Eur J Cancer Clin Oncol, Vol. 21, No. 4, pp. 525-530, 1985.
Printed in Great Britain.

0277-5379/85$3.00+0.00

Evaluation of an Amplified Enzyme-linked Immunoassay of Placental Alkaline Phosphatase in Testicular Cancer

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Abstract—*The levels of serum placental alkaline phosphatase (PLAP) have been examined in 81 male controls, 51 untreated testicular tumours (41 seminomas and ten non-seminomatous testicular tumours) and 34 patients in complete remission (11 seminoma and 23 non-seminoma). Smoking induced a significant rise of serum PLAP in the controls, with a median level of 0.055 U/l in non-smokers compared to 0.25 U/l in smokers. The levels found in pre-treatment seminoma (median 1.7 U/l) were significantly higher than in untreated teratoma (median 0.7 U/l). Treatment produced a significant fall in seminomas in remission (median 0.07 U/l). The role of PLAP in routine monitoring of seminomas was evaluated in 17 patients studied for 1-4 yr. PLAP shows similar trends to βHCG but is an independent variable. The main role of PLAP is to help determine that the response to treatment has been satisfactory and that there are no unexpected foci of tumour.*

INTRODUCTION

THE PLACE of tumour markers in the management of non-seminomatous germ cell tumours of the testis is no longer in doubt. Several studies have shown the combination of serum alphafetoprotein (AFP) and human chorionic gonadotrophin (βHCG) provide a guide to tumour recurrence and response to treatment [1-4]. By contrast, seminoma, even when in a very advanced state, produces no change in AFP and a rise in βHCG in only 20-30% [5, 6]. Placental alkaline phosphatase (PLAP), a heat-stable isoenzyme of alkaline phosphatase (EC 3.1.3.1), has recently been identified as a potential marker in seminoma [5-7]. This has resulted from improvement in the assay procedure, especially the development of radioimmunoassays [6-9], and in particular marked increase of specificity obtained by using monoclonal antibodies [10, 11].

The improvements in assay techniques, including the introduction of a fluorogenic substrate for the enzyme reaction [12], have lead to the discovery that serum PLAP levels are higher in smokers than in non-smokers [8, 12]. This paper describes the evaluation of an enzyme immunoassay for PLAP that uses a monoclonal antibody and a novel amplification procedure to enhance the signal from the enzymatic activity of the PLAP [13, 14]. The effects of smoking on PLAP are assessed in control subjects and the test is evaluated in the management of testicular tumours with emphasis on the long-term follow-up of seminomas.

MATERIALS AND METHODS

Controls

Serum samples were obtained during a survey of patients over 50 yr of age, attending their general practitioners for minor complaints. These controls were divided into 22 male cigarette smokers (>10/day) and nine male non-smokers. Also, a further 50 samples were obtained from male blood donors (25 smokers and 25 non-smokers).

Testicular tumours

Single serum samples from 55 patients with seminoma of the testis were investigated; 41 were pre-treatment samples, three were taken at the time of recurrence and 11 when in complete

Accepted 2 October 1984.
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remission. In addition, a longitudinal study was made for up to 4 yr duration with a sample frequency of every 1-3 months in 17 patients from the commencement of treatment for either primary or relapsed seminoma. A further 33 serum samples were taken from patients with non-seminomatous testicular tumours, ten from patients at presentation and 23 during surveillance.

Smoking history

The smoking histories of the controls and the 17 patients with seminomas observed in the longitudinal studies were known. The remaining serum samples came from several primary care centres and smoking history was not available.

Amplified enzyme-linked immunoassay (AELIA-PLAP)

The assay was in the form of a kit developed for research by IQ(Bio), Cambridge, U.K. A 96-well microtitre plate coated with monoclonal anti-PLAP antibody is used as the solid phase. Sixty microlitres of dilution medium and 20 μl of standard or serum sample were added to each of the wells and the plate was incubated for 2 hr at room temperature. The wells were then washed four times with washing solution, then 80 μl of substrate (NADP) were added and incubated for 20 min at room temperature. Finally, 220 μl of amplifier (a mixture of alcohol dehydrogenase and diaphorase containing ethanol and iodonitrotetrazolium violet) was added and the reaction stopped after 10 min, or earlier if the colour reaction was too intense, by adding 50 μl 0.2 M sulphuric acid [13]. The plates were then read by a Flow multiscan microplate reader at 495 nm and the results computed by a BBC microcomputer coupled to the plate reader using an IQ(Bio) software program. The coefficient of variation for the assay was around 6% and the sensitivity 0.06 U/l. The assay was checked for cross reactivity with human alkaline phosphatases from gut, liver, bone and kidney. Cross-reactivity was not detectable, that is , less than 0.003% for intestinal and less than 0.03% for the other phosphatases. The procedure took 3 hr total time and 30 min technician time, and was suitable for small batch running. All serum samples were stored at -20°C until assayed.

