A Radioimmunoassay for Placental-type Alkaline Phosphatase

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SUMMARY

Rabbit antiserum was raised against preparations of placental alkaline phosphatase (AP) extracted from human term placentae. The antiserum precipitated AP from the placenta and from the intestine, but it was inactive against AP from bone and liver. The concentration of antiserum required to precipitate intestinal AP was about 10 times higher than that necessary to precipitate placental AP.

Placental AP was labeled with iodide-$^{125}$I and was subjected to electrophoresis on polyacrylamide gel before use. A specific activity of about 100 μCi/μg protein was obtained. The isotopically labeled AP behaved somewhat like the unlabeled enzyme during electrophoresis and when subjected to Sephadex gel filtration. Both preparations were quantitatively and specifically bound by the antiserum when examined on polyacrylamide electrophoresis.

A radioimmunoassay of the solid-phase type was developed with the use of antiserum-coated plastic tubes. The assay was specific for placental-type AP when various phosphatase preparations and sera were tested, and it detected a minimum level of 20 ng protein per ml, which is equivalent to 0.03 enzyme unit/ml.

Sera from patients in the third trimester of pregnancy contained 60 to 320 ng of placental AP per ml, by radioimmunoassay. Radioimmunoassay and enzymatic assays gave comparable estimates of placental AP activity in the serum. Serum samples from approximately 100 patients with various forms of cancer were examined for placental-type AP by radioimmunoassay, by an enzymatic assay for heat-stable AP of comparable sensitivity, or by both methods. Placental-type AP was not detected in any instance.

Previous studies had revealed a placental-type AP in the serum of a patient with a bile duct adenocarcinoma. Both an AP purified from this tumor and the patient's serum gave parallel dose-response slopes to placental AP in the radioimmunoassay.

INTRODUCTION

Different forms of AP have been associated with a wide variety of human tumors (10, 14-17, 20, 21, 23, 27). Fishman et al. (6) found an AP variant in the serum of a cancer patient that had properties that resembled those of normal placental AP. This variant, which was of tumor cell origin, was named the "Regan isoenzyme" after that 1st patient. It was later detected in the serum of 4.6% of 590 cancer patients (26), but it has been detected more recently in 12% of 323 patients (17). The aims of this study were to develop a radioimmunoassay for serum placental AP and to evaluate this new assay as a means of detecting tumors that produce the enzyme.

Normal serum is known to contain AP's derived from bone, liver, and intestinal sources. Placental AP differs from these AP's by withstanding incubation at 65° and by its greater sensitivity to inhibition by certain L-amino acids, such as L-phenylalanine. This last property is shared by intestinal AP. Placental AP is itself heterogeneous; 6 different phenotypes have been identified electrophoretically (22), and these are thought to be determined by 3 autosomal allelic genes.

In a study of the AP activity of homogenates of 19 adenocarcinomas of the lung and colon, Kellen and Lustig (12) found that the tumor AP's were more strongly inhibited by several L-amino acids than were those of the corresponding normal tissue. They also noted that the pattern of inhibition of the tumor AP's was quite variable and that, by inference, there was no common type of tumor AP. Until the relationship between the different tumor and placental AP's is fully established, tumor AP's may best be considered to be a group of closely related, but not necessarily identical, phosphatases.

Although the relative heat stability of placental AP's can be made the basis of a discriminatory enzyme assay for their detection in serum, the length of the heat inactivation stage might be crucial to the detection of tumor AP's that might have properties intermediate between those of placental and nonplacental AP's. It seemed possible that an immunoassay system might avoid these difficulties, and so that adequate sensitivity could be obtained a radioimmunoassay was chosen.

MATERIALS AND METHODS

Disodium $p$-nitrophenyl phosphate and 2-amino-2-methyl-1-propanol were obtained from Sigma (London), London, England. Carrier-free iodide-$^{125}$I (1.0 mCi; 1MS-3) was obtained from the Radiochemical Centre, Amersham, England, and Freund's complete adjuvant was obtained from Difco Laboratories, East Molesey, England. Horse serum (No. 3, unheated) was obtained from Wellcome Reagents Ltd., Beckenham, England.

