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Screening of Sera and Tumor Extracts of Cancer Patients Using a Monoclonal Antibody Directed Against Human Placental Alkaline Phosphatase*

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Abstract—*A sensitive endogenous enzyme immunoassay involving an anti-human placental alkaline phosphatase (PLAP) monoclonal antibody was used in the screening of sera and tumor extracts of patients with various types of cancer. In sera of breast cancer patients an incidence of 5.2% was recorded. This value rose to 43% when tumor extracts were analyzed. For lung and bronchial cancers we found 11.2% seropositive patients. On several occasions a good correlation was observed between the PLAP determinations and the histopathological staging of tumor tissue.*

INTRODUCTION

HUMAN placental alkaline phosphatase (E.C. 3.1.3.1) is a member of a family of isozymes named for the tissue in which they predominate, such as placenta, intestine, bone, liver and kidney. They are distinguished from one another by electrophoretic and immunological methods, enzyme inactivation kinetics and peptide mapping (for reviews see [1, 2]). The bone, liver and kidney forms may be derived from a single gene locus, but the placental and the intestinal form seem to be encoded by different loci although both share a limited number of identical tryptic peptides [3]. Human placental alkaline phosphatase (PLAP) is normally produced in the microvilli of the syncytiotrophoblast. Different variant enzymes have also been described in various cancer tissues as oncodevelopmental proteins and can be detected in the sera of some cancer patients [4-12]. Recently, the enzyme has also been detected in very low amounts in certain types of normal tissue as shown by enzyme inactivation kinetics, and by electrophoretic and immunological methods using intensively adsorbed polyclonal antisera [13-15]. Previous studies on the level and frequency of expression of PLAP in tumor sera were hampered by difficulties in the differentiation of PLAP from the other isozymes, especially the intestinal one. We therefore constructed a hybridoma cell line that produces a PLAP-specific monoclonal antibody of the IgG2b, κ type [16]. This antibody shows an absolute specificity for PLAP and reacts with the three most abundant PLAP phenotypes [PLAP-PP(2), PLAP-PP(3) and PLAP-PP(1); previously termed F, I, S]. As such, it enabled us to establish an easy, sensitive immunoassay [17] to detect PLAP in sera and tumor tissue extracts from a variety of cancer patients.

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MATERIALS AND METHODS

Sera and tumor biopsies

Sera and tumor biopsies were collected over an 18-month period from mostly untreated patients entering the Academic Hospital of Antwerp or Ghent. All tumor biopsies were divided into two parts. One part was sent to the pathology department of the hospital while the other part was used for extraction of PLAP (see below). All samples were assayed in duplicate and scored double-blind.

Tumor tissue extracts

Tumor tissue was weighed and homogenized in 9 ml 50 mM Tris-Cl, pH 6.8, per g of tissue and

2/5 vol of *n*-butanol. The homogenate was rotated for 60 min at 37°C and centrifuged for 10 min at 9000 rpm in a Sorvall centrifuge in an SS 34 rotor. The aqueous phase was dialyzed overnight against 50 mM Tris-Cl, pH 6.8, at 0°C. Dialyzed extracts were kept frozen until used.

PLAP-specific endogenous enzyme assay

The mouse anti-PLAP monoclonal antibody E6, the endogenous enzyme assay and the different parameters involved have been detailed previously [17]. The assay is based on the fact that the monoclonal antibody E6 binds specifically but does not inactivate PLAP. In the final version the assay was performed as a direct immunoassay using the monoclonal antibody bound to polystyrene beads as solid phase to trap the PLAP activity from sera or tumor extracts whereupon the enzymatic activity of the bound enzyme was measured. Briefly, the method involved activation of the beads with glutaraldehyde, binding of a fixed amount of purified monoclonal antibody and saturation of remaining sites with fetal calf serum.

