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AMPLIFICATION OF ENZYME SIGNAL IN ENZYME - LINKED IMMUNOSORBENT ASSAY OF HUMAN ALPHAFETOPROTEIN.

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ABSTRACT

Enzyme linked immunosorbent assay (ELISA) is now used for the quantitation of a wide range of clinically important substances and have, in many cases, replaced radioimmunoassay though without improving its sensitivity. In this paper, the potential of enzyme amplification system for greatly enhancing both the sensitivity and the speed of ELISA is described. The principle of the new approach is that alkaline phosphatase was used as the labelling enzyme to catalyze the dephosphorylation of nicotinamide adenine dinucleotide phosphate ($NADP^+$). The nicotinamide adenine dinucleotide (NAD^+) formed catalytically activated a strictly NAD^+ –specific redox cycle which produced an intensely colored formazan dye as the end product. Each molecule of product from the first reaction took part in many cycles of the second reaction, thus amplifying the signal. As a result, the enzyme amplification increased the absorbance obtained from the commonly used enzyme label alkaline phosphatase by at least 250 fold. The application of this approach to ELISA made possible an assay for human alphafetoprotein (hAFP) which was rapid, precise and 40 times more sensitive than the conventional ELISA using p-nitrophenyl phosphate as the enzyme substrate. Moreover, the present method gave good correlation with the RIA method. Thus it is appropriate for routine assaying minute quantities of hAFP in any clinical specimens.

INTRODUCTION

Enzyme linked immunosorbent assay (ELISA) is now a widely used technique for the quantitation of a great variety of important analytes such as drugs, hormones, tumor markers.[1-3] As an assay method it competes with the well established technique of radioimmunoassay[4] and also with the non-isotope techniques of fluorescence[5] and luminescence.[6] Most ELISAs have certain practical advantages over these methods, namely

the stability of reagents, ease of use and the ready availability of cheap and simple colorimeters, However, the sensitivity of ELISA is often poor as they depend largely on the rate of detectable change catalysed by the enzyme label. This limit to sensitivity has not yet been overcome by the simple approach of extending the incubation time enzyme labels with their substrates. In a number of current assays further improvements in terms of sensitivity or speed of measurement would be of clinical value. Radioimmunoassay (RIA) has been the dominant method when maximum sentivity has been required. However, RIA has a numbers of drawbacks, including short shelf life, hazards of ionizing radiation, the need for complex measuring equipment, long assay times and the practical detection limit of an isotopically labelled molecule. This final point is of great importance in the limitation it causes to the achievable sensitivity of RIA.

Enzyme linked immunosorbent assay (ELISA) can in theory achieve even greater sensitivity since the label is catalytic. A central problem in the development of highly sensitive ELISA has been the difficulty of measuring low levels of enzyme label. The approach taken in this report to overcome this problem has been to choose an enzyme label which does not produce a directly detectable product, but forms a catalytic activator which in turn is capable of generating many molecules of coloured product. Thus by increasing the measurable change caused by the enzyme label it has been possible to develop rapid and more sensitive assays while retaining the advantages of ELISA. Of the wide variety of catalytic activators which can be generated by enzyme labels, cofactors that activate cyclic reactions are particular useful. There are a great number of enzyme combinations which can be used as labels and coupling reactions giving different sentivities and kinetics. Many coupling reactions have been applied in the quantitation of a great variety of clinically important biochemicals and enzymes for diagnostic purposes, such as glucose,[7] urea,[8] cholesterol,[9] triglycerides,[10] and creatinine kinase.[11] In these methods, the amplification is usually achieved by causing the enzyme to produce a catalytic activator for a coupling reaction, the activity of which is measured and used to quantify the enzyme. Amplification results from the combined catalytic effect of the enzyme producing the activator and the catalytic effect of that activator on the coupling reaction. Coupling reactions for detection systems may be based on enzymes which require a specific activator or on cyclic systems in which the activator takes part in the cycle, being changed and then reformed, during which an irreversible change occurs, such as the formation of a coloured product.

