The conditions for AFP measurement were set the same as mentioned previously and the AFP standard solutions were 0, 25, 100, 250 and 500 ng/ml.

Evaluation of the current system The correlation between the commercially available system and the newly developed system was compared. The kit used for comparison was the Genedia AFP ELISA Kit (Korea Green Cross Corp., Kyunggi-Do, Korea), which employs a two-step format. Intra-assay and inter-assay variations and the recovery of the system were determined.

To confirm the elimination of the hook-effect, 20 samples containing high concentrations of AFP, which were in the range from 800 ng/ml to 2.7 mg/ml and obtained from the Green Cross Medical Foundation, were measured on this system.

Results

Determination of McAb pair for ELISA Sixteen McAbs were prepared and 12 McAbs were conjugated to HRP by the two-step glutaraldehyde method. In the previous work, there were at least 4 major epitopes in AFP although little difference was observed in each group. Group **I** was recognized by clones **22**, **75**, **176**, **239**; group **II** by clones **1** and **11**; group **III** by clone **4**; group **IV** by clones **2**, **9**, **32**, **94** (Kim *et al.*, 1990). Each of the sixteen McAbs was immobilized to immunotubes and ELISA signals were measured using 500 ng/ml of the AFP solution against each of the 12 McAb-HRP conjugates. Signals were given by the 16 × 12 combination and summarized in Table I. Among the 192 combinations, 12 exhibited high signals (**4-2***, **22-2***, **4-9***, **19-9***, **22-9***, **176-9***, **75-32***, **4-94***, **11-94***, **22-94***, **9-176*** and **32-176***; the asterisk indicates HRP labeling). The group **IV** antibodies (**2**, **9**, and **94**) seem to be favorable for HRP conjugation and the clones **4** and **22** seem to be favorable for immobilization and those antibodies belong to group **III** and group **I** respectively (Table I).

Then clones **4**, **11**, **22** and their mixtures, **4** & **11**, **4** & **22**, **11** & **22** and **4** & **11** & **22**, were investigated for the suitability

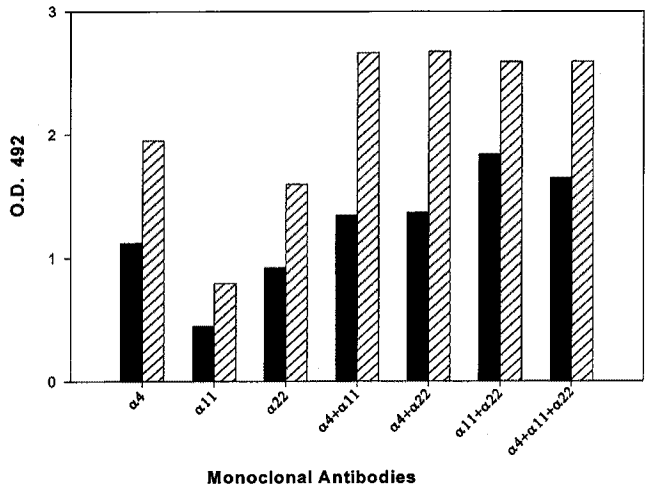

Fig. 1. ELISA signals according to different McAbs and their combination used for immobilization. The assay was performed as described in Materials and Methods. The black bar denotes the ELISA signal by 250 ng/ml of AFP and the hatched bar denotes the ELISA signal by 500 ng/ml of AFP.

of immobilization using 250 and 500 ng/ml of AFP and **94**-HRP conjugate. Each of the clones (**4** and **22**) gave a signal ratio of 1.74 (1.95/1.12 and 1.60/0.92 respectively) between 250 and 500 ng/ml AFP (Fig. 1). The clone **11** gave a signal ration of 1.78 (0.80/0.45) in that range of AFP, which is similar to those of clones 4 and 22, but the optical density (O.D.) values were lower than those of other clones (Fig. 1). The mixtures of **4** & **11** and **4** & **22** gave a similar signal ratio of 1.97 (2.66/1.35) and 1.95 (2.67/1.37) respectively between 250 and 500 ng/ml AFP. However, the mixtures of **11** & **22** gave a signal ratio of 1.40 (2.58/1.84) in that range of AFP (Fig. 1). Also the mixtures of **4** & **11** & **22** gave a signal ratio of 1.57 (2.59/1.65) in that range of AFP (Fig. 1). Although the mixture of **4** & **11** and **4** & **22** seemed to be a candidate for immobilizing antibodies, the mixture of **4** & **22** proved to be better in further comparison of ELISA readings in the whole range of the AFP standard (data not shown). Finally the mixture of **4** & **22** was chosen as the antibodies for immobilization.