For the enzyme assay, each bead was incubated overnight at room temperature with 200 µl serum or tumor extract. After incubation, the beads were washed four times with 1 ml of HBS (5 g/l HEPES, 8 g/l $Na_2HPO_4 \cdot 12H_2O$, 1 g/l dextrose, pH 7.5, supplemented with 2% fetal calf serum and 0.05% Tween 80) and rinsed once with 10% diethanolamine in water. Beads were then incubated with 200 µl of a solution of 10% diethanolamine and 5 mM *p*-nitrophenylphosphate for 2 hr at 37 °C. The reaction was stopped by the addition of 800 µl 1M NaOH and optical density was measured at 412 nm. Results are expressed as U/l for serum or mU/g tissue for tumor extracts, with 1 unit defining the liberation of 1 µmol *p*-nitrophenol/min at 37°C under the above conditions using $E = 14{,}600$ for *p*-nitrophenol at 412 nm. In each assay a number of controlled normals were included together with serial dilutions of a known standard containing purified PLAP.

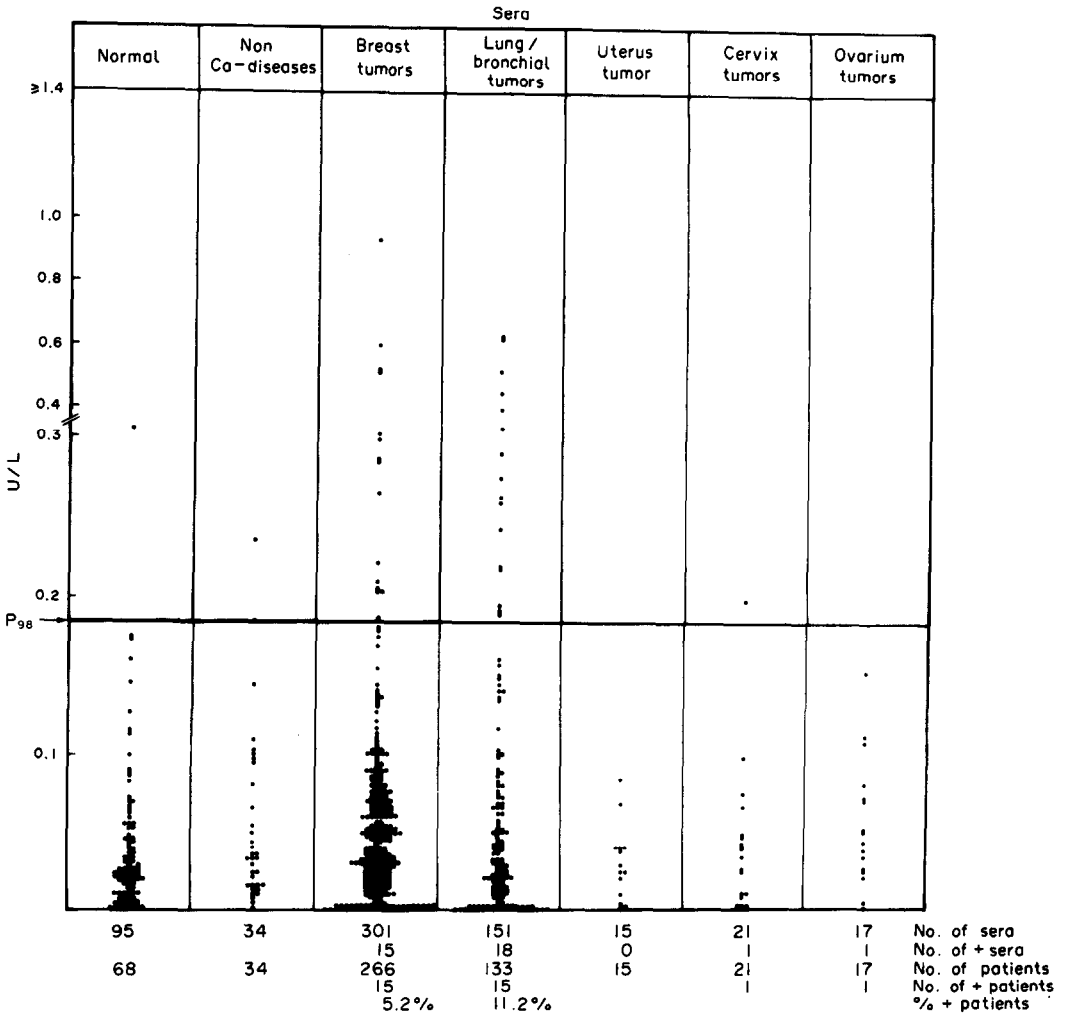

Fig. 1. Distribution of PLAP in sera of controls and patients suffering from different cancers. Note the switch in the ordinate scale above 0.3 U/l. Arrow denotes the 98th percentile. The number and percentage of positive patients was determined using the P_{98} *value as threshold.*