Protein was determined by the method of Lowry et al. (13), with bovine serum albumin as standard (Fraction V from bovine plasma; Armour Pharmaceutical Co. Ltd., Eastbourne, England).

Serum Samples. Serum samples were obtained from patients...
with histologically diagnosed malignant tumors, prior to surgery or other therapy. The sera were stored at -190°C in liquid nitrogen until required for use.

**Immunoelectrophoresis.** Double immunodiffusion was carried out as described by Ouchterlony (18). Immunoelectrophoresis was carried out in 2 stages. The 1st stage consisted of electrophoresis of samples of 0.01-ml volume on polyacrylamide gel. In the 2nd stage, the gels were removed from their glass cylinders and were placed in parallel about 2 cm apart on a square glass plate. A solution of agar (1% w/v) was poured over them and allowed to solidify. Troughs were cut in the agar parallel to the polyacrylamide gels and were filled with antiserum (0.1 ml). Gels were photographed unstained after 72 hr of diffusion.

**Purification of Placental AP.** AP was prepared from 3 term placentae, according to the method of Harkness (7). This involved 1-butanol extraction, methanol precipitation, ammonium sulfate fractionation, and chromatography on columns of DEAE-cellulose and Sephadex G-200. The final specific activity was 294 μmoles/min/mg protein at 25°C. No attempt was made to crystallize the enzyme. The product was homogeneous by column chromatography but, after polyacrylamide electrophoresis at pH 9.5, the gels could be stained for AP activity (25) or for protein (with naphthalene black) to show 3 closely spaced bands. The center band was the most intensely stained, and the pattern obtained might correspond with that of the triple banded structure characteristic of the FS phenotype of placental AP when subjected to starch gel electrophoresis (22). Alternatively, a mixture of different phenotypes might have been obtained from the 3 placentae.

The tumor AP described in this paper was extracted from secondary deposits of a bile duct adenocarcinoma removed at autopsy. The purification method, similar to that described above, has been reported (10). The tumor patient's serum had elsewhere been routinely screened for heat-stable (placental) AP and was positive (11).

**Polycrylamide Electrophoresis.** Disc gel electrophoresis and enzyme staining were performed according to the method of Smith et al. (25). The iodide-125I-labeled AP that was obtained after desalting was purified electrophoretically on each of 4 polyacrylamide gels. After electrophoresis for 15 to 20 min at 300 V, the gels were removed while an additional control gel, which had been loaded with unlabeled AP, was stained for enzyme activity. The substrate was p-nitrophenyl phosphate dissolved in the same buffer solution as was used for the enzyme assay. After 10 to 15 sec of development, all of the gels were aligned, and the section on the radioactive gels corresponding to the enzyme band on the control gel was cut out. These gel sections were further sliced, and the material was eluted overnight at 4°C in 2 changes of the assay diluent. More than 90% of the radioactivity was recovered from the gel, and the iodide-125I-labeled AP was diluted for the radioimmunoassay so that a volume of 0.05 ml would contain 30,000 to 40,000 counts/50 sec. The purification process described above was repeated prior to each radioimmunoassay.

**Antiserum Preparation.** Purified placental AP was dialyzed overnight at 4°C against 500 ml Tris-HCl buffer (0.1 M, pH 7.4). A 1.0-mg sample (0.12 ml or 1560 units/ml) was mixed in sterile 0.15 M NaCl with 1.0 ml Freund's complete adjuvant, with the use of a VIRTIS 45 homogenizer. The total volume was 2.0 ml. Half-lop rabbits (4 kg each) received 3 s.c. injections, each consisting of 0.3-mg of protein, at multiple sites at 2-week intervals. Adjuvant was omitted from the last injection; 3 weeks later, the serum was collected and stored at -13°C.

