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Serum placental-like alkaline phosphatase (PLAP): a novel combined enzyme linked immunoassay for monitoring ovarian cancer

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SUMMARY A new combined enzyme linked immunoassay (ELISA) was developed to measure both serum placental-like alkaline phosphatase (PLAP) activity (PLAP A) and concentration (PLAP C) in the same microtitre plate using an Imperial Cancer Research Fund monoclonal antibody, designated H17E2. PLAP A and PLAP C were determined together with an existing marker, CA125, in 397 serial samples from 87 patients with epithelial ovarian cancer. Retrospective assessment showed the sensitivity to increase from 73% with CA125 alone, to 88% using CA125 and PLAP A, and to 93% with all three markers in 261 samples from the patients with known active disease at the time of sampling. When the results for all 397 samples were included in the analysis, however, the specificity, sensitivity, accuracy and predictive powers of this monoclonal antibody were not sufficiently high to assist in the prospective follow up of patients with ovarian cancer. This was due to a significant number of false positive and false negative results.

Our data indicate that PLAP A or PLAP C estimation with H17E2 may, therefore, only be of value in the management of those patients with known active disease who are already known to be "marker positive" for this antigen.

Ovarian cancer produces few local symptoms and presents late in most cases; consequently the mortality is high. Attempts to improve the detection of early stage disease have foundered on the lack of a reliable clinical or radiological screening test. In the past five years screening work has focused on the identification of serological tumour markers which could facilitate earlier diagnosis and disease monitoring, a vital step towards improving the survival of these patients. Although numerous tumour markers have been identified to date,[1] none is specific enough to warrant its use as a primary diagnostic tool, but several have proved useful for monitoring the course of disease.[2] Since the publication of the initial report by Bast *et al*,[3] CA125 has become the accepted test with which other markers are compared.

There has been considerable interest shown recently in the application of placental-like alkaline phosphatase (PLAP) as a marker of epithelial ovarian cancer (EOC). Ectopic expression of PLAP was first discovered in a patient with squamous cell carcinoma of the lung[4] and has subsequently been found in various malignancies[5] including ovarian cancer. Raised serum concentrations of this oncofetal antigen have been found in 44%,[5] 35%,[6] and 40%[7] of patients with ovarian cancer.

PLAP is normally produced by the syncytiotrophoblast of the placenta and has been detected in sera as early as 9 weeks' gestation, increasing considerably during the second half of pregnancy.[8] It is normally undetectable in the sera of healthy subjects and it is this difference between normal adults and patients with cancer which affords it marker potential. Smoking, however, is an established cause of false positive results.[9]

Serum PLAP activity (PLAP A) and concentration (PLAP C) were determined in 387 healthy volunteers and 397 serial samples from 87 patients using a novel combined enzyme linked immunoassay (ELISA), developed by modification of two existing separate assays for PLAP A[10] and PLAP C.[11] Serum CA125 was also determined in all samples and evaluated with PLAP A and PLAP C in the patients with ovarian cancer. Both PLAP A and PLAP C were measured to investigate a recent report[11] that PLAP A decreased and PLAP C increased simultaneously with progression of disease.

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Subjects and methods

The Imperial Cancer Research Fund (ICRF) murine monoclonal antibody H17E2 used in this study was produced by immunisation with term placental membranes.[12] It reacts with a heat stable alkaline phosphatase that is more resistant to inhibition by L-Leu than Phe-Ala-Gly-Gly, confirming its recognition of term PLAP as opposed to other isoenzymes of the same family.[12]

Three hundred and ninety seven serial blood samples were collected from 87 patients with EOC over three years. The samples were separated by centrifugation at 1500 *g* for 10 min at 20°C and each serum sample stored in 0·5 ml portions at −20°C. Each sample was thawed once and assayed for PLAP A and PLAP C. A separate portion was assayed for CA125 using CIS ELSA-CA kits (CIS (UK) Ltd, High Wycombe, Buckinghamshire).

Upper limits of normal, defined as the 95th centile for PLAP A and PLAP C were established by assaying samples from 387 healthy blood donors after obtaining informed consent. Smoking habits were noted. All samples were tested in duplicate using a near term pregnancy serum pool as quality control material.