RESULTS

PLAP determination on serum samples

In order to establish the background values for the PLAP content of normal sera we analyzed 95 serum samples from 68 healthy, non-pregnant individuals. The results (Fig. 1, panel 1) indicated a range of 0–0.334 U/l, with a mean of 0.04 U/l. We also assayed 34 sera from 34 patients suffering from non-malignant diseases (hepatitis A and B, sinusitis, kidney failure, metabolic diseases, arthritis, urinary tract infection, gastroenteritis, nephrotic syndrome and persons with reproductive failure). All these sera scored between 0 and 0.236 U/l, with a mean of 0.05 U/l (Fig. 1, panel 2). Combining the data for normal individuals and patients with non-cancerous diseases, we found a 98th percentile value of 0.175 U/l. Using this threshold value, we found three control sera that had higher values (0.334, 0.236 and 0.180 U/l). Since these sera are derived from supposedly cancer-free patients or from healthy individuals, the raised values may possibly be due to the smoking habits of these individuals (see Discussion).

The results of the determination of serum PLAP values in sera of patients with various forms of cancer are summarized in panels 3–7 of Fig. 1. So far we have analyzed 301 serum samples derived from 286 patients with a proven record of breast cancer (including one male patient) and we detected 15 positive patients. The highest PLAP value reached was 0.92 U/l. The results of determinations on consecutive samples of the same patients were internally consistent.

For lung and bronchial cancers (Fig. 1, panel 4) we obtained 151 serum samples from 133 patients (8.7% women) suffering from adenomas, oat cell carcinomas and epidermoid epitheliomas, with a median age of 64 yr (range 52–80 yr). Of these, 15 patients (11.2%) and 18 sera scored positive. The highest value recorded for this group was 1.89 U/l. Like the sera of breast cancer patients, all doubles gave consistent results.

We analyzed 15 serum samples of 15 patients with various uterine tumors and 21 samples (21 patients) with cervical tumors (Fig. 1, panels 5 and 6). Only one patient was found to be clearly positive.

For ovarian cancers we obtained 17 samples from 17 patients (Fig. 1, panel 7) and we could detect only one positive sample, which is considerably lower than previously reported [11, 18].

PLAP determinations on tumor tissue extracts

As control samples for the interpretation of our results, 25 fragments of healthy tissue from 24 individuals were acquired either as post-mortem material or by surgery, deemed necessary on clinical and/or histological grounds. These tissues included 19 fragments of uterus, three of breast, one of ovary, one axillary lymph node and one fallopian tube fragment. All fragments were extracted as described for tumor tissue and run in identical assays. Their values ranged from 0 to 0.722 mU/g, with a mean of 0.23 mU/g and a 98th percentile of 0.60 mU/g (Fig. 2, panel 1). The latter value was retained as threshold and all results scoring higher were counted as positive.

In contrast to the rather low number of positive sera, we found a much higher incidence of positive values in tissue extracts. We obtained 150 samples of breast cancer tissue from 143 patients (median age 58 yr, range 30–93 yr). Of these, 67 samples from 62 patients (43%) scored positive (Fig. 2, panel 2) and four samples had values above 15 mU/g, with the highest value scoring 63.1 mU/g.

For several patients more than one tissue fragment was provided. In general, we found a good correlation between the status of the tissue fragment (normal or tumor tissue) and the presence or absence of PLAP (Table 1), although for patient 2397 a presumed normal tissue fragment scored higher than our background value.

PLAP levels of tissue fragments from 60 patients with positive samples of breast cancer tissue were compared with the levels of estrogen and progesterone receptor, expressed in fmol/mg tissue [19]. However, close inspection could not reveal any correlation between the two sets of data.