The purpose of this paper is to describe the application of the enzyme amplification system to ELISA and demonstrate that it is possible to develope ELISA of high sentivity and speed whilst retaining all their practical advantages. The enzyme amplification system was composed of alkaline phosphatase (AP) as the label enzyme to dephosphorylates $NADP^+$ to NAD^+, and NAD^+ as the catalytic activator for the secondary system which operates by cyclical redox interconvertion with NADH. The reduction of NAD^+ was brought about by alcohol dehydrogenase (ADH) and the subsequent oxidation of NADH by the action of diaphorase which simultaneously reduced a tetrazolium salt to a coloured product, "formozan", which was determined spectrophotometrically. The NAD^+ and $NADP^+$ redox

cycle of Lowry *et al.*[12] has long been known. However, an important aspect of the secondary system used here is that it was selected to be very strictly NAD^+-specific. This is particularly important because of the relatively high concentrations of $NADP^+$ required by AP. Making the cycle strictly specific allows small amounts of NAD^+ formed from low concentrations of AP to be accurately determined without the complication of having to remove the much higher amounts of $NADP^+$ before allowing the cycling to commence.

The clinically important analyte selected here for demonstrating the application of the enzyme amplification on ELISA is human alphafetoprotein (hAFP). Measurement of hAFP is now widely used in the antenatal diagnosis of fetal neural tube defect[13-14] and as a tumor marker in the management of patients with primary carcinoma of the liver,[15] and embryonal carcinoma of the gonads.[16]

MATERIALS AND METHODS

Materials

Mouse monoclonal antibody to hAFP (Mab-hAFP) was obtained from Zymed, USA. The rabbit polyclonal antibody to hAFP (Rab-hAFP) was from DAKOPATTS, Denmark. hAFP international reference standard, World Health organization (WHO) preparation no.72/227, was obtained from the National Institute for Biological Standards and Control, London, U.K. Alkaline phosphatase (AP) from bovine intestinal mucosa, diaphorase NADH : dye oxidoreductase (EC 1.6.4.3), alcohol dehydrogenase:NAD^+ oxidoreductase (EC 1.1.1.1) (ADH), bovine serum albumin-fraction V (BSA), p-nitrophenyl phosphate (pNPP) and p-iodonitrotetrazolium violet (INT-violet) were obtained from Sigma Chemical Co. Ltd, USA. Microplates (96 well Immunoplate I) were from Nunc, Denmark. All other reagents were of analytical grade or equivalent purity.

Human amniotic fluids, maternal sera during the second trimester and sera of patients with liver cancer were obtained from Siriraj Hospital, Mahidol University, Bangkok, Thailand.

METHODS

Conjugation of AP to Rab-haFP

Conjugate of Rab-hAFP was made by the one-step glutaraldehyde method using a solution of 2 mg/ml of the Rab-hAFP.[16] The conjugate was made up at 500 ng/ml AP in 0.1 M triethanolamine buffer, pH 7.5, containing 6% (w/v) BSA, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.05% (v/v) Triton X-100, 0.1% (w/v) NaN_3 and 0.15 M. NaCl.

Enzyme assays

The enzyme activity of conjugated AP was initially measured by both the conventional assay using pNPP as substrate and the amplified method. This was done by adding 10 μl samples containing a range of concentrations of the conjugate to microplate wells and assaying them as follows.

Conventional assay

80 μl of 5 mM pNPP in 0.9 M diethanolamine buffer, pH 9.8, containing 0.5 mM $MgSO_4$ was added to each well and incubated for 15 min at 25°C. The enzyme reaction was then stopped by addition of 270 μl of 2M NaOH and the increase in absorbance at 405 nm over a reagent blank recorded using a Titertek Multiskan MCC microplate reader.

Enzyme amplified assay

80 μl of substrate consisting of 0.2 mM $NADP^+$ in 50 mM diethanolamine buffer, pH 9.5, containing 1.0 mM $MgCl_2$ were added to each sample to be assayed by the amplified method and incubated for 15 min at 25°C. Further phosphatase activity was then inhibited and cycling commenced by the addition of 220 μl of a solution consisting of 0.4 mg/ml ADH, 0.4 mg/ml diaphorase and 0.55 mM INT-violet in 25 mM sodium phosphate buffer, pH 7.2, containing 4% (v/v) ethanol. The color development was stopped after a further 15 min incubation by addition of 50 μl 0.4 M HCl and the increase in absorbance at 495 nm over a reagent blank recorded.

