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Carcinoplacental Alkaline Phosphatase in Malignant and Premalignant Conditions of the Human Digestive Tract

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Summary. The presence of carcinoplacental alkaline phosphatase (CP Alk P) was demonstrated in the cells of malignant and premalignant states of the human stomach, colon and rectum using the immunoperoxidase technique.

It was shown to be present in 7 out of 18 carcinomas of stomach and 7 of 17 cases of carcinoma of colon and rectum. In the putative premalignant states it was demonstrated in 4 of 15 cases of intestinal metaplasia associated with gastric carcinoma, and 9 of 12 tubulovillous adenomas of colon. However, it was also demonstrated in 5 of 8 metaplastic polyps of colon which are not neoplastic and in 9 of 17 cases of intestinal metaplasia not associated with cancer of the stomach. It was not seen in normal gastric mucosa and only faintly in 1 of 11 samples of normal colon.

CP Alk P has been shown to be a specific marker of malignancy in a wide range of human cancers when studied in sera from patients or in tissue culture of tumour cells. In this study however, although a statistical difference exists between normal and diseased tissue the marker appears as frequently in non-neoplastic states. It is concluded that CP Alk P is, in tissues, a marker of proliferative activity in cells, rather than neoplastic or malignant change.

In this respect it is similar in some respects to carcinoembryonic antigen, but not other markers of placental origin such as pregnancy specific β, glycoprotein.

Key words: Carcinoplacental alkaline phosphatase – Precancer and cancer – Gastrointestinal tract

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Introduction

Although the incidence of carcinoma of stomach is falling in developed countries, it is still significant (in South Australia the rate in 1977/78 was 15.2 per 10^5 in males and 9.2 per 10^5 in females, South Australian Cancer Registry, 1980) especially as the diagnosis is generally made when the disease is well established and the prognosis is poor. In South Australia the cumulative two year survival is 16%; a low figure when compared with breast cancer where the cumulative two year survival is over 80%. The long postulated link between intestinal metaplasia and cancer of the stomach (Morson 1955) is by no means proven. Nevertheless the hypothesis is supported by the presence of extensive metaplasia in cancer bearing stomachs (Correa et al. 1970), and by the fact that widespread metaplasia occurs many years in advance of cancers in certain postoperative high risk groups (Stalsberg and Taksdal 1971) and cases of pernicious anaemia (Shearman et al. 1966). However, it is clear that most intestinal metaplasia does not evolve into cancer and attempts have been made to delineate definite pre-malignant features. Recently Morson et al. (1980) have described increasing dysplasia in relation to the development of cancer, but as in all studies of this type, the interpretations are subjective. Dysplasia is found in most cases in stomachs with established cancer and the putative malignant potential of dysplastic areas is merely an unproven hypothesis.

A similar problem exists in the diagnosis of colonic carcinoma in colonic polyps. It is now generally accepted that a polyp-cancer sequence exists, although only 1–2% of truly neoplastic polyps (tubulovillous adenomas) give rise to cancer. Despite the claims that the most "severe" of the various degrees of dysplasia is diagnostically important the only really reliable indicator of malignancy is invasion through the muscularis mucosa. Even this feature can be difficult to interpret as polyps are often removed piecemeal and frequently traumatised: pseudoinvasion may occur as the result of epithelial displacement secondary to trauma.

The development of immunohistochemical techniques to a high level of refinement (Burns 1975; Heyderman 1979) has permitted the study of various tumour markers in tissue sections, even in material collected and processed to paraffin wax in the routine manner, often many years previously.

These markers are for the most part not found in normal tissues in detectable amounts, and reflect a phenotype which is different from normal.

A large number of these markers are of foetal or placental origin. Skinner and Whitehead (1981) have recently shown that the identification of tumour antigens in cells offers some chance of increasing the detection of their malignant potential. One such marker placental alkaline phosphatase has been studied in cells of carcinoma and supposedly premalignant conditions of the stomach and large intestine and is referred to hereafter as carcinoplacental alkaline phosphatase (C.P. Alk P).

Material and Methods

Stomach

Eighteen cases of carcinoma of stomach were studied, nine were resection specimens and in another nine the tissue was obtained by endoscopic biopsy. In each case at least two blocks were from

the carcinoma and at least one each from cardiac, body and pyloric region. There were 17 cases of atrophic gastritis available for study, usually from cases of healed benign gastric ulcer. In each case biopsies were available from the major regions of gastric mucosa as above.