An independent comparison of the IQ(Bio) PLAP assay and an immunoassay for PLAP described by Haije *et al.* [9] was made in 13 sera from patients with ovarian cancer, which gave a correlation coefficient of $r = 0.97$ over the range 0.1-10 U/l IQ(Bio), 0.15-13 IU/l (Haije) (the upper limit of normal for Haije's assay is also 1.0 IU/l).

The beta subunit of human chorionic gonadotrophin (βHCG) was measured by a commercial radioimmunoassay supplied by Beckton-Dickinson, and alphafetoprotein (AFP) by radioimmunoassay as described by Ward and Bates [15]; these assays were made at the Supraregional Protein Reference Unit, Department of Immunology, Royal Hallamshire Hospital, Sheffield (AMW). The upper limits of normal for the assays used in this paper are 1.0 U/l for PLAP, 10 IU/l for βHCG and 10 g/l for AFP.

Statistical analysis

In the statistical calculations non-parametric methods were used due to the skewed distribution of the data. The results are reported as the median, range and percentage above the upper limit of normal, and differences between groups were calculated using the Wilcoxon two-sample test and the Kruskal-Wallis test, both of which gave the same result.

RESULTS

Controls

As age was not a significant factor the two age groups (controls aged over 50 yr and younger blood donors) were combined, and their joint PLAP distributions are shown in Fig. 1, according to their smoking habits. It is evident that smoking increases the median level of PLAP, the median level in non-smokers being 0.055 U/l compared to 0.52 U/l for smokers, and this difference was highly significant ($P = 0.0001$). The percentage with levels greater than 1.0 U/l was 0% in non-smokers and 14.6% in smokers.

Testicular tumours at presentation

A comparison of the distribution of serum PLAP levels in testicular tumours at presentation and follow-up is also shown in Fig. 1. The median PLAP levels were 1.7 U/l in untreated seminoma and 0.4 U/l in untreated non-seminomatous testicular tumours. Using an upper limit of normal of 1 U/l, raised levels were found in 7/81 (9%) of the male controls (smokers + non-smokers), 21/35 (60%) of untreated seminomas and 1/10 (10%) of untreated non-seminomatous testicular tumours. The pre-treatment seminoma samples had significantly elevated PLAP levels when compared to the follow-up seminoma patients in complete remission ($P = 0.0014$), the pre-treatment non-seminomatous testicular tumours ($P = 0.0150$) and the male controls when taken as a whole ($P = 0.0001$). The pre-treatment non-seminomatous testicular tumours did not show a significant elevation when compared to either the follow-ups in complete remission or the control male group.

