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A Sensitive Immunochemical Method for the Determination of the Regan Isoenzyme in Serum

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SUMMARY

An enzymatic, immunochemical test system has been developed which allows quantitation of minute amounts of the Regan isoenzyme of alkaline phosphatase. The basis of the method is the concentration (tenfold) of the Regan isoenzyme by reaction with a monospecific antiserum to placental alkaline phosphatase, insolubilized by polymerization with ethyl chloroformate. This concentration is achieved by incubating heat-inactivated serum with the antibody and by subsequent centrifugation. The pellet containing the active enzyme-antibody complex is immediately assayed for enzyme activity.

We studied sera from 91 normal healthy adults and 112 cancer patients to determine the presence of the Regan isoenzyme. Detectable Regan isoenzyme activity was found in 89 of 91 normal healthy adults. Three of the normal sera contained a level of the isoenzyme that fell above 2 S.D. from the mean. In the case of patients with neoplastic disease, 106 of 112 sera had detectable Regan isoenzyme activity and only 11 sera showed elevated activities. Cancer sera with abnormal Regan levels contained 3 to 300 times the average normal value.

INTRODUCTION

The Regan isoenzyme of alkaline phosphatase was first reported in 1968 by Fishman *et al.* (7). This enzyme has been found in the serum and tumor of patients with various cancers. It is identical to the placental isoenzyme with respect to L-phenylalanine sensitivity, heat stability, cleavage by neuraminidase, starch gel electrophoretic pattern, and immunological reactivity (4, 6, 7). Originally, the incidence of the serum isoenzyme was reported as 4.6% in a screening study of cancer patients (12). Later work (11) demonstrated the presence of the isoenzyme in the serum of 12% of cancer patients. The discovery of this isoenzyme raises again the very basic question of the possibility of antigenic reversions in human cancer tissue. By antigenic reversion, we mean the production by tissues of primitive embryonic constituents upon malignant transformation. Two other examples of possible antigenic reversions have been investigated, *i.e.*, the carcinoembryonic antigen and α_1-fetoprotein. The carcinoembryonic antigen of the human digestive system (9) has been isolated from entodermally derived neoplasms and also appears in embryonic digestive tissue. α_1-Fetoprotein is an embryonal serum α-globulin appearing in the sera of patients suffering from primary hepatomas (1).

The determination of the Regan isoenzyme presented a challenge because of the minute amounts of enzyme present in the sera of some cancer patients (12). The reported low incidence of the Regan isoenzyme in malignant disease could possibly be due to lack of sensitivity of the procedures used for its determination. Published methodology consists of electrophoresis in starch gel or cellulose acetate of heat-inactivated specimens that have been treated with and without antibody to placental alkaline phosphatase (10, 12). A positive identification of Regan isoenzyme was made if the isoenzyme-antibody complex appeared (shown by a decrease in electrophoretic migration). Naphthol ASMX, a fluorogenic substrate, has been used to increase the sensitivity of the procedure (10). We used the very sensitive immunochemical technique described in this report to quantitate the Regan isoenzyme in both normal individuals and patients with neoplastic disease.

MATERIALS AND METHODS

Antiserum to Placental Alkaline Phosphatase. The antiserum against a highly purified preparation of placental alkaline phosphatase (M. Usategui-Gomez, F. M. Yeager, and P. Tarbutton. Purification of Placental Alkaline Phosphatase by Isoelectric Focusing, in preparation) was produced in rabbits by a primary footpad injection of the antigen in Freund adjuvant, followed by i.v. boosters according to a procedure previously described (M. Usategui-Gomez, F. M. Yeager, and A. Fernandez de Castro. The Use of Polymerized Antisera for the Determination of Placental Alkaline Phosphatase in Pregnancy Sera, in preparation). We polymerized the monospecific antiserum with ethyl chloroformate, using a technique first described by Avrameas *et al.* (3) to successfully polymerize a number of proteins and antisera.

Assay Procedure. To 0.5 ml of the heated serum (65°, 10 min) are added 50 μl of an appropriate dilution of polymerized antisera. Enough antibody should be present to bind 100 to 300 times the amount of Regan isoenzyme found in normal sera. The mixture is gently shaken for 1 hr in an Ames aliquot mixer and centrifuged at 2000 × *g*.

After carefully removing the supernatant, we resuspended the precipitate in 100 μl of 0.01 M Tris, pH 7.0. The

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substrate, 0.5 ml of 72 mM phenyl phosphate in 0.1 M carbonate-bicarbonate buffer (pH 10.7) is then added. After a 2-hr incubation, the pH is adjusted to 9.0 with 20 μl of 1.7 N HCl. Color development takes place after the addition of 100 μl of 1.5% aminoantipyrine and 100 μl of 4% potassium ferricyanide. The absorbance of the wine-pink solution is read at 5 min at 505-nm wavelength.