Colon

Seventeen cases of carcinoma of colon were obtained as operative specimens. In addition there were 12 tubulovillous adenomas (TVA's) which were removed in entirety with stalk and/or base, either at operation or by endoscopy snare, depending on their size. Eight metaplastic polyps (hyperplastic polyps) were included in the study.

In each instance between two and six blocks of tissue were available for examination including, in the case of carcinomas and TVA's, a margin of histologically normal mucosa adjacent to the tumor, or from the same specimen.

Processing of Tissues

Blocks of tissue were fixed in 4% phosphate buffered formaldehyde solution (10% "formalin") at pH 7.0 for between 12 and 20 h. These were then processed in the routine manner through graded alcohols and xylol and finally embedded in "Paraplast Plus" – melting point 52° C.

Sections were cut at a microtome setting of 5 μ, dewaxed in xylol and washed in graded alcohols at 4° C. They were then washed three times in 0.2 M phosphate buffered saline (PBS) pH 7.4; 10 min for each wash. One section from each block was stained by the routine Haematoxylin Eosin method, and in the case of the gastric material, by Alcian Blue (pH 2.5) and periodic-acid Schiff (PAS) combined, to pick out areas of intestinal metaplasia.

Immunohistochemical Methods

Antisera. Antibodies to placental alkaline phosphatase were raised in rabbits in the laboratory. The antigen used was alkaline phosphatase of placental origin. This material has a specific activity of 150 international units/mg. It is supplied in a purified form and when separated by column chromatography on Sephacryl S100 gel gives a major peak containing all the alkaline phosphatase activity and a number of small peaks which were discarded before immunising rabbits. The column eluates with activity were concentrated to the original volume by dialysis against polyethylene glycol molecular weight 6,000 at 4° C overnight. Rabbits were immunised in the standard manner. The first injection, given subcutaneously, consisted of 10 mgs of CP Alk P in Freunds complete adjuvent. This was followed after 14 days by a further injection of 2 mgs CP Alk P as a booster. The animals were bled after a further 10 days. The serum was separated at room temperature (approximately 25° C) in glass vessels and then fractionated on Whatman DEAE 52 cellulose with 0.2 M phosphate buffer pH 8 as eluent. The IgG fraction was collected and concentrated by dialysis against 10% polyethylene glycol of molecular weight 6,000 overnight at 4° C.

Absorption of Antisera. Each batch of antisera was absorbed successively with packed, unwashed red cells three times, then with washed human buffy coat cells, and human liver cells in the same manner. The early batches of antibody were further purified on an immunoabsorbent column consisting of gel separated CP Alk P bound to sepharose-4B (Pharmacia) and eluted with phosphate buffer. The results obtained by this procedure were not a material improvement on the earlier absorptions and as more loss of antibody occurred to the column and later batches were not processed through this step.

The antibody thus prepared gave a single precipitation band on immunodiffusion against the original placental alkaline phosphatase.

The antibody to CP Alk P was then used in the unlabelled antibody peroxidase antiperoxidase (PAP) immunohistochemical method (Burns 1975).

The specificity of this antibody was checked by prior adsorption with pure CP Alk P (Miles). Staining of human placental syncytiotrophoblast was abolished. Prior adsorption with a suspension of liver cells or small intestinal cells, sources of other alkaline phosphatase isoenzymes of the human digestive system had no effect on placental staining.

There was no cross reactivity with antibodies to two other placental markers used in our laboratory, namely, pregnancy specific β glycoprotein and human β chorionic gonadotrophin and these antigens did not in any way diminish anti CP Alk P activity in the PAP Method.

The optimal dilution of material was determined using the control placentas as substrate and a dilution of CP Alk P antibody to 1/200 was found to be optimal. The swine anti-rabbit antibody was used at 1/20 and the PAP reagent at 1/50. All the reagents were allowed to react for 30 min at 25° C with three washes of PBS (pH 7.4) between steps.

Controls

Positive control tissue was obtained from human placenta from normal term deliveries. Syncytiotrophoblast stained consistently and gave a good dark brown precipitate with diaminobenzidine (DAB).

Negative control tissue was obtained from small intestine, liver, gastric mucosa, both body and pyloric, judged to be histologically normal and normal colonic mucosa from the ends of resection specimens from cases of diverticular disease and carcinoma. These tissues failed to stain with each batch of anti CP Alk P tested.