ELISA procedure of hAFP

Microplates were coated by incubating them overnight at 4°C with 100 μl of Mab-hAFP diluted to 2 μg/ml in 50 mM sodium bicarbonate buffer, pH 9.6, in each well. After the coating antibody was discarded, the wells were washed three times (400 μl/well) in phosphate buffered saline (PBS)/ Tween (0.05% v/v) and then blocked with 0.5% BSA in PBS/Tween for 30 min at room temperature. The blocking solution was discarded and 25 μl of each hAFP standard (0, 10, 20, 30, 40, 50 IU/ml) followed by 75 μl of the conjugate were added. The plate was then incubated for 1 h at 37°C before washing with the PBS/Tween to remove excess conjugate. The activity of the bound enzyme was measured by both the conventional and enzyme amplified methods (see Enzyme Assays).

Accuracy of the enzyme amplified ELISA method

Various concentrations (20, 40, 60 IU/ml) of hAFP standards were added into known pooled human serum (10 IU/ml). Each concentration was measured 10 times and the percent recovery of hAFP was calculated.

Precision of the enzyme amplified ELISA method

Both within-run precision and between-run precision were determined at three hAFP concentrations by distributing 20 replicates of each sample in a diagonal matrix across a microplate.

Correlation between the enzyme amplified ELISA method and the RIA method.

The hAFP concentrations of 100 specimens including amniotic fluids, maternal sera and sera from patients with liver cancer were determined by the enzyme amplified ELISA method and by the RIA method using the Dainabott AFP obtained from Abbott Lab., Ltd, Canada. Correlation between the two methods was plotted.

RESULTS

A sandwich ELISA for hAFP was carried out, with and without enzyme amplification. The enzyme activity of the bound conjugate was measured in 2 ways. In the conventional assay, the enzyme substrate, p-nitrophenyl phosphate, was partly dephosphorylated in an alkaline buffer to p-nitrophenol, which was then measured by its adsorbance at 405 nm (Fig. 1a). In the enzyme-amplified assay (Fig. 1b) a small proportion of the substrate, nicotinamide adenine dinucleotide phosphate ($NADP^+$) was dephosphorylated to nicotinamide adenine dinucleotide (NAD^+) which catalytically activated a secondary enzyme system consisting of a redox cycle driven by the enzymes alcohol dehydrogenase and diaphorase. In the cycle NAD^+ was reduced by ethanol and the NADH so formed reduced a tetrazoleum salt to regenerate NAD^+ and to produce an intensely coloured formazan dye. The rate of reduction of the tetrazoleum was directly proportional to the concentration of NAD^+ originally formed by the enzyme in the bound conjugate. The high specificity of this redox cycle for NAD^+ prevent interference by high $NADP^+$ concentrations, which obviates the necessary separation steps of redox cycles described previously.[12]

The AP activity of the conjugate was determined both by the enzyme amplification and by the conventional assay. After addition of cycling reagents, the rate of color development in the amplified method was constant at each AP concentration used. Furthermore, as shown by Fig.2, while the absorbance obtained after a total incubation time of 30 min was proportional to AP concentration with both methods, the enzyme amplified method gave a 250-fold increase in absorbance over the conventional method.

When enzyme amplification was compared to the conventional method for AP determination in an ELISA for hAFP, the dose-response curves shown in Fig.3 were obtained. In this study, the hAFP standards ranged between 0-50 IU/ml were determined in 8 replicates by each method over the same period. The sensitivities of the 2 assay methods (defined as that concentration of hAFP which corresponds to an absorbance that was 2 standard deviations from the mean absorbance of the 8 replicates of the zero standard) were as follows : the conventional method, 5 IU/I ; the amplified method, 0.1 IU/l. This showed the amplified method to be approximately 50 times more sensitive than the conventional assay.

Analytical variables of the enzyme-amplified ELISA method for hAFP were determined. The results were as follows :

Accuracy : Recovery data for hAFP, added to known pooled normal human serum were 97.64-105.00% (average=102.38%) as shown in Table 1.

Precision : For within-run precision, the coefficients of variation of low, medium and high values were 4.92%, 5.43%, 6.54% respectively (Table 2). For between-run precision, the coefficients of variation of low, medium and high values were 5.52%, 6.59% and 6.98% respectively (Table 2).

Correlation to the other method : Results from the enzyme amplified ELISA method compared against those from the RIA method yielded a good correlation coefficient of $r=0.99$ and linear regression equation : $y=0.99\times-3.47$ (Fig.4).