Patients. Sera from 91 healthy individuals who were undergoing preemployment or annual physical examinations were obtained. In all 112 serum samples from patients with cancer, the diagnosis was confirmed by histological examination of tumor tissue. The sera were kept frozen at −20° until ready to be used.

RESULTS

A typical standard curve is shown in Chart 1. Reliable and reproducible quantitation of minute amounts of enzyme is possible by the above-described procedure. Since total levels of alkaline phosphatase in normal sera range from 30 to 80 i.u./liter, the present method can accurately quantitate an isoenzyme that accounts for less than 1% of the total alkaline phosphatase found in serum.

The sera of 91 normal individuals were assayed for the presence of the Regan isoenzyme. The unexpected results obtained are presented in Chart 2. All but 2 of the normal sera contained detectable levels of a heat-stable isoenzyme that combines with antibody to placental alkaline phosphatase. The average value obtained in the normal group was 0.29 ± 0.21. Three of the normal sera contained a level of the Regan isoenzyme that fell above 2 S.D. from the mean.

Among the patients with neoplastic disease, 106 had detectable Regan serum levels (Chart 3). However, only 11 serum samples showed isoenzyme values above 2 S.D. from the mean normal value. Cancer sera with abnormal levels can contain from 3 to 300 times the average normal value.

In Table 1, a summary of the data obtained from the 112 neoplastic disease patients is presented. It is important that, in this series of cancer patients, one-half of the sera with abnormal levels came from tumors of the female genital tract. The percentage elevation of the Regan isoenzyme in this type of cancer is 29, compared to 11 in tumors of gastrointestinal or breast origin and 9 in tumors of the respiratory tract. In the total group of neoplastic disease patients, the incidence of occurrence of the Regan isoenzyme was 10%. A wide range of values was observed in cases with abnormal levels (Table 2).

DISCUSSION

Previous studies (4, 6, 7, 11, 12) have demonstrated the existence in neoplastic disease sera of an isoenzyme identical biochemically and immunochemically to placental alkaline phosphatase, namely, the Regan isoenzyme. In a separate report (M. Usategui-Gomez, F. M. Yeager, and A. Fernandez de Castro. Regan Isoenzyme in Normal Human Sera, in preparation), we demonstrated that a heat-stable isoenzyme detectable in normal sera by reaction with monospecific antibody to placental alkaline phosphatase was indeed the Regan isoenzyme. The heat-stable isoenzyme present in a pool of 50 normal sera was isolated by reaction with polymerized antibody to the placental isoenzyme, and its properties were studied. The pH optimum and L-phenylalanine sensitivity of the isoenzymes isolated from heated pregnant sera, cancer sera, and a normal pool were found to be identical. Other investigators have observed residues of heat-stable intestinal alkaline phosphatase which frequently cross-reacted with their antisera to placental alkaline phosphatase (11). The pH optimum curves for intestinal and placental isoenzymes are