Results

Histological Interpretation

Stomach. The classification of gastric carcinoma into diffuse and intestinal types was made according to the criteria outlined by Laurén (1965). There were four cases of diffuse cancer, 12 intestinal and two unclassified. The diagnosis of atrophic gastritis and intestinal metaplasia was made using criteria outlined by Whitehead (1979).

The diagnosis of carcinoma of the colon and tubulovillous adenocarcinoma was made using standard criteria (WHO 1976). They were graded A to C using Dukes classification.

The tubulovillous adenomas varied in size from 1.5 to 5.5 cms in diameter and histologically varied from a predominantly tubular pattern to a more obviously villous pattern. However, in no case was one pattern exclusively seen. The colonic carcinoma consisted of 4 cases Grade A, 8 grade B and 5 grade C.

Immunohistochemistry Staining Patterns

The distribution of CP Alk P positive staining varied greatly. In some cases not all tumour cells were positive (Fig. 1), often as few as 10%, in others all were positive (Fig. 2). The staining often involved clumps of tumour cells (Fig. 3) or collections of gland-like spaces, or in some instances individual cells within a rudimentary acinus (Fig. 4). The distribution of stain within cells showed variation as is the case with many other markers. In some instances the apical membrane was stained (Fig. 5) and in these instances there was usually quite strong staining of luminal "secretion". In other instances the cells showed granular staining within cytoplasm; throughout the cell or just above the nucleus.

No attempt was made to estimate the intensity of staining but in general it was much less intense than in syncytiotrophoblast. A further dilution of anti-CP Alk P of between four and tenfold was required to bring the intensity within the range seen in the majority of tumours. The so-called premalignant conditions showed a similar staining intensity and variation in stain distribution.

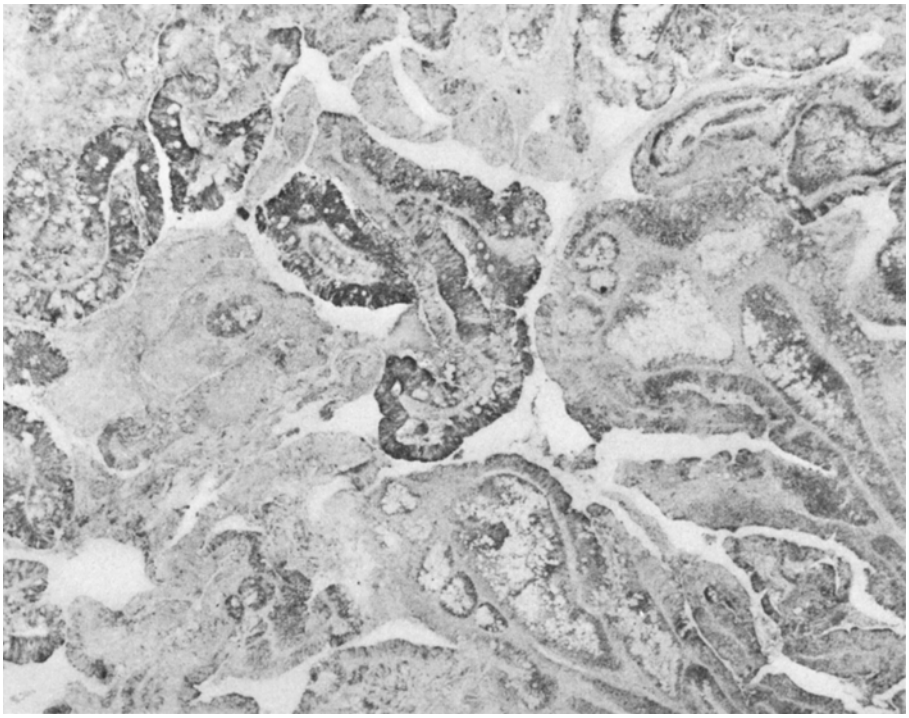

Fig. 1. A human colon adenocarcinoma stained for CP Alk P showing darkly stained positive tumour cells, and similar unstained cells, pale in the photograph