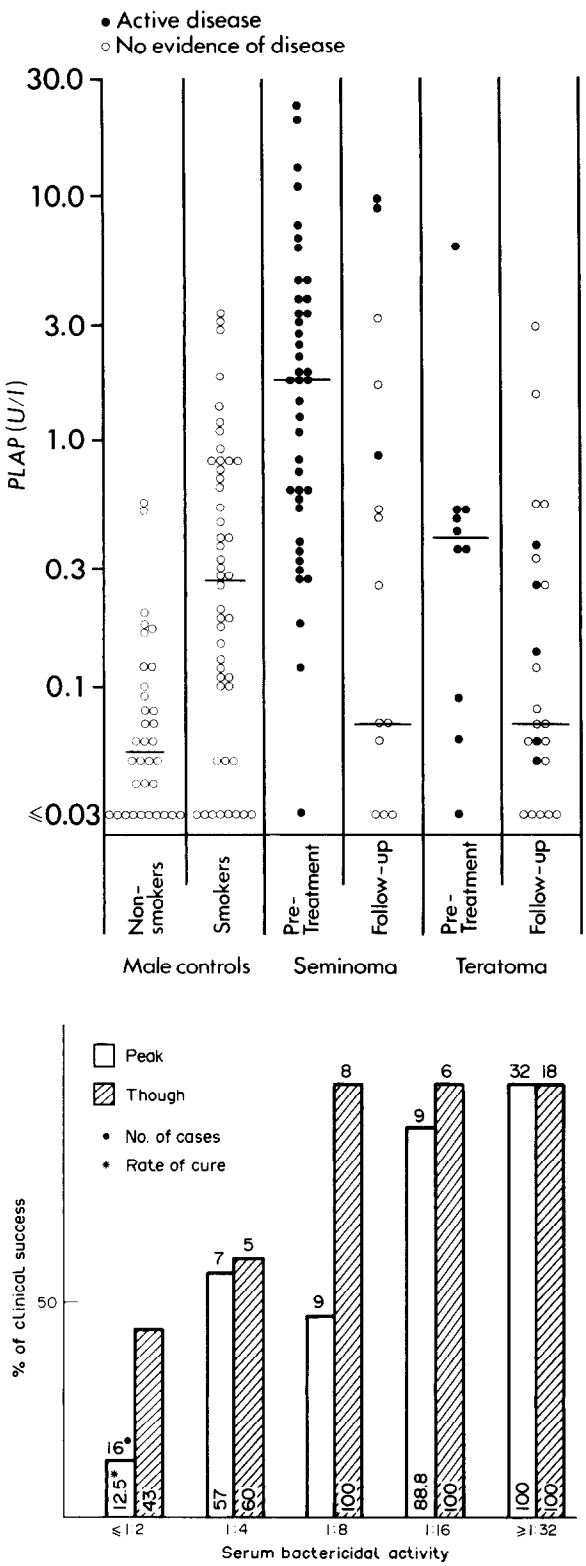

Fig. 1. Distribution of PLAP levels in controls, seminoma and teratoma of the testis.

There was no relationship between the levels of βHCG and PLAP in the seminoma patients at presentation. The Spearman rank correlation coefficient was 0.318, giving a probability that the βHCG and PLAP levels were related in less than 5%. All the seminomas had levels of AFP <10 g/l at presentation.

Longitudinal studies

Serial studies were performed in 17 patients with seminoma where specimens were available from the commencement of treatment of either primary or recurrent disease.

Three cases are illustrative:

Case 1 (*Fig.* 2). This patient had a right orchidectomy at the age of 36 yr for a classical seminoma seen to be invading the tunica, epididymis and vascular pedicle. Metastatic deposits were seen in the para-aortic nodes on CT scan. Post-operative radiotherapy to the para-aortic and bilateral pelvic nodes was given at another centre (30.00 Gy in 20 fractions over 27 days).

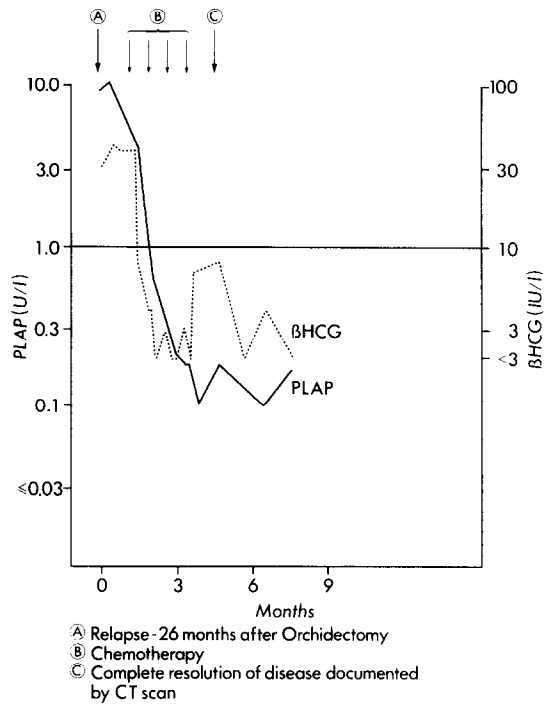

Fig. 2. Serial PLAP and βHCG levels in a patient with a relapsing seminoma (case 1).

Twenty-six months later the patient developed left loin pain and a palpable mass in the epigastrium. CT scan revealed a huge posterior mass (12.5 cm maximum diameter) continuous with enlarged retrocural and para-aortic nodes extending from the tracheal carina down to the second lumbar vertebra. Bilateral basal pleural effusions and a pericardial effusion were present. Four courses of chemotherapy with *cis*-platinum (25 mg/m², days 1-3) and etoposide (120 mg/m², days 1-3) repeated at 3-weekly intervals were given with complete resolution of disease documented by CT scan.