DISCUSSION

In this paper the potential of enzyme amplification in the field of enzyme linked immunosorbent assay (ELISA) is described. The enzyme amplification can increase the absorbance obtained from the commonly used enzyme label alkaline phosphatase by at least 250-fold over the conventional assay. The sensitivity of this particular example of the amplification is apparent from Fig.2 which shows the absorbance increase obtained in 30 min as a function of AP. The results show that 10^{-16} mole of AP gave an absorbance increase of about 1.7 absorbance unit; and therefore, as the absorbance increase is linear with AP concentration, this can be measured with ease and thus allows a sensitivity significantly below any practical detection limit for iodine-125. Furthermore, even greater sensitivity than demonstrated here is possible by increasing the extent of the enzyme amplification.

There are a great number of enzyme combinations which can be used as label and secondary detection systems giving different sensitivities and kinetics. Here, a simple system with a AP enzyme label giving rise to NAD^+, the catalytic activator for a specific redox cycle has been illustrated, The important features of this system are (i) high specificity of the redox cycle for NAD^+ compared with $NADP^+$, (ii) a high turnover number for the AP/$NADP^+$ combination, and (iii) a low Km of the ADH and diaphorase for NAD^+ and NADH.[12]

A specific example to demonstrate the enhancement of the enzyme amplification on ELISA has been demonstrated by an enzyme amplified ELISA of hAFP. Its application allowed the development of hAFP assay which gave rise to much higher rates of absorbance change than obtainable with the conventional assay employing pNPP as the substrate. The large difference between the two methods is shown in Fig.3. The result also shows that the amplified method was quantitative and gave an essentially linear relationship between hAFP concentration and rate of absorbance change over a useful working range. The sensitivity of the amplified assay was found to be approximately 50 times more sensitive than the conventional assay and approaches that of the radioimmunoassay,[17] but without the need to invest in advanced instrument and facilities. Apart from increased sensitivity the other main benefits of enzyme amplification for hAFP-ELISA are increased assay speed and when used to provide colorimetric end points, qualitative visual reading of results. This combined with the highly sensitive nature of the test enables as little as 0.1 IU/l of hAFP to be detected visually.

Such sensitivity and simplicity of the enzyme amplified ELISA for hAFP should offer many possibilities of diagnostic importance especially for antenatal diagnosis of fetal neural tube defect and also for monitoring primary hepatomas and non-seminomatous germ cell tumors. The enzyme amplified ELISA of hAFP presented here has been found to be accurate, precise and well-correlated to the RIA method as shown in Table 1, Table 2 and Fig.4 Thus it would seem to be the most powerful and convenient method for routine determining minute quantities of hAFP in amniotic fluids, maternal sera and the tumor patient sera.[13-16]

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บทคัดย่อ

ปัจจุบันมีการใช้เทคนิคเอ็นไซม์อิมมูโนเอสเสย์ (ELISA) สำหรับวัดปริมาณสารสำคัญทางการแพทย์หลาย ๆ ชนิดแทนที่การตรวจวิเคราะห์ด้วยเทคนิคเรดิโออิมมูโนเอสเสย์ ทั้ง ๆ ที่ยังไม่มีการปรับปรุงความไวของการวิเคราะห์ให้สูงขึ้นเท่าเทียมกัน รายงานนี้จึงนำเสนอผลงานวิจัยที่อธิบายถึงศักยภาพของระบบการขยายสัญญาณของปฏิกริยาเอ็นไซม์เพื่อช่วยเพิ่มทั้งความไวและความเร็วของการตรวจวิเคราะห์ด้วยเทคนิค ELISA หลักการของกลวิธีดังกล่าวนี้ ใช้อัลคาไลน์ฟอสฟาเทสเป็นเอ็นไซม์ติดฉลาก ซึ่งจะเร่งปฏิกิริยาการกำจัดกรุ๊ปฟอสเฟต (de-phosphorylation) จาก nicotinamide adenine dinucleotide phosphate ($NADP^+$) ให้เกิดเป็น nicotinamide adenine dinucleotide (NAD^+) แล้วเข้าสู่วงจรจำเพาะ (redox cycle) ที่ให้ผลิตผลตัวสุดท้ายเป็น formazan ซึ่งเป็นสารมีสีเข้ม แต่ละโมเลกุลของผลิตผลจากปฏิกริยาแรกจะสามารถเข้าสู่วงจรของปฏิกริยาที่สองซ้ำได้หลาย ๆ ครั้ง จึงเป็นผลให้เกิดการเพิ่มขยายสัญญาณ ผลที่ได้ก็คือช่วยเพิ่มค่าการดูดแสงที่เกิดจากปฏิกริยาของอัลคาไลน์ฟอสฟาเทสที่เป็นเอ็นไซม์ติดฉลากได้อย่างน้อย 250 เท่า รายงานนี้นำเสนอการประยุกต์กลวิธีใหม่มาใช้ในการตรวจวิเคราะห์ปริมาณแอลฟ่าฟีโตโปรตีนของคนด้วยเทคนิค ELISA ทำให้ได้ผลการวิเคราะห์ที่รวดเร็ว มีความแม่นยำสูง และมีความไวสูงขึ้นกว่าวิธีการเดิมที่ใช้สาร p-nitrophenyl phosphate เป็นซับสเตรทของเอ็นไซม์ได้ถึง 40 เท่า นอกจากนั้นวิธีการที่นำเสนอยังให้ผลการวิเคราะห์ที่มีความสัมพันธ์อย่างดีกับวิธีเรดิโออิมมูโนเอสเสย์ จึงน่าจะเป็นวิธีที่เหมาะสมสำหรับงานประจำเพื่อวิเคราะห์ปริมาณ hAFP น้อย ๆ ที่อยู่ในสิ่งส่งตรวจทางการแพทย์