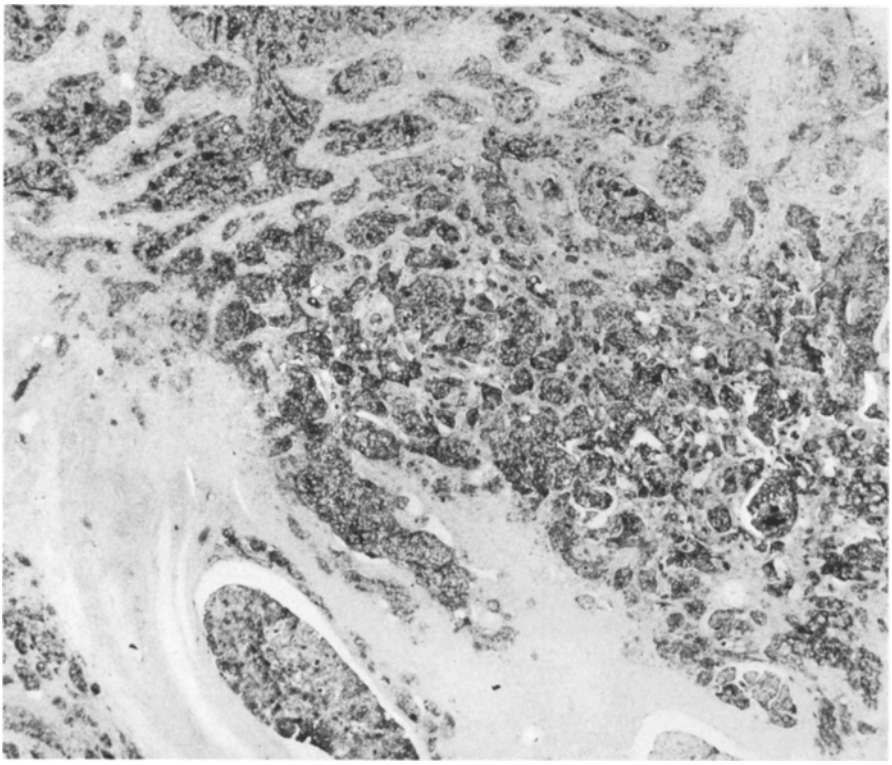

Fig. 2. A human gastric adenocarcinoma showing staining of all tumour cells for CP Alk P

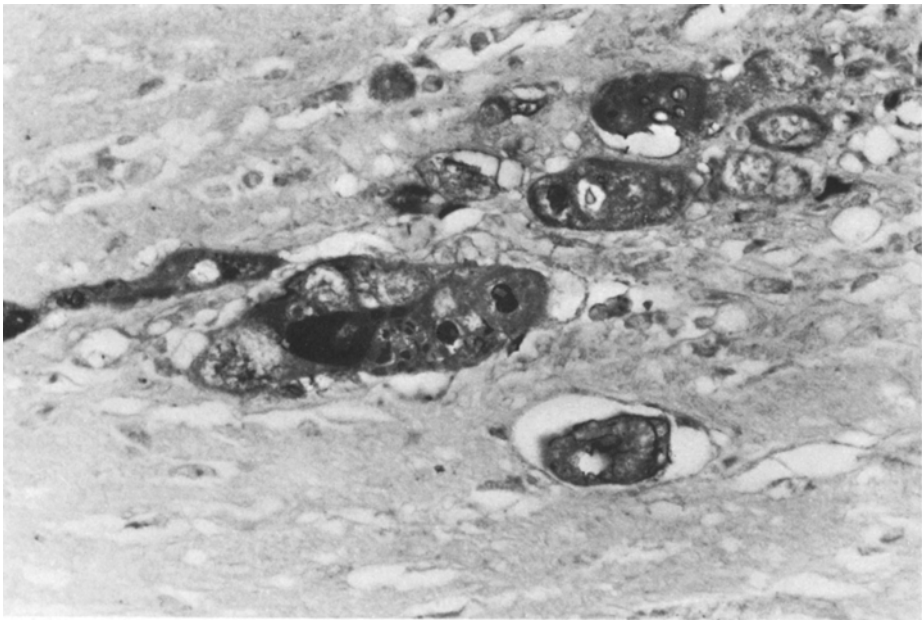

Fig. 3. Individual groups of tumour cells stained positively for CP Alk P (dark cells)