Serum βHCG and PLAP levels fell rapidly with chemotherapy and have remained within normal limits since. The patient remains well without evidence of disease 12 months after chemotherapy.

Case 2 (*Fig.* 3). A left orchidectomy was performed for classical seminoma of the testis when this man was 37 yr old. Postoperative megavoltage radiotherapy to the para-aortic and ipsilateral pelvic nodes was given at another centre (30.00 Gy in 15 fractions over 21 days). A left supraclavicular node mass appeared only 8 months later and was treated at the same centre with orthovoltage (250 kV) radiotherapy (22.50 Gy in 10 fractions over 14 days) with complete resolution of the mass.

Eighteen months from orchidectomy the patient developed shortness of breath and hemoptysis. Bronchoscopy was normal. Right thoracotomy revealed involvement of the right pleura, parenchymal involvement of the right lower lobe and nodes in the mediastinum and paratracheal regions. Partial tumour resection was performed and the patient referred for further therapy. CT scan confirmed residual disease in the thorax. A course of megavoltage radiotherapy was given to a 'chest bath' with careful screening of the previously irradiated zones (30.00 Gy in 20 fractions over 30 days). Because of a high risk of disseminated disease, three courses of chemotherapy as above were given starting 1 month after the completion of radiotherapy. Since then the patient has developed signs and symptoms of slight pulmonary fibrosis which improved with steroid therapy. He remains well without evidence of disease 30 months from the completion of chemotherapy. Retrospective analysis of PLAP and βHCG assays show that both markers rose markedly with the massive thoracic relapse and fell with therapy.

Case 3 (*Fig.* 4). This 60-yr-old man presented with pyrexia of unknown origin and was found to have a mass in the right side of the abdomen. At laparotomy a large oedematous retroperitoneal mass was found to be extending from the right kidney to the right inguinal ligament. A biopsy was suggestive of seminoma. Because of the patient's poor condition no further active intervention was undertaken for another month, when irregularity of the right testis was evident and an orchidectomy performed. Histology confirmed a classical seminoma.

At that time there was a large abdominal mass, the right kidney was not functioning and there were signs of inferior vena cava obstruction. Mediastinal involvement was evident on chest X-ray.

A course of megavoltage radiotherapy was given to the whole abdomen—15.00 Gy in 10 fractions over 14 days. This was followed by three courses of chemotherapy as above. This therapy caused great improvement in the patient's general condition. The leg oedema and palpable mass

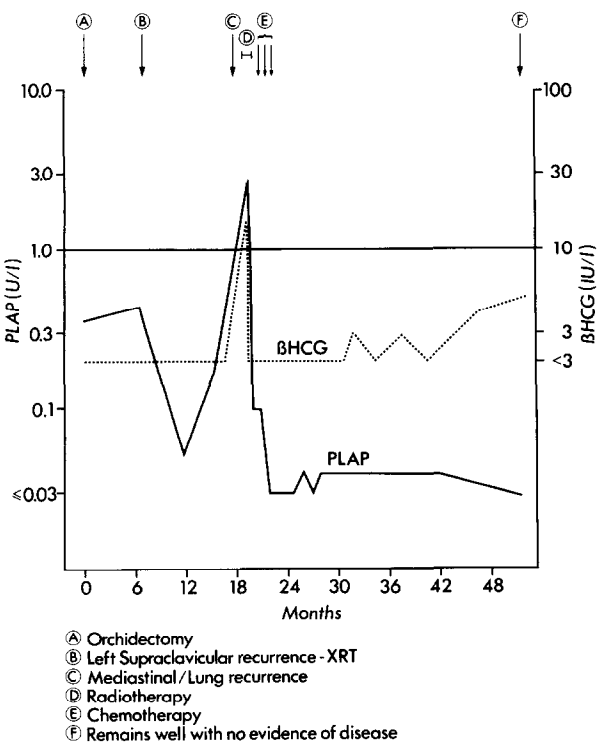

Fig. 3. *Serial PLAP and βHCG levels in a seminoma patient with pulmonary metastases (case* 2).