For lung and bronchial tumors we received 11 tissue samples from ten patients and found nine patients with PLAP values between 1.7 and 32.6 mU/g tissue, while only one patient scored negative (Fig. 2, panel 3). We received normal tissue and tumor tissue, without histopathological confirmation, from three patients. Tumor tissue resulted in values of 8.33, 4.28 and 32.6 mU/g, while the corresponding 'normal' tissues yielded 1.41, 2.36 and 4.57 mU/g (Table 1). The latter values are considerably higher than our threshold values established on the basis of 25 normal extracts which did not include normal lung, but it should be noted that the readings for three normal tissue extracts remained consistently lower than the corresponding tumor tissue. Also, it should be taken into account that we were unable to confirm the absence of malignancy indications in the 'normal' tissues by histopathology and if the latter were removed in the vicinity of the tumor, the raised values could easily be explained by microscopic invasion of tumor cells in the surrounding tissue.

Table 1. Correlation between histopathological and hPLAP determinations on tissue fragments

Patient	Cancer	Histopathology of Tissue Fragment	hPLAP(mU/g) in Tissue Extract
2236	breast	duct cell carcinoma	1.09
		lymph node w/ invaded tissue	1.12
2251	breast	duct cell carcinoma	3.25
		lymph node, invasive tissue	3.25
		lymph node, normal tissue	0.002
2286	breast	carcinoma	1.31
		axillary lymph node, normal	0
2396	breast	duct cell carcinoma	14.1
		normal tissue	0.59
2539	breast	duct cell carcinoma	0.15
		lymph node, normal	0.017
2229	breast	biopsy, suspected	63.22
		Halstead frag.: duct. adenocarcinoma	9.44
2254	breast	biopsy apex: adenocarcinoma	2.34
		Halstead fragment: adenocarcinoma	1.96
2397	breast*	tumor tissue	5.14
		normal tissue	1.41
2407	lung*	tumor tissue	32.58
		normal tissue	4.57
2413	lung*	tumor tissue	4.28
		normal tissue	2.36
2415	lung*	tumor tissue	8.33
		normal tissue	1.41
2290	uterus	cornu myometrium, adenocarcinoma	11.89
		fundus myometrium, invasive tissue	0.87
		normal myometrium, cervical side	0.15
2522	uterus	myometrium invasion, cervix side	1.50
		endomet., adenocarc., right tubal side	0.64
		myometrium invasion, right tubal side	0.55
		endometrium adenocarc., left tubal side	0.07
2269	ovary	adenocarcinoma, left	0.73
		adenocarcinoma, right	0.54
2279	ovary	adenocarc., ampulla & left fallop. tube	0.86
		normal isthmus, left fallopian tube	0.24
		normal myometrium (surgical section 1)	0
		normal myometrium (surgical section 2)	0

* indicates that no histopathological data on the tissue fragment was available

In the case of uterine tumors, we determined PLAP values on 14 extracts from eight patients. Here six extracts (four patients) scored positive, the highest being 11.9 mU/g (Fig. 2, panel 4). In Table 1 we have summarized the results of the two patients where different fragments of the same patient were available.

So far we have obtained only eight samples of ovarian tumors (Fig. 2, panel 5 and Table 1) from seven patients and seven patients had PLAP values above our threshold, five of which had levels above 100 mU/g.

DISCUSSION

In the PLAP assay developed by us [17] an optical density of 0.01 (blank subtracted) corresponds to 0.02 U/l or 0.25 mU/g. If we take this value as our lowest accurate detection limit and assume a specific activity for PLAP of 380 U/mg, the sensitivity amounts to 52 ng/l for serum and 0.6 ng/g tissue. These values compare favorably with methods used in previous studies based on enzyme inhibition kinetics [4–7] or polyclonal anti-PLAP antibodies in RIA- or ELISA-based assays [8, 10, 11]. They are as