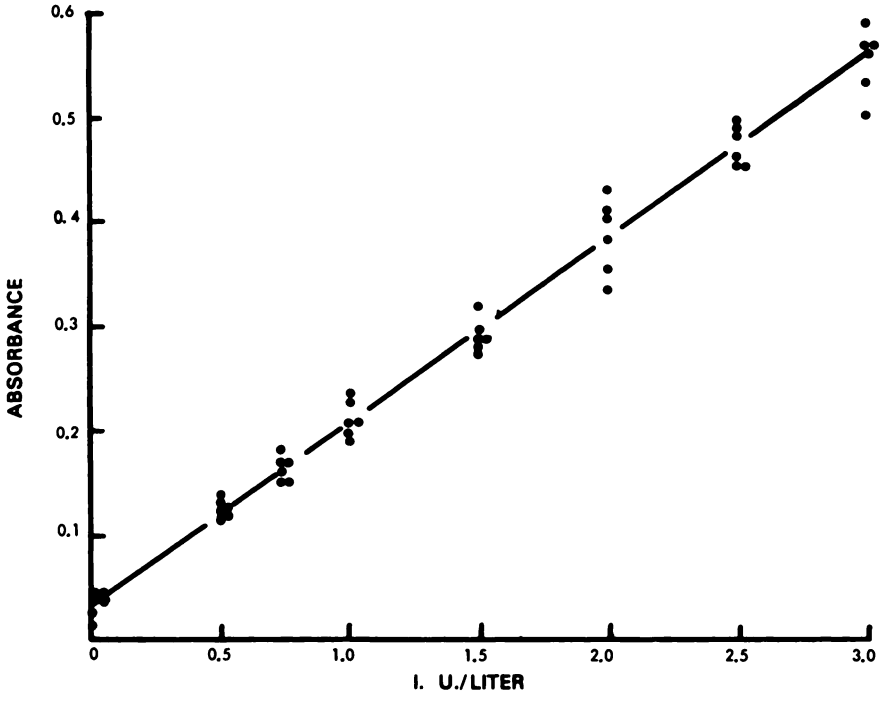

Chart 1. Standard curve for the determination of the Regan isoenzyme. Relationship between absorbances and phosphatase activity of placental enzyme standards. At each enzyme level, 6 determinations were performed. The standard solutions are assayed at 30° by a continuous spectrophotometric method with the use of 4m M paranitro phenyl phosphate as the substrate in 0.75 M 2-amino-2-methyl-1-propanol buffer, pH 10.15, at 30° (9).

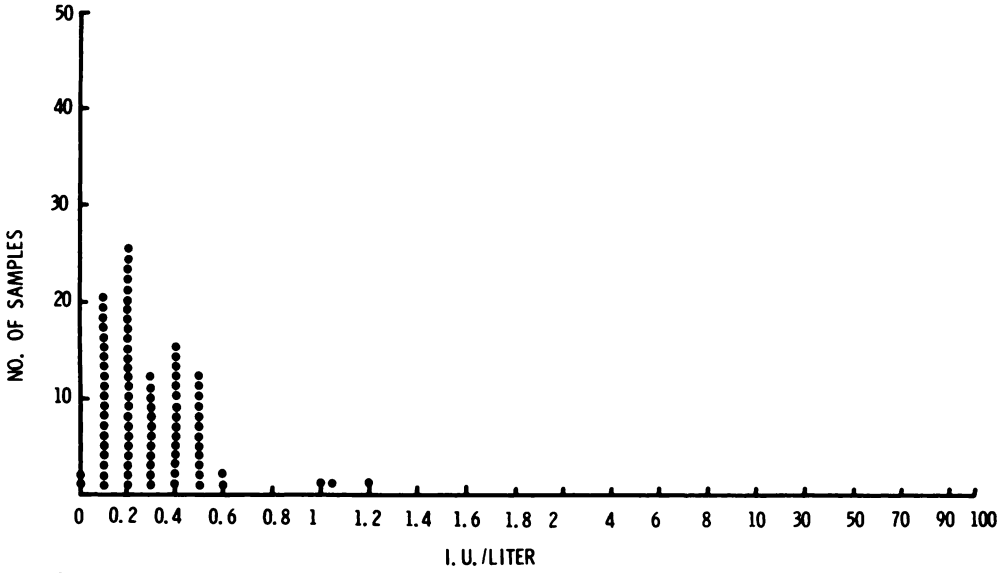

Chart 2. Distribution of the Regan isoenzyme among a group of normal healthy individuals.

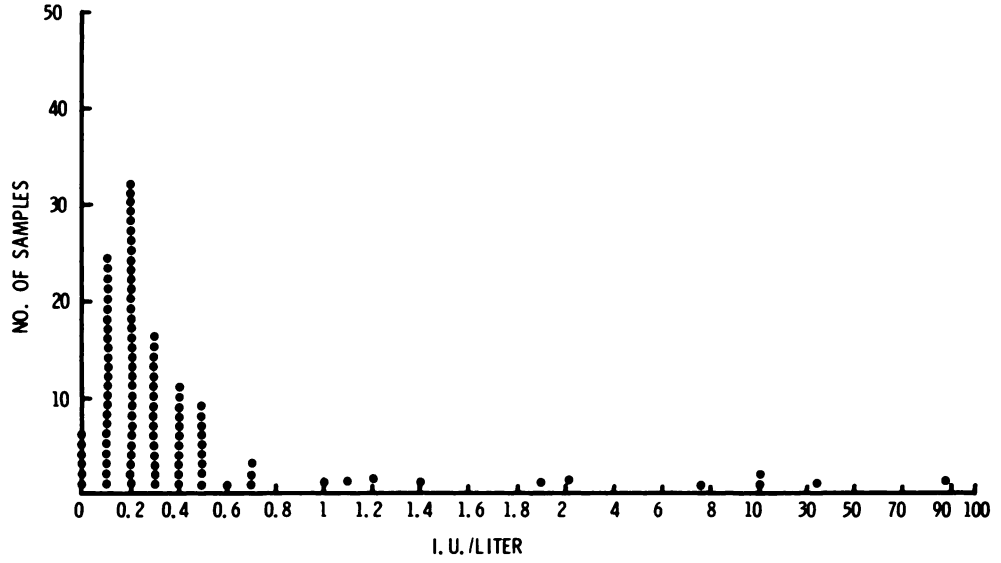

Chart 3. Distribution of the Regan isoenzyme among a group of patients with a variety of neoplastic diseases.

quite different (5, 8), ruling out the possibility of intestinal origin for the heat-stable isoenzyme, currently isolated from most normal sera by specific placental antibody.