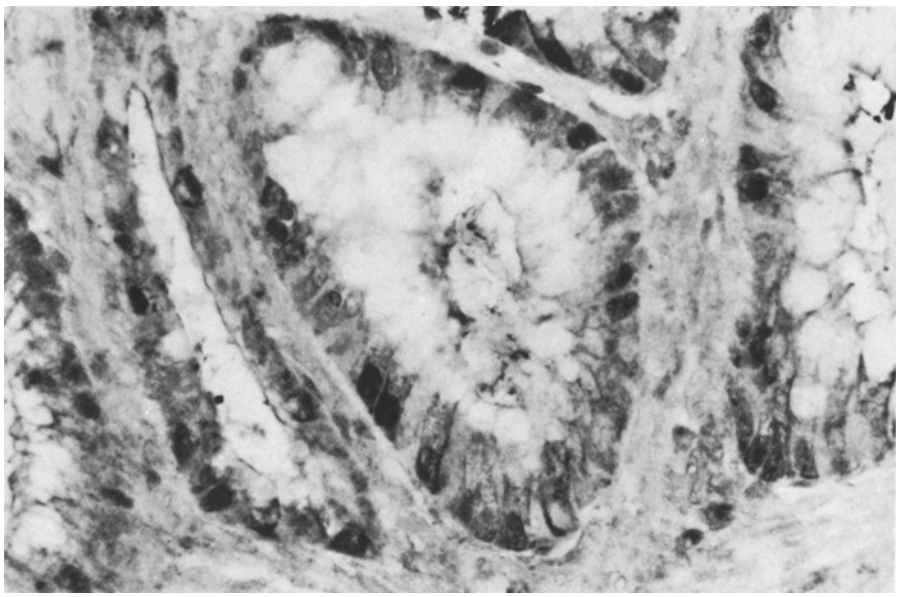

Fig. 4. Individual cells within an acinus showing positive dark staining for CP Alk P

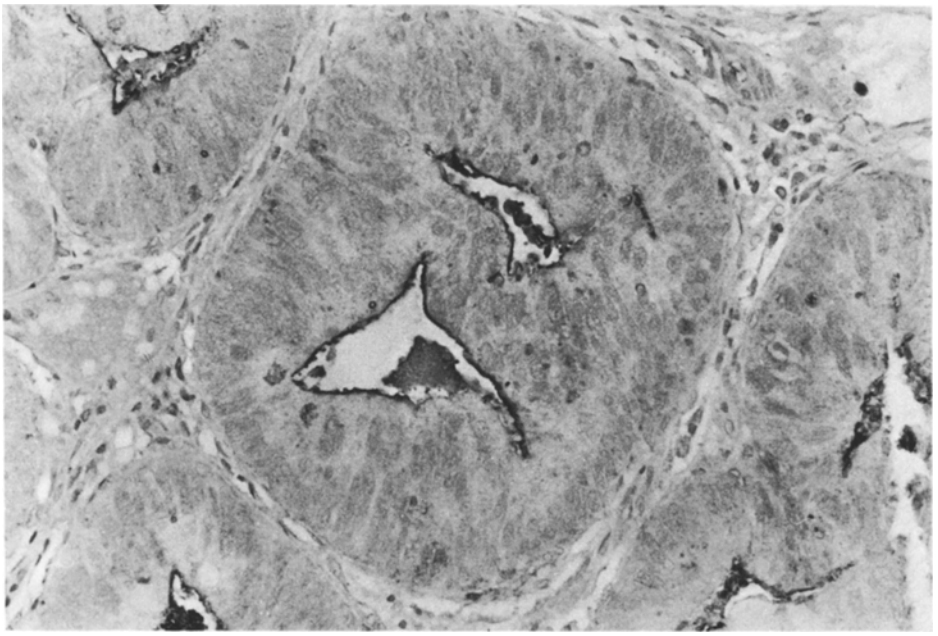

Fig. 5. Shows apical membrane staining in glandular structure in a human colon adenocarcinoma

Table 1. CP Alk P in colon

	Positive	Negative	Total
Normal	1	10	11
Metaplastic polyp	5	3	8
TNA	9	3	12
Cancer	7	10	17

Table 1 shows the number of cases in which CP Alk P was identified in the colon, including the normal cases. Using the two-way χ^2 contingency test the value of $\chi^2 = 11{,}137$ with three degrees of freedom gives a P value of 0.011. This demonstrates a difference between the normal controls and the test cases. Examining only the three test categories the χ^2 value is 3.18 which for two degrees of freedom gives a P value of 0.181 i.e. there is no significant difference between the CP Alk P positive and negative abnormal groups.

Table 2 shows a similar analysis for the gastric cases.