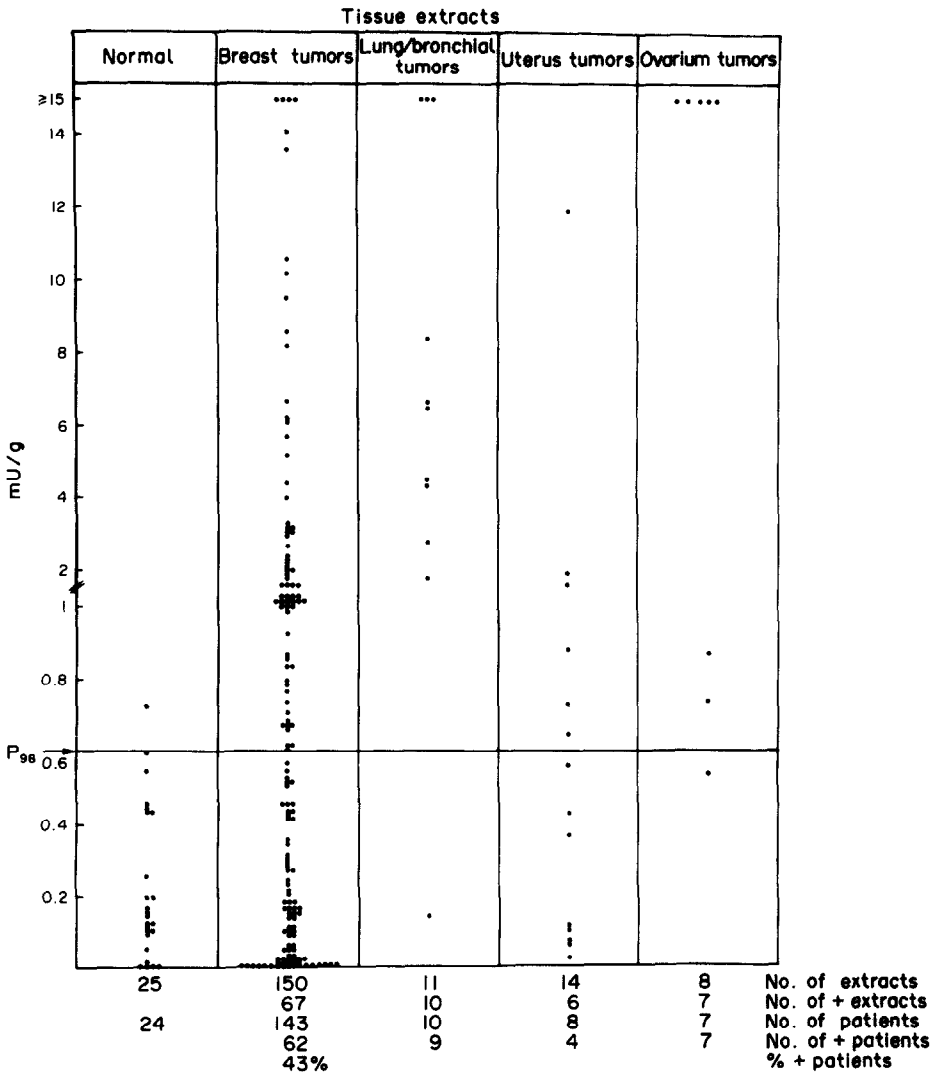

Fig. 2. Distribution of PLAP in extracts of normal and tumorous tissue. Note the switch in the ordinate scale above 1.0 mU/g. *Arrow denotes the 98th percentile. The number and percentage of positive patients was determined using the* P_{98} *value as threshold.*

sensitive as an assay using rabbit anti-mouse Ig-coated microplates as solid phase-bound antibody followed by an anti-PLAP monoclonal antibody for fixation of the amount of PLAP to be determined [20].

For breast cancer a study [10] on 11 metastatic breast tumors and 16 primary tumors indicated the presence of PLAP in 20% of the sera for metastatic patients, while only 3% of the primary cancer patients had positive values. We screened 286 patients, mostly in the early stages of the disease but without discriminating for the disease status, and found 5.2% positive sera, in contrast to a study [20] where no positive plasma samples were detected but where only 32 samples were analyzed. The low amount of positive sera for breast cancer is, however, not indicative for the amount of breast cancers that do express PLAP since out of 143 patients, 43% proved to have positive tissue extracts. This value may represent a better assessment of the true incidence of PLAP in breast cancer tissues than previous studies on smaller numbers of patients where positivity was found in 7/7 patients [21] and another study [22] where 2/5 patients had positive immuno-peroxidase staining of primary breast cancer sections treated with polyclonal anti-PLAP.