COMBINED PLAP A AND C ELISA

MicroELISA plates (M129B, Dynatech, Billingshurst, Kent) were coated overnight at 4°C with 100 μl/well 1·0 μg ml^{-1} H17E2 monoclonal antibody (supplied by courtesy of the ICRF, Lincoln's Inn Fields, London) in 50 mM carbonate buffer, pH 9·6. The plates were washed three times in 0·15 M phosphate buffered saline (PBS), pH 7·4, containing 0·05% v/v Tween 20 (PBS/Tween 20) to remove unbound antibody; 100 μl serum were then added and incubated for two hours at room temperature. After washing four times in PBS/Tween 20 100 μl phosphatase substrate: 5 mmol l^{-1} disodium p-nitrophenylphosphate (Sigma, Poole, Dorset) in 0·2 mmol l^{-1} diethanolamine buffer (BDH, Glasgow, Scotland) containing 0·5 mmol l^{-1} $MgCl_2$ (pH 9·8) were added and incubated for two hours at 37°C. Optical density was measured at 405 nm using a Titertek Multiskan MCC/340 spectrophotometer (Flow Laboratories, Irvine, Scotland) to determine PLAP A. The plates were then washed four times and 100 μl rabbit anti-human PLAP (Dakopatts, Denmark) at 1/250 dilution in PBS containing 0·5% w/v bovine serum albumin (BSA) were added and incubated for 30 minutes at room temperature. After washing three times in PBS/Tween 20 100 μl peroxidase-conjugated goat anti-rabbit IgG (Sigma, Poole, Dorset) at 1/1000 dilution in PBS/0·5% BSA were added and incubated for 30 minutes at room temperature. The plates were finally washed three times and incubated at room temperature for 45 minutes with 100 μl peroxidase substrate: 0·04% w/v o-phenylenediamine and 0·012% v/v H_2O_2 in 0·15 M citrate-phosphate buffer (pH 5·0) H_2SO_4 (50 μl 2·5 M) were added to stop the reaction and PLAP concentration determined by measuring optical density at 492 nm using a Titertek Multiskan MCC/340.

All patient samples were scored true or false by correlating the clinical state at the time of sampling with the antigen titre. Presence of disease was defined on clinical, radiological, or surgical grounds (laparotomy). Clinical disease activity was defined temporally as declared disease progression or reactivated disease within six months of assay. From this the sensitivity, specificity, accuracy and predictive values of PLAP were determined, alone and in combination with CA125.

DISCRETE PLAP C ASSAY

PLAP C was determined separately to show that preincubation with phosphate substrate for the activity assay did not have any deleterious effects on subsequent PLAP C assay performance in the combined assay.

The plates were coated in the same manner as in the combined assay. After the serum incubation step the plates were washed three times and rabbit anti-human PLAP added. The remainder of the assay was identical with that of the combined concentration assay.

Results

ASSAY PERFORMANCE

The correlation between the combined and discrete PLAP C assay was high ($n = 34$, $r = 0{\cdot}97$, $y = 1{\cdot}4\,x$, $p < 0{\cdot}001$) (fig 1), supporting the use of a combined assay.

Based on results from the near term pregnancy serum pool, the between ($n = 160$) and within ($n = 46$) assay coefficients of variation were: 16% and 7·5% for PLAP A assay, 8·2% and 3·8% for combined PLAP C assay, and 13% and 4·4% for discrete PLAP C assay, respectively.

CORRELATION BETWEEN PLAP A AND C IN BLOOD DONORS

Using the results from 397 blood donors, PLAP A and C were poorly associated ($n = 387$, $r = 0{\cdot}56$, $y = 0{\cdot}16\,x$, $p < 0{\cdot}001$), although still significantly correlated.

PLAP A did not seem to be influenced by smoking in either male or female blood donors ($p > 0{\cdot}05$). PLAP C, however, did seem to be increased by smoking in both male and female donors ($p < 0{\cdot}001$).