**Quantitative Immunoprecipitation.** Serum or extract (0.04 ml) and antiserum at the required dilution were made up to a total volume of 0.15 ml in 0.15 M NaCl with the use of tubes of 0.4 ml capacity. Antiserum was omitted from the controls. The overall serum concentration of each incubation sample was adjusted to 10% v/v by the addition of appropriate amounts of normal rabbit serum. After being incubated, first for 1 hr at 37°C, then for 96 hr at 4°C, the tubes were centrifuged cold for 15 min at 28,000 x g on a microhematocrit centrifuge (Griffin-Christ; London, England) with an angle head. The supernatants (0.14 ml) were removed by syringe and assayed for enzyme activity. No correction was made for the volume (0.01 ml) remaining with the precipitate.

**Antiserum Homogeneity.** Antiserum that had been absorbed out against normal liver tissue gave a single precipitin line when examined either by double diffusion or by immunoelectrophoresis (Fig. 1). The areas bounded by the precipitin lines stained positively for AP activity (not shown).

**Enzyme Assay.** AP was assayed in a system consisting of 2-amino-2-methyl-1-propanol-hydrochloride buffer, pH 10.2, at 24°C (0.073 M); MgCl2 (0.005 M); disodium p-nitrophenyl phosphate (0.002 M); and, when indicated, L-phenylalanine (0.005 M). The final volume was 2.85 ml. (The figures in parentheses refer to final concentrations.) The reagents were maintained in their cuvets at 30 ± 0.2°C with the use of an SB-2 water bath with a circulating pump (Grant Instruments Ltd., Cambridge, England), which was linked to the cell housing of a Unicam SP 800 recording spectrophotometer. The reaction was started by the addition of serum or extract (0.05 ml, or more where indicated), and was followed by the increase in absorption over a 10-min period, with the Unicam SP 250 scale expansion accessory set at a 10-fold magnification. Enzyme was omitted from the blank that was routinely included in the assay. The absorbance of the blank was subtracted from that of the test samples prior to calculation of results. One enzyme unit is defined as the amount of enzyme that causes an increase in absorption, at 410 nm, of 0.10/min, or the transformation of 0.158 μmole of substrate per 2.85 ml cell per min, under the above-described conditions. Specific activity is given as the number of μmoles of substrate transformed per min per mg of protein.

**Preparation of Isotopically Labeled Placental AP.** Iodination with 1.0 mCi iodide-125I was performed, as described by Hunter and Greenwood (8), with the use of purified placental AP (5 μg or 6.91 enzyme units in 0.01 ml). The iodination was terminated after 10 sec by the addition of sodium metabisulfite solution. The protein was separated from unreacted iodide (desalted) before further use on a column of Sephadex G-50 (8). The peak samples were combined and stored undiluted at -25°C. About 36% of the labeled protein was identified by polycrylamide gel electrophoresis as iodide-125I-labeled AP. The remaining isotopically labeled components were assumed to comprise material damaged during iodination. Examination by electrophoresis showed that they were no longer quantitatively and specifically bound.
Radioimmunoassay for Placental AP

Radioimmunoassay. The solid-phase assay was modified from that of Catt and Tregear (4). Disposable plastic tubes (12 x 75 mm; Falcon Plastics, Oxnard, Calif), were coated overnight with antiserum (0.5 ml; diluted 1:15,000 in carbonate:bicarbonate buffer, 0.05 M, pH 9.6) with the use of a Standard No. 50 automatic syringe (Techne Ltd., Cambridge, England). The liquid was then aspirated, and the tubes were washed twice in an equal volume of NaCl (0.15 M), and once in bovine serum albumin (0.6 ml, 3% w/v). The tubes were then ready for use.

For convenience, a 2-day assay was chosen, and the addition of the iodide-\(^{125}\)I-labeled AP was delayed until the 2nd day. During the 1st day, the iodide-\(^{125}\)I-labeled AP was eluted from the polyacrylamide gels. The diluent used throughout contained NaCl (0.15 M), sodium Merthiolate (final dilution, 1:10,000) and bovine serum albumin (0.2% w/v) in sodium phosphate buffer (0.075 M, pH 7.25). Both phosphate and Merthiolate inhibit the enzymatic activity of AP.