The clone **2-HRP** and **94-HRP** conjugates were compared. Both exhibited similar dose-response curves, but the hook effect was observed in the **94-HRP** conjugate. The **94-HRP** conjugate showed ELISA readings of 1.62 and 1.01 for 500 ng/ml of AFP and the sample higher than 500 ng/ml of AFP. However, the **2-HRP** conjugate showed ELISA readings of 1.60 and 2.16 for 500 ng/ml of AFP and the sample higher than 500 ng/ml of AFP respectively.

Evaluation of the system The one-step ELISA system was fixed as clones **4** & **22** for immobilization and clone **2** for HRP conjugation. The typical standard curve of the system (using standards of 0, 25, 100, 250 and 500 ng/ml AFP) is shown in Fig 2. The curve is linear to 500 ng/ml of AFP.

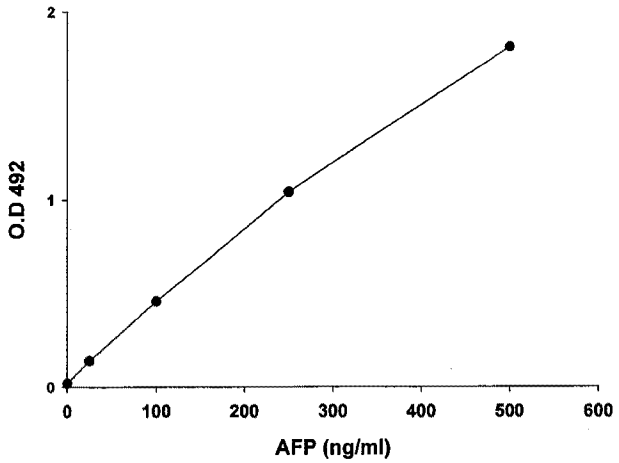

Fig. 2. Standard curve of the one-step ELISA. The used standards are 0, 25, 100, 250 and 500 ng/ml and the O.D was measured at 492 nm.

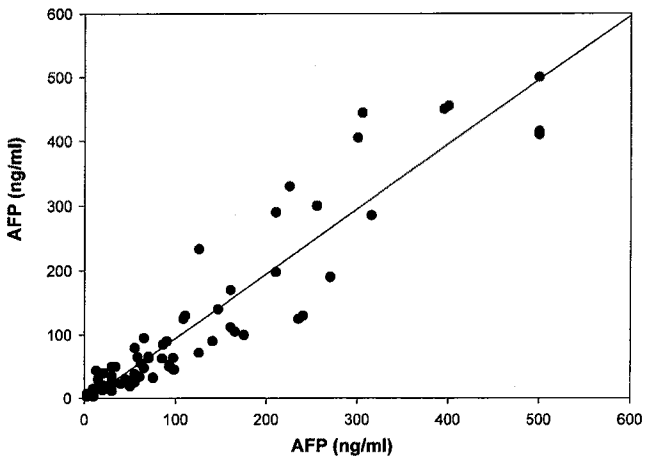

Fig. 3. Comparison of correlation between the current system and commercial product. Eighty samples were compared and X axis denotes the value obtained by current system and Y axis denotes the value obtained by commercial product. The correlation between two systems is $y = x-5.2$, $r^2 = 0.95$.

The correlation of the system with other commercial one was compared for 80 samples and exhibited 95% correlation (Fig 3). The recovery of the system was estimated between 94% and 102%. The intra- and inter-assay variations were in the range of 95~104% and 97~105% respectively.

The measurement of AFP in 20 samples containing high concentrations of AFP, which were in the range from 800 ng/ml to 2.7 mg/ml, did not exhibit a lower ELISA signal than that of the highest standard 500 ng/ml AFP (data not shown). This indicates the elimination of the hook-effect.

Discussion

The advantage of the one-step sandwich immunometric assay is the convenience and rapidity of the assay. However, the system could not be applied to every two-site sandwich immunometric assay because of the hook-effect. In samples with increasing analyte concentrations, the excess antigen progressively saturates both the solid-phase and the detection antibodies. This prevents them from forming the sandwich and consequently gave false low values when samples with high concentrations were measured (Khosravi, 1990). The hook effect is gaining importance as more and more McAbs are being used in modern test systems designed as sandwich assays (Dahlmann *et al.*, 1990).

Table 1. ELISA signals given by 16 12 combinations of McAbs*.