TABLE 1 Accuracy on hAFP determination by the enzyme amplified ELISA.

hAFP in pooled serum (IU/ml)	hAFP added (IU/ml)	Found (IU/ml)	Expected (IU/ml)	Recovery (%)
10	20	31.50	30	105.00
10	40	52.25	50	104.50
10	60	68.35	70	97.64
			Average=	**102.38**

TABLE 2 Precision on hAFP determination by the enzyme amplified ELISA.

Precision test	n	Mean	SD	% Condition variance
Within-run	20	10.37	0.51	4.92
	20	20.64	1.12	5.43
	20	40.96	2.68	6.54
Between-run	20	10.85	0.60	5.52
	20	20.79	1.37	6.59
	20	41.38	2.89	6.98

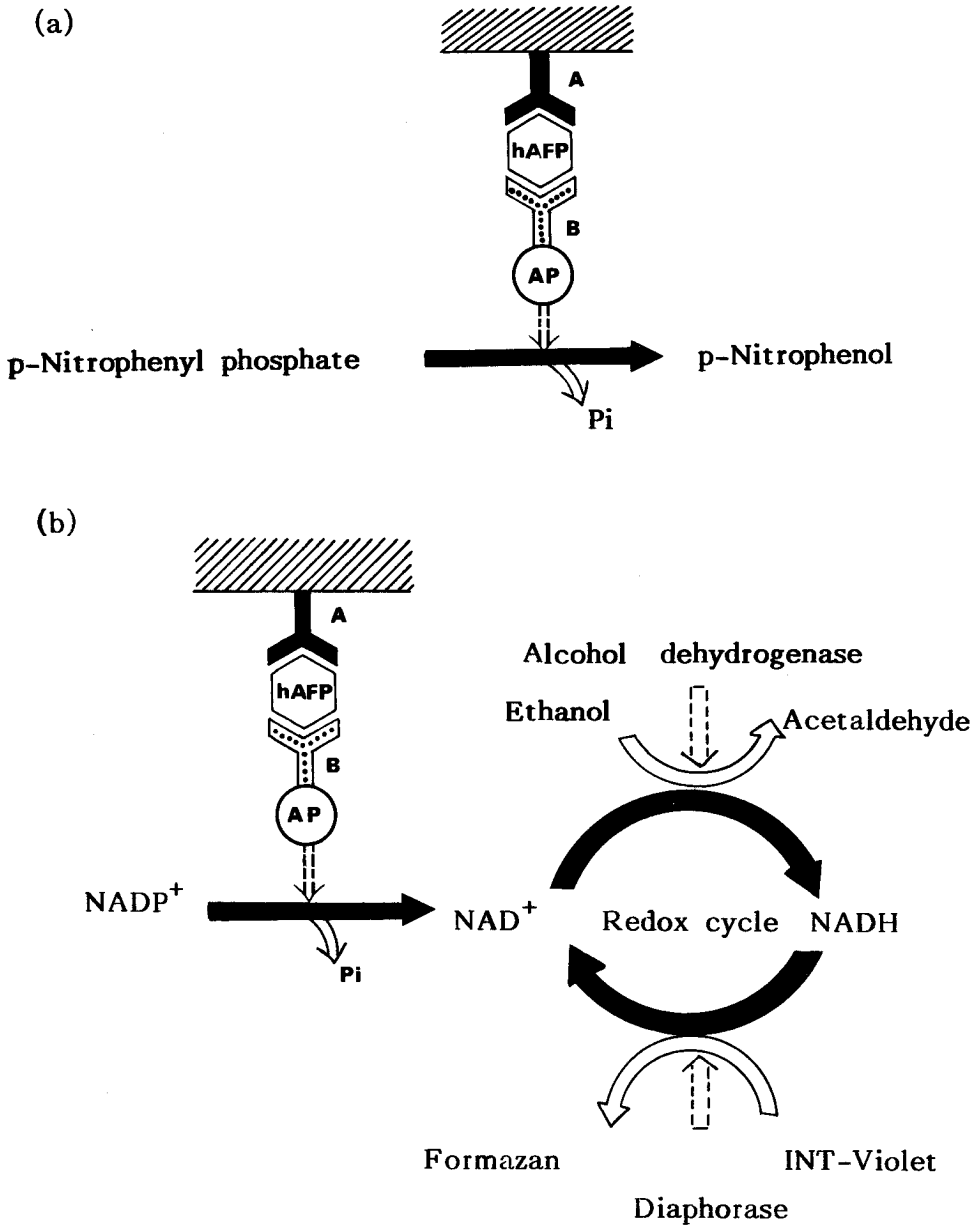

Fig.1 Principles of alkaline phosphatase (AP) assay methods in ELISA of hAFP : (a) conventional assay; (b) enzyme amplified assay. Mouse monoclonal antibodies (A) specific to hAFP are coated to polystyrene surface of microtitre wells. Rabbit polyclonal antibodies (B) specific to hAFP are conjugated with AP as the marker enzyme. By the conventional assay, the enzyme activity of the bound AP was measured involving the dephosphorylation of p-nitrophenyl phosphate to p-nitrophenol. By the enzyme amplified method, the enzyme activity of the bound AP was measured involving the initial dephosphorylation of $NADP^+$ to NAD^+, and the cycling of NAD^+ and NADH in a redox cycle generating a coloured formazan dye. Pi is inorganic phosphate, INT-Violet is a tetrazolium salt and AP is alkaline phosphatase.