Standard curves were prepared by the inclusion of tubes containing from 0.1 to 10.0 ng (1.38 to 138.00 \(\times 10^{-5}\) enzyme units) of purified placental AP in 0.5 ml 10% v/v horse serum. The protein concentration of the AP standard was determined according to the method of Lowry et al. (13). Satisfactory results could also have been obtained by the use of pooled normal human serum in place of horse serum. Controls, in which unlabeled AP was omitted, were prepared. The test samples contained 0.05 ml patient’s serum in place of horse serum and were made up to 0.05 ml in diluent and mixed. After 1 day at room temperature, iodide-\(^{125}\)I-labeled AP (0.05 ml containing 3 to 4 \(\times 10^{4}\) counts/50 sec) was added to all of the tubes with a 2-ml Repette (Jencons, Ltd., Hemel Hempstead, England), and the tubes were left for a further day. They were then counted for radioactivity (preshash count), washed twice with water, and re-counted (postwash count). Radioactivity was determined with an Auto-Gamma Counter (Packard Instrument Co., La Grange, Ill.). In different assays, from 25 to 45% of the added radioactivity was bound by the control tubes. The percentage count was calculated as postwash count/preshash count \(\times 100\).

RESULTS

Specificity of Antiserum. Normal serum is known to contain AP that originates in tissue such as bone, liver, and intestine. The latter enzyme is immunologically related to that of the placenta (3, 24) and could possibly interfere with estimations of placental AP. For investigation of the interaction between intestinal AP and the antiplacental antiserum, enzyme aliquots were incubated with graded dilutions of antiserum for 5 days, as indicated in “Materials and Methods” and, after centrifugation to remove the precipitate, the supernatant activity was measured. Liver AP that had been extracted from the tissue with 1-butanol and sera that were shown by electrophoresis to contain a band of liver or bone AP were not precipitated by antiserum within the range of dilution 1:15 to 1:3000. The samples had activities ranging from 0.28 to 0.67 unit/ml. In contrast to this result, the heat-stable AP of pregnancy serum was precipitated at an antiserum dilution of 1:1500 (Table 1). Intestinal AP was precipitated at an antiserum dilution of 1:150 but not at a greater dilution. In the comparison of these AP's, the activities used in the incubation aliquots (Table 1) were not equal. Precipitate was not detected when 2 sera, shown by electrophoresis to contain intestinal AP, were incubated with graded dilutions of antiserum (Table 1).

Antiserum Binding of Iodide-\(^{125}\)I-labeled AP. Iodide-\(^{125}\)I-labeled AP which had not been desalted was fractionated on a column of Sephadex G-200. Samples of the column eluate were tested for antigen uptake on antiserum-coated plastic tubes (Chart 1). The incubation buffer was that used in the radioimmunoassay. The 1st peak of radioactivity was asymmetrical, suggesting heterogeneous material, with the
Table I
Immunoprecipitation of AP samples

After being incubated with antiserum for 96 hr at 4°, the precipitates were removed by centrifugation, and the supernatants were assayed for AP activity. The details are described under "Materials and Methods."

<table>
<thead>
<tr>
<th>AP activitya with antiserum dilution</th>
<th>Control (no antiserum)</th>
<th>1:1500</th>
<th>1:750</th>
<th>1:150</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal AP (1-butanol extract of human colon)</td>
<td>0.499</td>
<td>0.445</td>
<td>0.426</td>
<td>0.095</td>
</tr>
<tr>
<td>Intestinal AP serum No. 1b</td>
<td>0.269</td>
<td>0.267</td>
<td>0.216</td>
<td>0.251</td>
</tr>
<tr>
<td>Intestinal AP serum No. 2b</td>
<td>0.255</td>
<td>0.267</td>
<td>0.247</td>
<td>0.236</td>
</tr>
<tr>
<td>Pregnancy serum, heatedc</td>
<td>0.318</td>
<td>0.064</td>
<td>0.050</td>
<td>0.047</td>
</tr>
</tbody>
</table>

a Units/ml of serum or extract.
b Shown by electrophoresis to be rich in intestinal AP.
c Serum preheated for 20 min at 65° to destroy nonplacental AP's.