Including the normal category the value for $\chi^2 = 5{,}535$ ($F=3$, $P=0.136$). Excluding the normal category $\chi^2 = 2.306$ ($F=2$; $P=0.316$). No difference has been demonstrated between CP Alk P positive and negative groups and none between normal and test groups though it is noted that the normal controls number only five and all are negative.

Table 2. CP Alk P in stomach

	Positive	Negative	Total
Normal	0	5	5
Intestinal metaplasia with Ca.	9	8	17
Intestinal metaplasia with atrophic gastritis	4	11	15
Cancer	7	11	18

The mucosa adjacent to cancer in the colon was positive in one case and next to TVA's in two.

In the stomach, mucosa adjacent to tumour without intestinal metaplasia was negative, though only three cases fell into this category. In two cases oesophageal squamous epithelium was included in a section and it was also negative.

There was no relationship to tumour type in the stomach and in the colon no difference was demonstrated between the least and most invasive (Dukes A to C) carcinomas.

Discussion

Since Fishman et al. (1968) demonstrated an isoenzyme of alkaline phosphate, with characteristics identical to placental alkaline phosphatase in the serum and tumour cells of a patient with carcinoma of the lung, the enzyme has been demonstrated in a number of different neoplasms (Nathanson and Fishman 1971). Attention has largely focussed on the detection of this enzyme, usually designated carcinoplacental alkaline phosphatase as a tumour marker in sera of patients with cancer (Haije et al. 1979; Wada et al. 1979; Wahren et al. 1979). It appears to be of particular value in the follow-up of patients and in the detection of recurrences.

Though first detected in a lung tumour the greatest attention has been given to its use in gynaecological and breast cancers and in testicular tumours. In gynaecological and testicular tumours it appears to be a somewhat better marker than carcinoembryonic antigen (CEA) (Haije et al. 1979) though not in breast. Nevertheless it is not present in the sera of the majority of cancer patients (Nathanson and Fishman 1971).

As with other tumour markers such as CEA and alpha fetoprotein (AFP) it is assumed that the tumour cells produce and shed or secrete CP Alk P. It has been demonstrated in cytological smears of seminomas (Wahren et al. 1979), in osteosarcoma cells in tissue culture (Singh et al. 1978) and in gastric carcinoma cells, both in culture (Tokumitsu et al. 1979) and in cells metastatic to liver (Kojuma et al. 1979). However, in an extensive study of tumour cell lines in tissue culture Neuwald et al. (1980) failed to detect CP Alk P (or non-placental alkaline phosphatase) in cells or culture supernatants from normal intestine or colonic tumours. It is possible that the enzyme is not shed in large amounts by tumour cells and therefore not easily detected in serum.

The pattern of its staining within the cells suggests a manufacture in the rough endoplasmic reticulum and a transport along secretory pathways as occurs with mucins, rather than an incorporation into lysosomes. It is even conceivable that some pinocytosis of secreted material occurs either returning the product to the cell or allowing entry into adjacent cells.

In this study CP Alk P was demonstrated in less than half the cases and in only one normal mucosa from colon, albeit weakly. Nor is it particularly associated with gastrointestinal carcinoma unlike markers such as pregnancy specific β glycoprotein (Skinner and Whitehead 1981). In fact the finding of CP Alk P in "reactive" and hyperplastic states such as the intestinal metaplasia with atrophic gastritis and metaplastic polyps of the colon would point more to an association with any high cell turnover state, analogous to the production of high levels of CEA in inflammatory bowel disease. It would thus have little or no use in the diagnosis of malignant potential.

About 50% of positive cases overall is quite high in relationship to the published literature on CP Alk P. This may be due to the fact that previous studies have concerned serum levels or production by cultured cell lines. There is, however, another possible explanation. Though the antisera used were extensively adsorbed and gave negative results in normal intestinal tissues and liver it remains a possibility that rapidly dividing intestine derived cells (including those in metaplasia in the stomach) produce larger amounts of intestinal alkaline phosphatase and even a weak cross reaction with anti CP Alk P gives a positive result in these instances. Immunohistochemical methods are to some extent dependent on the number of antigen binding sites available and accessible to the antibody used. An increase in antigen would statistically increase the chance of an antibody antigen reaction occurring on the surface of the tissue sections. The greater the amount produced, the more likely and intense the reaction would be. Such cross reactions were not detected in immunodiffusion studies, but the PAP method on tissues is somewhat more sensitive.

It would appear that a study of CP Alk P as a tumour marker is better confined to tissues in which alkaline phosphatase is not present in normal circumstances.

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