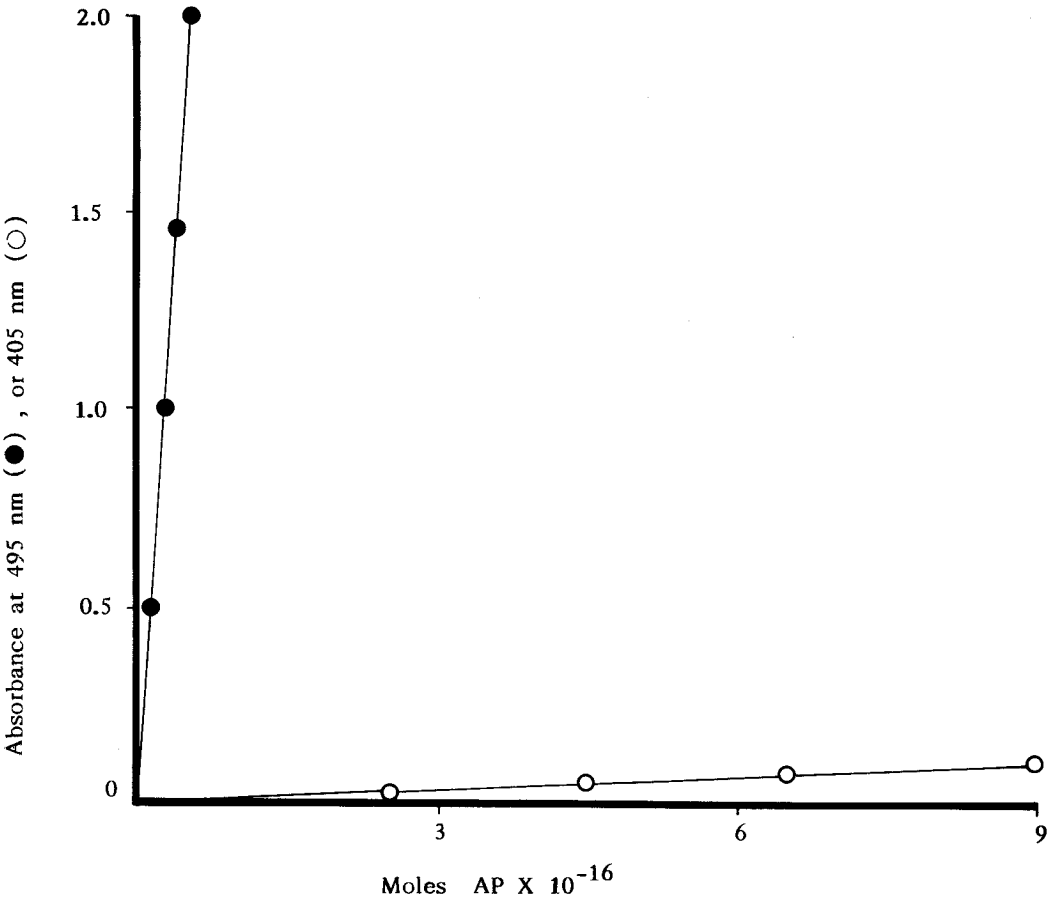

Fig.2 The absorbance obtained by the enzyme amplified (●) and the conventional (○) assay of alkaline phosphatase conjugate. The conjugate was diluted in substrate in buffer containing 0.5% BSA. Ten microlitre samples containing the amounts indicated were assayed as described in "Materials and Methods" and the resulting absorbance increases plotted as a function of the amount of alkaline phosphatase.