Chart 1. Gel filtration of iodide-125I-labeled AP. A 1.6- x 60-cm column of Sephadex G-200 was equilibrated with Tris-HCl buffer (pH 7.4, 0.01 M) containing 0.15 M NaCl. Iodide-125I-labeled AP (1.0 ml containing 1.0 x 10^6 counts/50 sec), obtained prior to desalting, was prepared in the Tris buffer. Column elution was by a flow pumped downward at 8.0 ml/hr, and 3.0-ml fractions, were collected. •, radioactivity of 0.03-ml aliquots from each of the column eluate tubes; ○, uptake of antigen on antiserum-coated plastic tubes. A 1:5000 dilution of antiserum was used to coat the tubes. Samples from the column eluate (0.015 ml each, in triplicate sets) were incubated overnight in the coated tubes, washed, and recounted. The bound count is expressed as a percentage of the total count ± S.D.

higher-molecular-weight material preferentially bound. Enzymatic activity was not determined.

Electrophoresis on polyacrylamide gel gave a better resolution than that obtained on the Sephadex column. A sample of iodide-125I-labeled AP, purified electrophoretically as described in "Materials and Methods," was incubated for 1 hr with antiserum at a final dilution of 1:75. The sample was run on the gel at pH 9.5, stained for AP activity, and sliced into sections by hand. The sections were counted for radioactivity (Chart 2). The control gel was run with a sample that had been incubated with normal rabbit serum at the same dilution. On this control gel, 51.1% of the total counts were located coincident with the enzyme stain while, on the antiserum gel, 48.1% of the total counts were found coincident with the enzyme stain but at the gel origin. This enzymatically active material was thought to be the antigen:antibody complex.

Influence of Antiserum Concentration on Binding of Iodide-125I-labeled AP. Plastic tubes were coated with graded dilutions of antiserum and, after incubation for 24 hr with iodide-125I-labeled AP obtained after desalting, the uptake of radioactivity was measured (Chart 3). From a maximum binding level of 28% of the added counts at a 1:2,000 dilution of antiserum, the percentage binding fell on both sides of the curve. In fact, with electrophoretically purified iodide-125I-labeled AP, satisfactory counts were obtained with antiserum dilutions of from 1:5,000 to 1:15,000. The controls with normal rabbit serum bound only 0.5% of the total counts and, in subsequent experiments, no correction was made for this factor. The reduction of binding at high antiserum concentration, which is seen in Chart 3 at a 1:1,000 dilution, has been reported for other solid phase systems (1, 2).

Specificity of Radioimmunoassay. Horse serum and normal human sera, typed for ABO blood group, were examined for inhibition of uptake of labeled antigen. Serum was added (0.05 ml each, to duplicate tubes) and incubated with iodide-125I-labeled AP. The sera were of Group O (5 samples),
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for heat-stable AP. These included 75 of the sera sampled by radioimmunoassay. The controls consisted of 17 sera from women in the 3rd trimester of pregnancy and samples of the placental standards in heated normal serum. The tumor sera differed from the controls in that no heat-stable AP (> 0.03 unit/ml) was detected in any instance. The activities of the unheated tumor sera also differed from the controls in that they were relatively insensitive to inhibition by L-phenylalanine (Table 2). In contrast, the serum from the single cancer patient described above gave results which were similar to those of the controls.

The sera were obtained from patients with a wide variety of malignant tumors (Table 3), and the results indicate that in this series there was no detectable placental-type AP. The single positive sample was identified as a result of other studies (10, 11).

Eight pregnancy sera that had earlier been assayed for total and heat-stable AP activity were quantitated by radioimmunoassay. The values (ng/tube) were read from the standard curve. The correspondence between enzymatic and radioimmunoassay estimates was close, and there was no correlation with the values for the total AP activity (Chart 5). This is to be

Chart 4. Radioimmunoassay: comparison of 2 different antisera. A, Antiserum 1; B, Antiserum 2. The radioimmunoassay technique is described under “Materials and Methods.” The percentage bound count is shown as the mean value ± S.D. of triplicate determinations. The standard curves ranged from 8,380 to 2,230 counts/50 sec (A); and 10,740 to 4,840 counts/50 sec (B). •, purified placental AP standards; ○, pregnancy serum obtained at 39 weeks gestation (0.292 unit/ml heat-stable activity); ◆, serum from tumor patient +ve for placental-type AP (0.750 unit/ml heat-stable activity). Aliquots of 0.002 to 0.006 ml of the serum were assayed. The data for the sera are plotted on the assumption that 0.03 enzyme unit/ml is equivalent to 20 ng protein/ml.