REFERENCE RANGE FOR PLAP A AND C

PLAP A and PLAP C were not normally distributed in

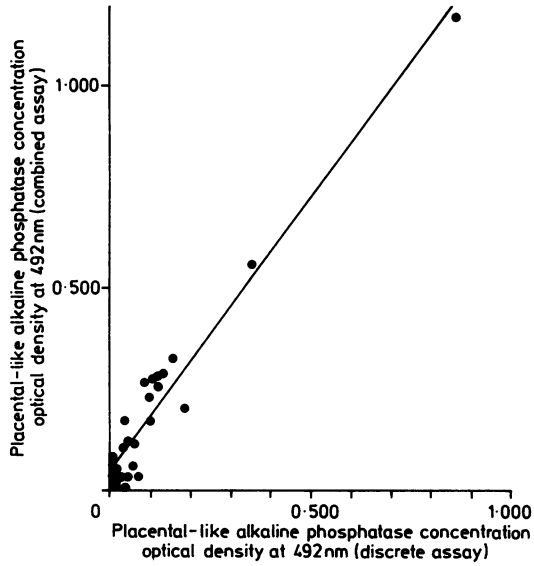

Fig 1 *Correlation between PLAP concentration measured alone and after activity in combined technique.*

the control population, neither did they normalise following logarithmic transformation as tested by Kolmogorov-Smirnov Goodness of Fit Test ($p < 0.05$). Therefore, the 95th centiles of the control population, 0·400 and 0·085 for PLAP A and PLAP C, respectively, were used as cut off values. In smokers, however, the 95th centile of the PLAP C reference interval was 0·185; for PLAP A it was unchanged from the non-smokers' value. Therefore, PLAP C abnormality was defined as a value greater than halfway between the 95th centile for non-smokers and smokers because the smoking habits were known in only 10% of the patient population. Assigned cut off values were therefore 0·400 and 0·135 optical density units for PLAP A and PLAP C, respectively.

CORRELATION BETWEEN PLAP A AND C IN PATIENTS WITH CANCER

The correlation between PLAP A and C in patients with ovarian cancer ($n = 397$, $r = 0.18$, $p < 0.001$) was poorer than that for the blood donors, although still significant.

PLAP A, PLAP C AND CA125 CONCENTRATIONS

The proportion of samples with raised values of PLAP A, PLAP C, and CA125 are shown in table 1. Patients were divided according to the Féderation International de Gynecologie et Obstetrique (FIGO) stage and histopathology. Abnormal values were seen in all stages, but were more numerous in advanced (FIGO stages III and IV) disease.

The sensitivity, specificity, and accuracy of PLAP A, PLAP C, and CA125 for each stage and histopathological type are shown in table 2. Using these criteria PLAP A was more sensitive but less specific than PLAP C for stages III and IV disease, although both performed less well than CA125.

The sensitivity, specificity, accuracy and predictive values of a positive result (PVP) and of a negative result (PVN) for each marker are shown in table 3: all three markers were tested in all 397 samples. This would be the case in a prospective analysis of all sample results.

Table 4 shows the above indices when the combination of all three markers are considered together; A

Table 1 *Proportion of samples with raised marker values*

	No of subjects	*No of samples*	*No of sample results evaluated (TP and FP)*		
			PLAP A (OD > 0·4)	*PLAP C (OD > 0·15)*	*CA125 (35 U/ml)*
Blood donors	387	387	18 (5%)	5 (1%)	
Patients:					
Stage I:					
Serous	5	20	11	9	1
Mucinous	1	5	2	0	0
PDA	1	3	1	2	0
Stage II:					
Serous	4	19	6	2	4
Mucinous	1	3	2	0	0
Stage III					
Serous	40	204	122	81	108
Mucinous	3	12	9	6	3
PDA	12	47	21	5	22
Stage IV:					
Serous	15	64	44	24	44
PDA	5	20	15	4	14
Total	87	397	233 (59%)	133 (33·5%)	196 (49%)

PDA—Poorly differentiated adenocarcinoma.

Table 2 *Marker performance as assessed by histopathology and FIGO staging*

Stage (FIGO)	*Tumour marker*	*Sensitivity (%)*	*Specificity (%)*	*Accuracy (%)*
I	PLAP A	20	43	39
	PLAP C	60	65	64
	CA125	20	100	86
II	PLAP A	0	60	55
	PLAP C	0	90	91
	CA125	100	90	91
III	PLAP A	61	51	58
	PLAP C	37	69	46
	CA125	70	96	78
IV	PLAP A	78	67	76
	PLAP C	38	93	48
	CA125	83	93	85
I + II	PLAP A	14	51	46
	PLAP C	43	77	72
	CA125	43	95	88
III + IV	PLAP A	66	53	63
	PLAP C	37	73	46
	CA125	74	96	80
Histological type (stages I–IV):				
Serous	PLAP A	65	51	60
	PLAP C	42	71	51
	CA125	74	95	81
Mucinous	PLAP A	71	38	50
	PLAP C	14	62	45
	CA125	43	100	80
Poorly differentiated adenocarcinoma:				
	PLAP A	63	65	64
	PLAP C	21	95	44
	CA125	73	95	80