Although the presence of PLAP was first detected in a patient with broncogenic carcinoma [4], since then referred to as 'Regan enzyme', a systematic study of occurrence of PLAP in sera of lung and bronchial cancer patients has, so far, not been reported. Here we document a rather high incidence of 11.2% PLAP seropositive patients in various stages of the disease. Recently, PLAP assays conducted on the sera of smokers and non-smokers, using a heat inactivation of 7 min at 65°C for the removal of contaminating isozymes, indicated that sera of smokers had a mean of 0.44 U/l PLAP [23]. Although we did not discriminate between smokers and non-smokers in our control group, we may tentatively conclude

that our P_{98} value is representative. Nevertheless, since smokers have a higher incidence of lung cancer, the possibility remains that for this particular group of cancers the values should rather be matched with a control group consisting of light and heavy smokers free from any evidence of cancer. In this respect the rather high value of 0.33 U/l for one healthy individual and 0.23 U/l for a non-cancer patient possibly could be attributed to smoking habits. This factor will be evaluated in future studies.

Although likely, it still remains difficult to assess whether smoking habits of patients could also influence the interpretation of our data on lung tumor tissue. We analyzed 11 extracts, all of elderly male patients whose smoking habits were unrecorded. We found nine patients with values between 1.76 and 32.6 mU/g, with a mean of 11.35 mU/g. One patient registered 0.14 mU/g and was thus considered negative. Even if we take into account that the composition of our control of normal tissues is not a true reflection of the tissues that were analyzed (breast tissue was under-represented and lung tissue was absent), we still can state that the threshold value deduced for normal tissues remains valid because, as shown in Fig. 2, all histopathologically confirmed normal tissues had PLAP values falling below our P_{98} cut-off value. Most striking aberrations, however, were found for lung tumor tissue extracts, where all presumed normal tissues scored higher than our P_{98} value (4.57, 2.36 and 1.41 mU/g) but were still well below the values reached by lung tumor extracts. Given previous results on the expression of low amounts of PLAP in normal lung tissue [15] and the apparent PLAP-inducing characteristics of smoking habits [23], more experimental data are needed to evaluate the presence of PLAP in normal and cancerous lung tissue.

In a careful study on the occurrence of PLAP in the sera of patients with cancer of the cervix, endometrium or the ovaries positive values of 22, 41 and 40% respectively were reported for patients with clear evidence of disease, dropping to 12, 6 and 2% respectively for patients with less clear symptoms [11]. Although we have so far only analyzed a limited number of patients without differentiating for the status of the disease, we found no positives for uterus (out of 15 samples), one positive for cervix (out of 21 samples) and only one positive out of 17 samples (5.9%) for ovarian cancer. As in the case of breast cancer, the incidence of positives could rise sharply if the assay is carried out on tumor tissue, as is suggested by the preliminary reults of our limited sampling (4/8 patients positive for uterine tumors and 7/7 positive for ovarian cancer). Here, again, we found that for positive patients a good correlation exists between the PLAP determination and the histopathological record.

The contrast between the degree of seropositivity and the degree of tissue positivity limits the use of PLAP as a marker for general screening of potential cancer patients. The discrepancy between the values of the tissue extracts and the serum determinations is clearly evident in the case of breast tumors, where the PLAP was determined both in the serum and in the tumor tissue of 33 patients with positive tissue extracts. Of these, only two patients had positive values both for serum and tumor tissue extracts. No patients were found with positive serum values and negative tumor tissue values. Nevertheless, for a limited number of patients the usefulness of the PLAP serum test for following the course of the disease may be promising and merits further investigation.

In the course of this study we have so far detected only three false positive values in serum analysis and scored two false positive results for tumor tissue extracts (one being a case of a woman with benign ovarian tumor). Therefore, despite the rather low overall detection frequency of PLAP in serum (7%), PLAP determinations should be considered for those patients already suspected of cancer. Moreover, considering the discrepancy between serum positivity and tissue positivity, an immunohistochemical assay based on the use of monoclonal antibodies might be useful as a complementary tool in pathological determination. Furthermore, given the low concentration of circulating antigen and the fact that PLAP is located on the surface of the cell membrane, the anti-PLAP monoclonal antibody should be useful for radioimmunolocalization and as a carrier molecule for drug-targeting.