Assay of Sera from Cancer Patients. Sera from 97 patients were examined in duplicate sets by radioimmunoassay. The counts measured in duplicate tubes did not differ by more than 10% from each other. There was no detectable immunologically active AP in any of the samples. The mean percentage bound was 38.42 ± 2.23% compared with 32.28 ± 0.97% for 1.0 ng/tube placental AP, the minimum concentration detectable in the radioimmunoassay. This difference in the percentage bound was highly significant (t test; p < 0.001). A single cancer patient described above gave results which were similar to those of the controls.

Chart 3. Uptake of iodide-125 I-labeled AP onto plastic tubes coated with graded dilutions of antiserum. The antiserum was diluted, as indicated, in carbonate/bicarbonate buffer (pH 9.6, 0.05 M) and allowed to coat onto plastic tubes for 2 hr. The contents were aspirated, and after washing the tubes were refilled with labeled AP (mean, 47 ± 10 counts/50 sec) in diluent. After 24 hr, the tubes were washed and counted. Tubes were prepared in duplicate sets except for those containing antiserum diluted 1:1,000 (prepared in triplicate) and those at antiserum dilutions of 1:32,000 and over (prepared singly). Duplicate control tubes were coated with normal rabbit serum in place of antiserum (serum dilution, 1:1,000). Antiserum (○) and control (●) tubes were counted for radioactivity (mean count ± S.D.).

Group A (3 samples), and Groups B and AB (1 sample each); all were rhesus +ve except for 1 sample each from Groups O and A. The mean percentage radioactivity bound by the tubes ± S.D. was 46.60 ± 2.58%, and the corresponding value obtained with horse serum was 44.97 ± 1.15%. The difference was not statistically significant (t test; p > 0.05). Five sera ranging in total AP activity from 0.476 to 0.825 unit/ml were shown by electrophoresis to exhibit a band of activity at the position of intestinal AP. The percentage bound was 43.80 ± 2.86%. For comparison, the value for the minimum detectable concentration of placental AP of 1.0 ng/tube was 35.56 ± 1.20%, a significantly lower binding value (t test; p < 0.001).

An experiment was performed to determine whether graded dilutions of pregnancy serum and serum from a patient with a tumor known to be positive for placental-type AP would give results showing the same inhibition curve as that for the purified standards. The experiment was performed with 2 different antisera (Chart 4). The standard curves for the 2 antisera showed different slopes, but the curves for both of the sera were coincident with that for the standards when assayed in Antiserum 1. With Antiserum 2, (Chart 4B), the pregnancy serum gave a slightly steeper dilution curve than that of the standards, while the dilution curve for the tumor serum was further displaced.

Assay of Sera from Cancer Patients. Sera from 97 patients were examined in duplicate sets by radioimmunoassay. The counts measured in duplicate tubes did not differ by more than 10% from each other. There was no detectable immunologically active AP in any of the samples. The mean percentage bound was 38.42 ± 2.23% compared with 32.28 ± 0.97% for 1.0 ng/tube placental AP, the minimum concentration detectable in the radioimmunoassay. This difference in the percentage bound was highly significant (t test; p < 0.001). The sera from 101 patients were examined by enzymatic assay