Table 3 *Individual CA125, PLAP A, and PLAP C results*

	CA125	*PLAP A*	*PLAP C*
Sensitivity	190/262 (73%)	169/262 (65%)	98/262 (37%)
Specificity	130/135 (96%)	71/135 (53%)	99/135 (73%)
Accuracy	320/397 (81%)	240/397 (60%)	197/397 (50%)
PVP	190/196 (97%)	169/233 (73%)	98/133 (74%)
PVN	130/201 (65%)	71/164 (43%)	99/264 (38%)

shows positive marker state where one or more than one marker in the panel was increased, and B shows the same indices when PLAP was tested only in the samples which were CA125 negative (so called "series testing").

SERIAL ANTIGEN TITRES IN TWO PATIENTS

Two patients were chosen after analysis of their serial antigen titres, (fig 2) to illustrate retrospectively if PLAP A and PLAP C had been useful for predicting relapse. In case 1 CA125 had been negative until well after clinically evident relapse, and in case 2 an increase in CA125, although preceding relapse, was a late event. An increase in PLAP A in case 1 and an increase in PLAP C in case 2 would have been earlier predictors of relapse. Both patients were followed up after a positive second look laparotomy. Case 1 was receiving chlorambucil when each sample was taken and case 2 received three cycles of Cis-platinum throughout the blood sampling period.

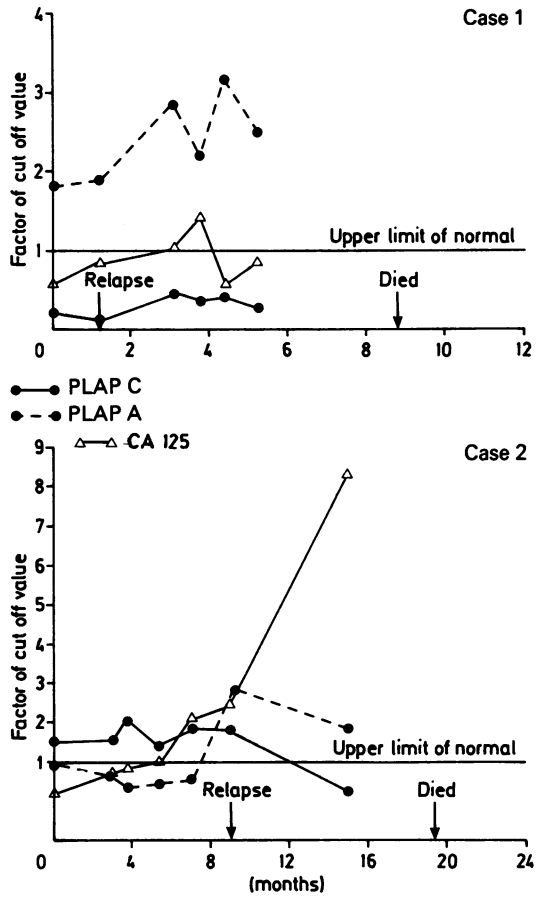

Fig 2 *Case 1: stage III adenocarcinoma. Case 2: stage III serous papillary adenocarcinoma.*

Table 4 *CA125, PLAP A, and PLAP C panel results*

	Sensitivity	*Specificity*	*Accuracy*	*PVP*	*PVN*
A: CA125, PLAP A and PLAP C where ⩾ 1 marker is positive	43/261 (93%)	57/136 (42%)	300/397 (76%)	243/322 (75%)	57/75 (76%)
B: PLAP testing in series on a negative CA125 result	53/71 (75%)	57/130 (44%)	110/201 (55%)	53/126 (42%)	57/75 (76%)

Discussion

Determination of both PLAP A and C in a combined assay has several advantages over separate assays. These include reduced expense (in particular halved monoclonal antibody costs) and reduced operator time and error due to sampling variation. The correlation between the combined and discrete PLAP C assay was high, supporting the use of the combined assay. The gradient of the slope was greater than 1·0, however, for which the reasons are unclear. The greater absorbance in the combined assay was not due to residual p-nitrophenyl phosphate substrate, which showed zero absorbance at wavelengths greater than 470 nm. In the combined assay the catalytic reaction may have induced a conformational change in PLAP which results in enhanced recognition and binding by the rabbit anti-human PLAP.