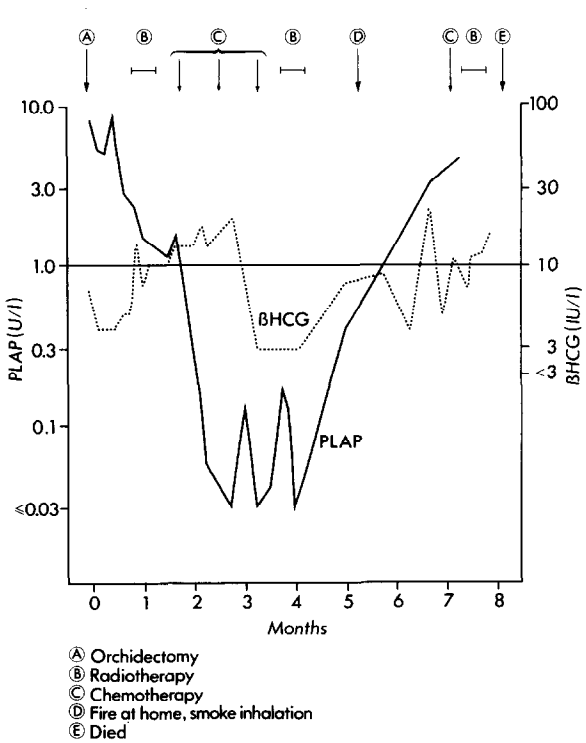

Fig. 4. *Serial PLAP nad βHCG levels in a patient with advanced seminoma (case* 3).

resolved, as did the mediastinal mass, on chest X-ray. A consolidation course of radiotherapy was given to the para-aortic and right iliac node areas (15.00 Gy in 10 fractions over 15 days). Following this the patient recovered. About 2 months later he suffered smoke inhalation from a house fire. Shortly after this his general condition worsened, with repeated chest infections and pleuritic pain. A palpable abdominal mass recurred with lower limb oedema, and despite further gentle chemotherapy with etoposide (100 mg/m^2, day 1 only) and palliative radiotherapy to the abdomen, he gradually deteriorated and died.

The serum PLAP levels reflect the initial good response to therapy and then recurrence, and appear to follow the course of the disease more closely than the βHCG levels, which were normal at the time of presentation and which fluctuated between 4 and 23 IU/l during relapse.

Of the remaining 14 patients studied longitudinally four had serum PLAP levels of <1 U/l (0.84, 0.54, 0.30 and 0.05 U/l) prior to treatment. In two of them the level fell on treatment, but in the two starting at 0.30 and 0.05 U/l there was no alteration of the PLAP or βHCG throughout the period of observation. Ten out of the 14 had elevated PLAP levels coincidental with untreated active disease, and all fell to reach normal levels within 1-2 months of treatment; there was a concordant fall of βHCG. Subsequently, whilst in complete remission, one patient maintained a level of about 1.0 U/l for 30 months, and in another it rose slowly to 3.0 U/l in 18 months. Both of these patients were smokers. One patient with very advanced disease had levels oscillating between 0.4 and 4.0 U/l for 2 yr prior to death.

DISCUSSION

This study has confirmed the observations by Maslow *et al.* [12] and Tonik *et al.* [8] that the serum PLAP levels are higher in smokers than non-smokers. The induction of higher levels resulted in an increase of the median level, suggesting that some effect was present in all smokers. This induction of PLAP has been detected by fluorometric assays of heat-stable PLAP [12], polyclonal RIA [8] and the current monoclonal AELIA test. This indicates that the induced enzyme shares many of the properties of PLAP in non-smokers. The IQ(Bio) PLAP assay gave a comparable performance in 13 samples from patients with ovarian cancer to an immunoimmobilization assay using antisera cross-reacting with PLAP and intestinal alkaline phosphatase (the latter removed by heat denaturation) described by Haije *et al.* [9]. These results suggest that the monoclonal antibody used in the IQ(Bio) PLAP assay reacts with a main epitope on PLAP that is common to PLAP of ovarian and testicular origin. It is of interest that bilateral orchidectomy does not produce a fall in PLAP levels, suggesting the normal blood levels are from sources other than the gonads.