10 -1.000 10 0.002 0.006

ALKALINE PHOSPHATASE (ng/tube) log scale

0 10

0.5 5 10

ALKALINE PHOSPHATASE (ng/tube) log scale

0.5 5 10

Chart 4. Radioimmunoassay: comparison of 2 different antisera. A, Antiserum 1; B, Antiserum 2. The radioimmunoassay technique is described under “Materials and Methods.” The percentage bound count is shown as the mean value ± S.D. of triplicate determinations. The standard curves ranged from 8,380 to 2,230 counts/50 sec (A); and 10,740 to 4,840 counts/50 sec (B). •, purified placental AP standards; ○, pregnancy serum obtained at 39 weeks gestation (0.292 unit/ml heat-stable activity); ◆, serum from tumor patient +ve for placental-type AP (0.750 unit/ml heat-stable activity). Aliquots of 0.002 to 0.006 ml of the serum were assayed. The data for the sera are plotted on the assumption that 0.03 enzyme unit/ml is equivalent to 20 ng protein/ml.
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Table 2

Heat-stable AP activity

The data refer, where more than 1 sample was measured, to the mean value ± S.D. Heat-stable activity was determined after the sera were heated for 20 min at 65°.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inhibition by 0.005 M L-phenylalanine (%)</th>
<th>Heat-stable activity (%)</th>
<th>Heat-stable activity (unit/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancy sera obtained from 17 women in the 3rd trimester of pregnancy</td>
<td>53.8 ± 10.4c</td>
<td>43.9 ± 16.5</td>
<td>0.228 ± 0.172</td>
</tr>
<tr>
<td>Purified placental AP (in heated normal serum)</td>
<td>88.4</td>
<td>90.5</td>
<td>0.294</td>
</tr>
<tr>
<td>Serum from single patient positive for placental-type AP</td>
<td>38.6</td>
<td>27.6</td>
<td>0.750</td>
</tr>
<tr>
<td>Sera from 101 patients with cancer</td>
<td>18.00 ± 4.05d</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a AP activity was determined as described in "Materials and Methods."
b Calculated as heat stable/total AP activity × 100.
c Mean ± S.D.
d Ten sera were assayed.

Expected if the heat-labile phosphatases are immunologically unrelated to placental AP. The sera were not examined for placental phenotype (22).

Estimation of a Tumor AP by Radioimmunoassay. Liver tissue was obtained at autopsy from a patient with a poorly differentiated adenocarcinoma of the bile duct with metastases throughout the liver. A placental-type AP was purified from this tissue by means of the procedure described above. Its properties, which were similar to those of the serum heat-stable AP, have been reported elsewhere (10). Graded dilutions of the concentrated extract were prepared in horse serum, assayed in quadruplicate, and compared with the standard curve (Chart 6). The measured enzymatic activity was plotted as ng protein/tube (0.03 enzyme unit/ml is equivalent to 20 ng protein per ml). The 2 curves were coincident, showing that the purified extracts displace iodide-125I-labeled AP from the antiserum in a like manner. Samples of the patient’s serum were diluted and assayed in triplicate over a limited range (Chart 4); the most concentrated sample contained 0.006 ml of serum. The results, which matched the standard curve closely, indicated a protein concentration of 425 ng/mg of placental-type AP. The binding level was reduced to a mean of 1600 ± 64 counts/50 sec, or 4.9% of the total counts added.

DISCUSSION

A convenient radioimmunoassay has been developed for the detection and estimation in serum of placental AP. The validity of the method for detecting placental-type AP in the sera of cancer patients was suggested by the positive identification of the single cancer patient, whose serum was known from other studies to contain this enzyme.

AP from an intestinal extract was precipitated at an antiserum concentration about 10 times higher than that necessary to precipitate placental AP. No immunologically active material was detected when 5 sera containing intestinal AP were examined by radioimmunoassay. A much higher serum concentration of intestinal AP would probably be necessary to produce a significant reduction of the bound count. The radioimmunoassay was free from interference by bone and liver AP.

In a survey of 323 patients with malignant tumors, Nathanson and Fishman (17) identified the Regan isoenzyme in the sera of 39 patients (12%) of the group. Heat-stable AP was confirmed as being of the Regan type if it was selectively bound by an antiserum to placental AP and therefore failed to migrate on starch gel electrophoresis. This test was performed on sera containing over 0.4 heat-stable placental isoenzyme unit and on some sera containing over 0.1 unit (or about 0.028 King-Armstrong unit/100 ml).

In this survey, placental-type AP was not detected in any of the patients’ sera.