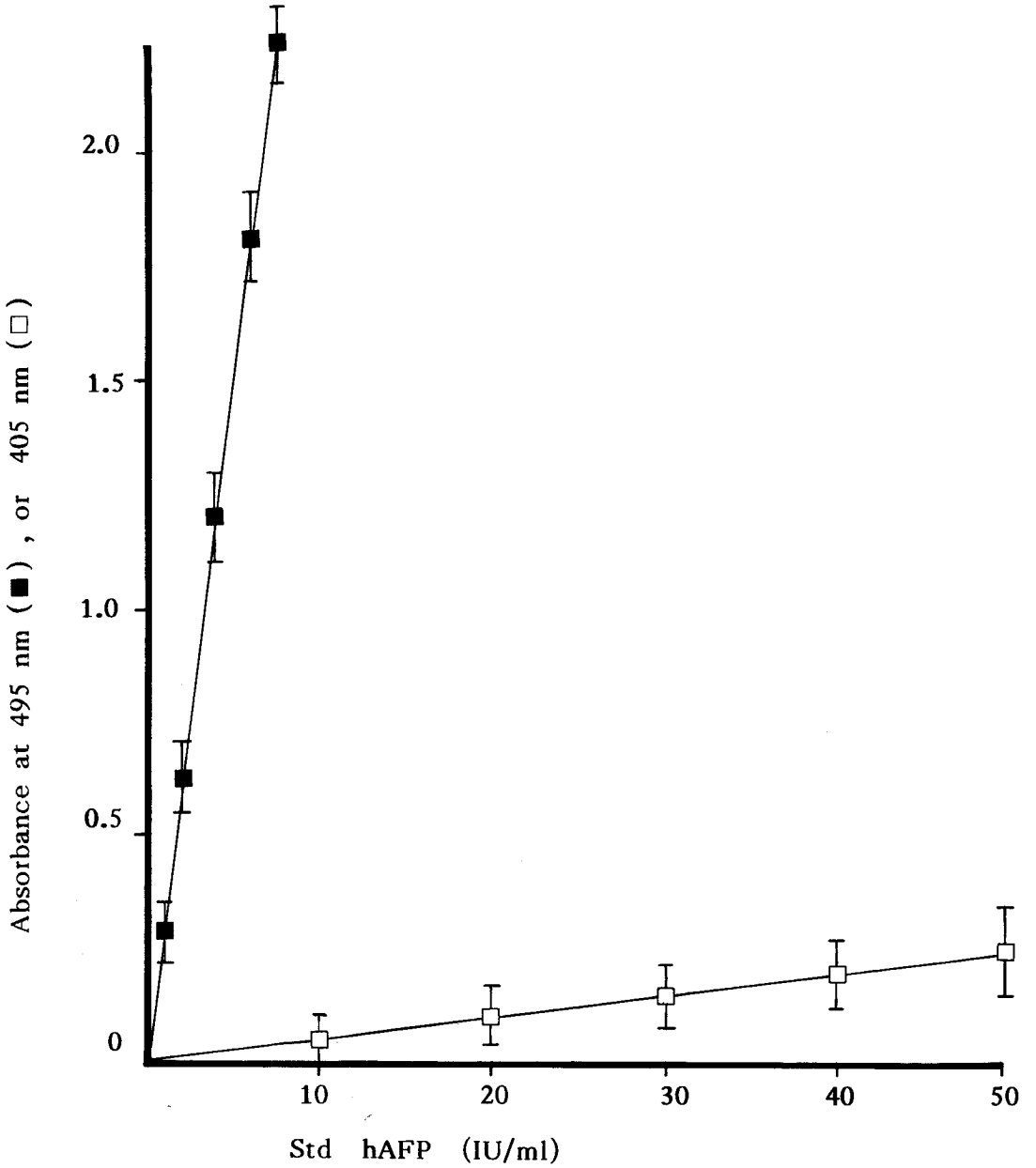

Fig.3 A comparison of conventional and enzyme amplified assay methods in hAFP-ELISA. The absorbance of the liquid in the wells over a reagent blank was measured at 495 nm for the enzyme amplified assay (■) and 405 nm for the conventional assay (□) in a Titertek Multiskan MCC plate reader. Each point on the graph represents an average of 8 readings.