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REFERENCES

1. Fishman WH. Perspectives on alkaline phosphatase isoenzymes. *Am J Med* 1974, **56,** 617-650.
2. Moss DE. Alkaline phosphatase isoenzymes. *Clin Chem* 1982, **28,** 2007-2016.
3. Seargent LE, Stinson RA. Evidence that three structural genes code for human alkaline phosphatases. *Nature* 1979, **281,** 152-154.
4. Fishman WH, Inglis NI, Stolbach LL, Krant MJ. A serum alkaline phosphatase isoenzyme of human neoplastic cell origin. *Cancer Res* 1968, **28,** 150-154.
5. Jacoby B, Bagshawe KD. Placental-type alkaline phosphatase from human tumor tissue. *Clin Chim Acta* 1971, **35,** 473-481.
6. Higashino K, Hashinostume M, Kang K-Y, Takahashi Y, Yamamura Y. Studies on a variant alkaline phosphatase in sera of patients with hepatocellular carcinoma. *Clin Chim Acta* 1972, **40,** 67-81.
7. Sasahi M, Fishman WH. Ultrastructural studies on Regan and non-Regan isoenzymes of alkaline phosphatase in human ovarian cancer cells. *Cancer Res* 1973, **33,** 3008-3018.
8. Cadeau Y, Blackstein HE, Malkin A. Increased incidence of placenta-like alkaline phosphatase activity in breast and genito-urinary cancer. *Cancer Res* 1974, **34,** 729-732.
9. Keller JA, Bush RS, Malkin A. Placenta-like alkaline phosphatase in gynaecological cancers. *Cancer Res* 1976, **36,** 269-271.
10. Wada HG, Shindelman JE, Ortmeyer AE, Sussman HH. Demonstration of placental alkaline phosphatase in human breast cancer. *Int J Cancer* 1979, **23,** 781-787.
11. Haije WG, Meerwaldt JH, Talerman A *et al.* The value of a sensitive assay of carcinoplacental alkaline phosphatase (CPAP) in the follow-up of gynaecological cancers. *Int J Cancer* 1979, **24,** 288-293.
12. Slack HH, Chu MT, Waysman LE, Murphy GP. Carcinoplacental isoenzyme (Regan) in carcinoma of the prostate. *Cancer* 1981, **47,** 146-151.
13. Chang CH, Angelis D, Fishman WH. Presence of the rare D-variant, heat-stable placental-type alkaline phosphatase in normal human testis. *Cancer Res* 1980, **40,** 1506-1510.
14. Goldstein DJ, Blasco L, Harris H. Placental alkaline phosphatase in non-malignant human cervix. *Proc Natl Acad Sci USA* 1980, **77,** 4226-4228.
15. Goldstein DJ, Rogers C, Harris H. A search for trace expression of placental-like alkaline phosphatase in non-malignant human tissues: demonstration of its occurrence in lung, cervix, testis and thymus. *Clin Chim Acta* 1982, **125,** 63-75.
16. De Waele P, De Groote G, Van De Voorde A *et al.* Isolation and identification of monoclonal antibodies directed against human placental alkaline phosphatase. *Arch Int Physiol Biochim* 1982, **90,** B21.
17. De Groote G, De Waele P, Van de Voorde A, De Broe ME, Fiers W. Use of monoclonal antibodies to detect human placental alkaline phosphatase. *Clin Chem* 1983, **29,** 115-119.
18. Benham FJ, Povey MS, Harris H. Placental-like alkaline phosphatase in malignant and benign ovarian tumors. *Clin Chim Acta* 1978, **86,** 201-215.
19. De Boever J, De Geest K, Van Moele G, Vandekerkhove D. Estrogen receptors in breast cancer. In: Adlercreutz H, Bulbrook RD, Van der Molen HJ, Vermeulen A, Sciarra F, eds. *Endocrinological Cancer: Ovarian Functions and Disease.* Amsterdam, Excerpta Medica, 1981, 41-47.
20. McLaughlin PJ, Gee H, Johnson PM. Placental-type 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.
21. McDicken IW, Stamp GH, McLaughlin PJ, Johnson PM. Expression of human placental-type alkaline phosphatase in primary breast cancer. *Int J Cancer* 1983, **32,** 205-209.
22. Miyayama H, Doelgast GJ, Memdi V, Gandbhir L, Fishman WH. Direct immunoperoxidase staining for Regan isoenzyme of alkaline phosphatase in human tumour tissues. *Cancer* 1976, **38,** 1237-1246.
23. 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* 1978, **29,** 201-215.