Immobilized McAb \ HRP-Conjugate	1	2	9	19	52	56	75	94	140	145	176	239
1		0.19	0.54	0.35	0.16	0.56	0.25	0.01	0.31	0.35	0.16	0.29
2	0.18		0.14	0.28	0.14	0.28	0.66	0.02	0.23	0.66	0.37	0.59
4	0.23	1.01	0.91	0.45	0.16	0.20	0.62	0.93	0.24	0.46	0.68	0.54
9	0.28	0.08		0.27	0.16	0.27	0.78	0.03	0.25	0.60	1.05	0.57
11	0.30	0.70	0.78	0.39	0.16	0.22	0.76	1.32	0.25	0.60	0.34	0.58
19	0.27	0.06	2.32		0.18	0.45	0.30	0.04	0.28	0.40	0.09	0.32
22	0.16	1.67	1.25	0.17	0.17	0.26	0.32	1.67	0.28	0.20	0.30	0.27
32	0.37	0.04	0.24	0.35	0.13	0.22	0.85	0.15	0.27	0.53	2.22	0.67
52	0.51	0.02	0.26	0.38		0.17	0.38	0.04	0.33	0.30	0.44	0.29
56	0.28	0.03	0.17	0.34	0.16		0.41	0.05	0.28	0.44	0.16	0.27
75	0.27	0.30	0.24	0.31	0.16	0.20		0.73	0.38	0.32	0.08	0.31
94	0.26	0.10	0.20	0.29	0.18	0.27	0.61		0.28	0.44	0.35	0.36
140	0.36	0.02	0.09	0.53	0.13	0.21	0.27	0.05		0.37	0.14	0.38
145	0.39	0.09	0.35	0.28	0.17	0.30	0.26	0.05	0.25		0.19	0.36
176	0.31	0.12	1.03	0.34	0.24	0.31	0.28	0.05	0.61	0.31		0.34
239	0.25	0.05	0.21	0.32	0.16	0.20	0.26	0.05	0.25	0.38	0.11	

*Sixteen McAbs were immobilized to immunotubes and 12 McAbs were conjugated to HRP. ELISA signals were measured for 500 ng/ml of AFP as described in Materials and Methods. Blank indicates the reaction by same antibody and signals over 0.80 are highlighted.

In previous studies, clones **22** and **32** were employed for radioimmunoassay, in which clone **32** was immobilized and clone **22** was labeled with I^{125} (Chung *et al.*, 1984). Also these two clones were employed for two-step enzyme immunoassay, in which clone **22** was immobilized and clone **32** was conjugated with HRP (Noh *et al.*, 1986). There seems to be a preference in antibodies for conjugation of small (I^{125} in this case) and bulky (HRP in this case) molecules.

Only two clones were employed in these studies, but more clones were produced for further systemic studies. In the epitope mapping of AFP using a group of McAbs, the group I McAbs (**22**, **75**, **176**, **239**) are more distant than the group II McAbs (**1** and **11**) from the group IV McAbs (**2**, **9**, **32**, **94**), and the group III McAb (**4**) is distinct from all of the others (Kim *et al.*, 1990). Clone **22** seems to be better than clone **32** for immobilization in enzyme immunoassay considering the previous study (Noh *et al.*, 1986) and Table I. Also by employing another McAb, which recognizes a different epitope from that of clone **22**, the hook-effect could be eliminated. Only clone **22** showed the hook-effect (data not shown).

Group **IV** McAbs were favorable for HRP conjugation (Table I) and clone **2** was chosen over clone **94** for conjugate preparation since clone **2** did not exhibit the hook-effect on the samples higher than 500 ng/ml AFP, instead clone **94** exhibited the hook-effect. So far it is impossible to explain the difference between clones **2** and **9** and what made the one McAb resistant to the hook-effect. The two McAbs are both IgG1, have kappa light chain, and recognize the same epitope. It will be an interesting approach to elucidate the difference between the two antibodies. An investigation is already underway.

One author proposed that the effect of excess antigen can be reversed by increasing the concentration of the detection antibody so that it is sufficient to saturate both the excess antigen and those captured by the solid-phase antibody. Under such conditions, the assay response would be expected to plateau at analyte concentrations that otherwise would result in the hook-effect (Khosravi, 1990). However the approach of increasing the concentration of the detection antibody might cause high background. Instead the approach of immobilization of more than one Ab, which recognizes different epitope and systemic comparisons of Abs for conjugation (presented in this article) might help the elimination of the hook effect of the one-step two-site immunometric assay.

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