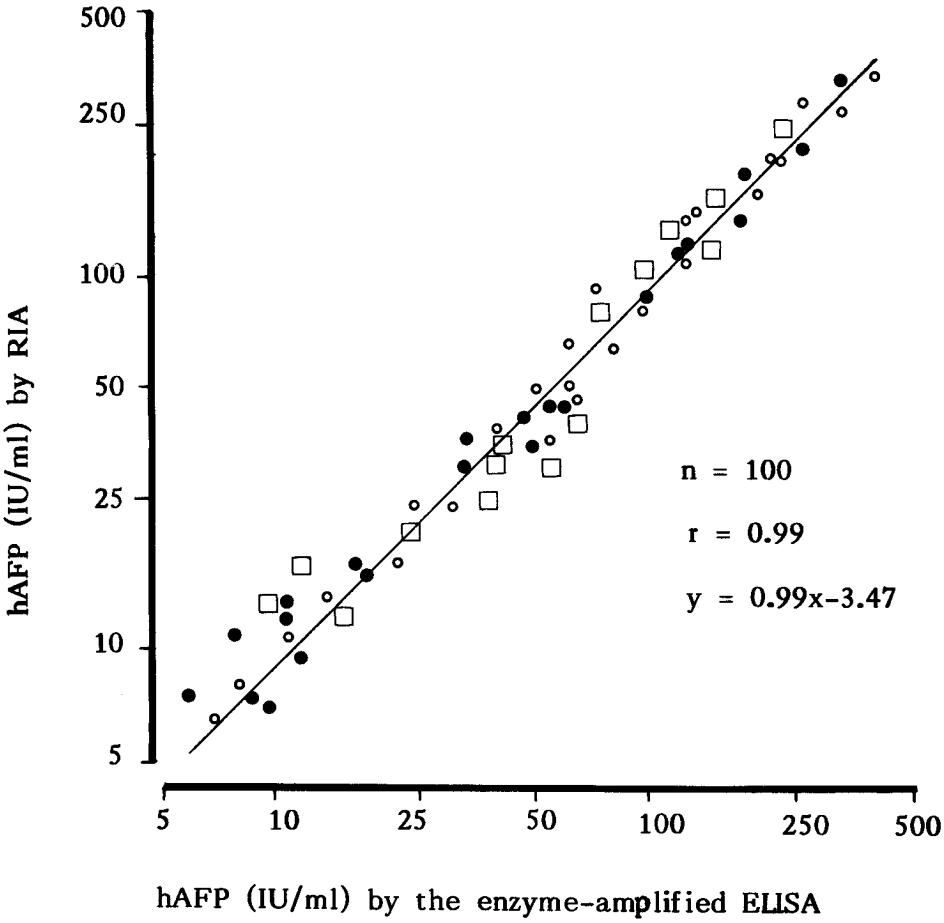

Fig.4 Correlation between hAFP values dertermined by the enzyme amplified ELISA and radioimmunoassay. The hAFP concentrations of 100 specimens including amniotic fluids (○), maternal sera (□) and sera from patients with liver cancer (●) were assayed by both methods.