Table 3

Diagnosis of patients whose sera were examined by radioimmunoassay and enzymatic assay

The number of patients in each category is listed. Sera from 75 of the patients were assayed by both methods shown.

<table>
<thead>
<tr>
<th>Diagnosis of patient</th>
<th>Radioimmunoassay</th>
<th>Enzymatic assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoma</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>Bladder</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Bronchus</td>
<td>11</td>
<td>15</td>
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<td>Cervix</td>
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<td>6</td>
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<tr>
<td>Breast</td>
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<tr>
<td>Stomach</td>
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<tr>
<td>Colon</td>
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<tr>
<td>Tongue</td>
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<td>1</td>
</tr>
<tr>
<td>Urethra</td>
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<td>1</td>
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<td>Hard palate</td>
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<td>1</td>
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<tr>
<td>Teratoma of testis</td>
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<td>1</td>
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<tr>
<td>Pancreas</td>
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</tr>
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<td>Larynx</td>
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<td>2</td>
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<tr>
<td>Retropertioneal sarcoma</td>
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<tr>
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<td>4</td>
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<tr>
<td>Melanoma of vulva</td>
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<td>Hodgkin's disease</td>
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<td>Myeloma of spine</td>
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No. of patients

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<th>36</th>
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<tr>
<td>Female</td>
<td>66</td>
<td>65</td>
</tr>
<tr>
<td>Total</td>
<td>97</td>
<td>101</td>
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Radioimmunoassay for Placental AP

Chart 5. Comparison of units. Eight sera, obtained from women in the 3rd trimester of pregnancy, were quantitated by radioimmunoassay and by enzymatic assay for both total and heat-stable AP activity. The estimate of placental AP obtained by radioimmunoassay is plotted against the enzymatic activity of the unheated sera (•) and that of the same sera after 20 min of incubation at 65° (○). The enzymatic activities of both heated and unheated sera are obtained in units/ml.

75 sera assayed by both radioimmunoassay and enzymatic assay for heat-stable AP. The minimum amount detectable in either case was about 0.03 enzyme unit or 1.0 King-Armstrong unit/100 ml. This is some 10- to 30-fold less than the sensitivity obtained by Nathanson and Fishman (17). From the published data of these authors concerning the serum AP of 323 cancer patients, we estimate that 14 Regan-positive sera contained more than 1.0 King-Armstrong unit/ml of heat-stable AP and would be detectable by our methods. In our series, none of the 101 sera examined by enzymatic assay contained detectable (≥ 1.0 King-Armstrong unit) heat-stable AP. This difference in incidence is not statistically significant ($\chi^2$ test, $p > 0.05$). In fact, there were substantial differences in the distribution of types of cancer in the 2 series, and there were differences in methods of enzymatic assay. These factors may have contributed to the difference in incidence recorded.

The ability to detect very low serum concentrations of placental-type AP specifically in the presence of AP's originating from other tissues presents considerable technical difficulties. Although sera have been heated for 5 min at 65° prior to enzymatic assay (17, 26), a longer period of heating (20 min at 65°) was used in our study [in common with the 20- to 30-min heating times used by other investigators (5, 9, 19)], in order to inactivate nonplacental AP's. Heat inactivation is, of course, unnecessary prior to estimation by radioimmunoassay.

The practical value of detecting placental-type AP in the sera of patients would seem to be in the area of cancer diagnosis and in monitoring the development of tumors producing this enzyme variant. Radioimmunoassay appears to offer a satisfactory method of monitoring placental AP's. Greater sensitivity can usually be achieved in radioimmunoassay, as in other methods, but there may be some loss of specificity, and it remains to be determined whether this course of development is desirable for clinical applications.

ACKNOWLEDGMENTS

We are indebted to Dr. R. C. Jennings, Consultant Pathologist at the General Hospital, Altrincham, Cheshire, England, for the provision of serum and tumor tissue from the patient with a bile duct adenocarcinoma.

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A Radioimmunoassay for Placental-type Alkaline Phosphatase

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*Cancer Res* 1972;32:2413-2420.

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