The data show that PLAP A and PLAP C assays, individually and in combination, are insufficiently sensitive and specific (tables 2 and 3) for the management of women with epithelial ovarian cancer. Several reasons for the failure of PLAP to fulfil expectations may be postulated. Changes in antigen expression during disease progression and increasing tumour dedifferentiation are complex, substantiated by the lack of close correlation we have found between PLAP A and PLAP C in cancer patients when compared with normal controls. These findings contrast with reports from another group who used a different monoclonal antibody raised against PLAP.[14]

Numerous factors influence the expression of PLAP, including smoking, which induces PLAP-like alkaline phosphatase synthesis and secretion by lung alveoli.[15] H17E2 recognises this isoenzyme,[16] which may in part account for a high proportion of false positive results in our series of patients. Whether a "smoking effect" was a source of error in this series is uncertain. The control sera showed a significant increase in the reference interval only in the PLAP C assay; in the patients' sera many more false positive results were seen with PLAP A than with the PLAP C assay. For PLAP C the use of a correction factor based on observations in the large control group might reasonably be expected to have reduced the false positive results associated with smoking. Unfortunately, information on the smoking habits in most patients was unobtainable, hence the effect of smoking on the patients' PLAP values could not be assessed properly.

PLAP has not previously been evaluated in terms of sensitivity, specificity, accuracy and predictive power,[16] although numerous reports advocate its use as a tumour marker in ovarian cancer.[5-8 11 14] When results for PLAP A and PLAP C were combined with CA125 results the overall sensitivity increased from 73% with CA125 alone to 93% with all three markers, where at least one gave a positive result (table 4a). Combining results in this way, however, resulted in a considerable loss of specificity, from 96% for CA125 alone to 42% for the combined results. This loss of specificity is better seen in terms of the relative predictive powers of the test. The PVP of CA125 alone was 97% with a PVN of 65% (table 3), whereas the combined results showed a fall of PVP to 75% and only a relatively small rise in PVN to 76% (tables 4a and b).

PLAP may be assayed in series[17] with CA125. This reduces the total number of PLAP assays required as only CA125-negative samples would require retesting, allowing the PVP of CA125 alone to be retained. Serial analysis of the patients' data (table 4b) showed that the use of PLAP A and PLAP C on samples negative for CA125 (n = 201) gave a PVP of both assays together of only 42%, with PVN remaining unchanged at 76%. It is clear, therefore, that assay of PLAP A and PLAP C did not add significantly to the predictive value of CA125 in these negative samples in which the active disease prevalence, as assessed clinically, was 71/201 (35%).

These data indicate that PLAP, as measured with this monoclonal antibody, confused the interpretation of CA125 results in this cohort of patients. CA125 assay used on its own would seem to be more helpful in clinical decision making. The results of this study agree with those of a recent report by Haije *et al*,[18] who also assayed PLAP activity and concentration by immunoreactivity, but found neither to be useful for general patient follow up and management.

Tucker *et al* have found determination of PLAP activity using H17E2 useful in the follow up of testicular germ cell tumours, particularly seminomas.[10] The applicability of the simple combined assay deserves to be tested in this and possibly other cancers.

The combined assay, using a more specific monoclonal antibody, one which does not react with PLAP induced by smoking,[6] may prove useful in the follow up of patients with ovarian cancer. Increased specificity would then be obtained, possibly at the expense of sensitivity—an acceptable modification in the context of a panel of markers where specificity of each marker is the most important criterion.

Although measurement of PLAP was not found to be generally helpful, a few patients may have benefited from additional PLAP assay (fig 2). It is impossible at this stage to judge which patients will benefit most from prospective serial measurement of PLAP. Appropriate patient selection is essential if PLAP is to be of use in future as an adjunct in monitoring ovarian cancer.

Unfortunately, insufficient preoperative samples were available in this study to assess the value of PLAP

assays in the untreated patient. Preoperative measurement of PLAP may provide a helpful indicator of patients who will benefit from further serial measurement in a manner analogous to CA125 where assay at the time the disease presents helps in the selection of "secretors".[19]

Despite initial promising investigations of PLAP as a tumour marker in ovarian cancer this study and others[18 20] have failed to corroborate them. Work is currently under way using various monoclonal antibodies to investigate further the PLAP molecules and epitopes in conjunction with assay development for other promising markers such as mucin antigens.[13]

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