The results support the opinion that serum PLAP can be a test in the monitoring of seminoma, as has recently been described by Jeppson *et al.* [7], Lange *et al.* [6] and Javadpour [5], and that the combination of HCG and PLAP is better than either alone. It was of interest that in patients with massive tumour burdens the levels were only modestly increased compared to that seen in stage I disease. The pre-treatment PLAP levels may also be of value in drawing attention to combined seminoma-teratoma tumours of the testicle. The influence of smoking provides difficulties in the interpretation of the level of serum PLAP, especially in presentation samples. The range of values in a healthy smoker can overlap those in patients with proven seminomas. The majority of seminoma patients (18/23, 78%) when in complete remission have PLAP levels <0.4 U/l, smoking probably accounting for residual higher activity. However, it is also clear that, using a sensitive assay, the levels in patients in complete remission were significantly lower than at presentation, and initial treatment is followed by a fall of previously raised PLAP levels. A progressive rise of PLAP during the period of surveillance is indicative of a recurrence, as shown in this study and those reported by Lange *et al.* [6] and Jeppson *et al.* [7]. However, it must be stressed that in a well-managed patient the incidence of recurrence is low, as about 70% of patients will have stage I disease at presentation. Nevertheless, not all patients are treated correctly from the outset and it would seem advisable that repeated PLAP measurements for 2 months after orchidectomy for a seminoma is a wise practice as a high value could be an indication of unexpected metastatic spread.

Acknowledgements—We are grateful to Dr W. Haije of the Rotterdam Radiotherapeutisch Instituut for supplying some of the serum samples used for evaluating this assay. We wish to thank IQ(Bio), Cambridge for donating the kits for evaluation and Drs C. Keightley and A. Johannson for their advice and interest.

REFERENCES

1. Javadpour, N. McIntire KR, Waldmann, TA. Immunochemical determination of human chorionic gonadotrophin (βHCG) and alphafetoprotein (AFP) in sera and tumours of patients with testicular cancer. *Natl Cancer Inst Monogr* 1978, **49**, 209-213.

2. Kohn, J, Orr AH, McElwain TJ, Bentall M, Peckham MJ. Serum alphafetoprotein in patients with testicular tumours. *Lancet* 1976, **ii,** 433-436.
3. Schultz H, Sell A, Nogaard-Pedersen B, Arends J. Serum alpha-fetoprotein and human chorionic gonadotrophin as markers for the effect of post-operative radiation therapy and/or chemotherapy in testicular cancer. *Cancer* 1978, **42,** 2182-2186.
4. Bosl GJ, Lange PH, Nochomovitz LE *et al.* Tumor markers in advanced nonseminomatous testicular cancer. *Cancer* 1981, **47,** 572-576.
5. Javadpour N. Multiple biochemical tumour markers in seminoma: a double blind study. *Cancer* 1983, **52,** 887-000.
6. Lange PH, Millan JL, Stigbrand T, Vessella RL, Ruosiahti E, Fishman WH. Placental alkaline phosphatase as a tumor marker for seminoma. *Cancer Res* 1982, **42,** 3244-3247.
7. Jeppson A, Wahren B, Stigbrand T, Edsmyr F, Anderson L. A clinical evaluation of serum placental alkaline phosphate in seminoma patients. *Br J Urol* 1983, **55,** 73-78.
8. Tonik SE, Ortmeyer AE, Shindelman JE, Sussman HH. Elevation of serum placental alkaline phosphatase levels in cigarette smokers. *Int J Cancer* 1983, **31,** 51-53.
9. Haije WG, Meerwaldt JH, Talerman A *et al.* The value of a sensitive assay of carcino-placental alkaline phosphatase (CPAP) in the follow-up of gynecological cancers. *Int J Cancer* 1979, **24,** 288-293.
10. De Groote G, De Waele P, Van de Voorde A, De Broe M, Fiers W. Use of monoclonal antibodies to detect human placental alkaline phosphatase. *Clin Chem* 1983, **29,** 115-119.
11. McGlaughlin PL, Gee H, Johnson PM. Placental-like alkaline phosphatase in pregnancy and malignancy: plasma specific estimation using a monoclonal antibody in a solid phase enzyme immunoassay. *Clin Chim Acta* 1983, **130,** 199-209.
12. Maslow WC, Muensch HA, Azama F, Schneider AS. Sensitive fluorometry of heat-stable alkaline phosphatase (Regan enzyme) activity in serum from smokers and nonsmokers. *Clin Chem* 1983, **29,** 260-263.
13. Self CH. Enzyme amplification—a general method applied to provide an immunoassisted assay for placental alkaline phosphatase. *J Immunol Methods* In press.
14. Self CH. European Patent Application 80303478.4 (15.04.81).
15. Ward MF, Bates G. Serum AFP and apparent half life estimates in the management of endo-dermal sinus tumours. *Prot Biol Fluids* 1979, **27,** 365-368.