Our work confirmed the elevation of the Regan isoenzyme in about 10% of cancer patients. In this study, however, by using a very sensitive immunochemical assay, we also found the isoenzyme at very low levels in most of the normal sera tested, as well as in sera from patients with a wide variety of cancers. The results obtained in this investigation give strong support to the idea that the Regan isoenzyme is not a tumor-specific antigen. The suggested concept of a stage- or phase-specific antigen (2) seems more appropriate for this isoenzyme of alkaline phosphatase.

A published study by Nathanson *et al.* (11) of sera of 140 hospital personnel undergoing preemployment or annual physical examination showed only 4 Regan isoenzyme-positive patients, when the assay was performed with the use of a less sensitive electrophoretic technique. Likewise, in a series of 102 unselected patients with elevated alkaline phosphatase, only 9 patients with nonmalignant disease had the Regan isoenzyme in their sera. The authors offered as a possible explanation for the above findings the actual production of the Regan isoenzyme by nonmalignant tissue.

The immunochemical test system described herein allows the quantitation of the Regan isoenzyme by a simple procedure without the need for any special equipment. By this technique, a Regan isoenzyme determination can be performed in any clinical laboratory. In certain types of tumor with a high incidence of Regan isoenzyme, including tumors of the female genital tract, this assay may be particularly useful (note that 2 squamous cell carcinomas *in situ* of the cervix showed small but definite elevations of the Regan isoenzyme). The increased sensitivity of this assay could provide an early diagnosis of cancer. Additional clinical studies may find a use for Regan isoenzyme levels as a measurement of the success of tumor removal after surgery or the effectiveness of antitumor

Table 1
Results obtained with the Regan isoenzyme assay in 112 cancer patients

Diagnosis	Abnormal levels	Normal levels
Gastrointestinal		
Adenocarcinoma, colon and rectum	1	8
Adenocarcinoma, stomach	0	9
Squamous cell carcinoma, esophagus	1	0
Metastatic leiomyosarcoma of the liver, primary stomach	0	1
Biliary		
Adenocarcinoma, bile duct	0	3
Adenocarcinoma, gall bladder	0	2
Adenocarcinoma, liver	0	1
Female genital		
Squamous cell carcinoma *in situ*, cervix	2	2
Squamous cell carcinoma invasive, cervix	0	5
Adenocarcinoma, cervix	0	2
Adenocarcinoma, endometrium	2	1
Adenocarcinoma, ovary	1	2
Breast		
Adenocarcinoma	2	18
Male genital		
Embryonal carcinoma, testis	0	1
Adenocarcinoma, prostate	0	8
Urinary		
Adenocarcinoma, kidney	0	2
Transitional cell carcinoma, kidney	0	1
Adenocarcinoma, bladder	0	1
Transitional cell carcinoma, bladder	0	2
Respiratory		
Squamous cell carcinoma, lung (bronchus)	1	11
Adenocarcinoma, lung	0	3
Undifferentiated carcinoma, lung	0	1
Oat cell carcinoma, bronchus	0	1
Thyroid		
Undifferentiated carcinoma, thyroid	0	1
Adenocarcinoma, thyroid	0	1
Reticuloendothelial system		
Hodgkin's disease	0	1
Leukemia, chronic lymphocytic	0	1
Lymphoma, histiocytic	0	2
Lymphoma, lymphocytic nodular	0	1
Miscellaneous		
Squamous cell carcinoma, larynx	0	3
Transitional cell carcinoma, pelvic wall	0	1
Adenocarcinoma, abdominal wall	0	1
Squamous cell carcinoma, skin (1 scalp, 1 cheek)	0	2
Adenocarcinoma, primary unknown	0	1
Undifferentiated adenocarcinoma, primary unknown	1	0
Large cell, undifferentiated carcinoma in lymph gland	0	1

therapy. Preliminary reports are encouraging (11), but further work is necessary to assess the value of Regan isoenzyme testing as an aid in cancer prognosis.

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Table 2
Cases with abnormal levels of the Regan isoenzyme

Diagnosis	i.u./liter
Squamous cell carcinoma, esophagus	92.1
Adenocarcinoma, colon	7.8
Squamous cell carcinoma *in situ*, cervix	1.9
Squamous cell carcinoma *in situ*, cervix	1.0
Adenocarcinoma, ovary	12.0
Adenocarcinoma, endometrium	11.6
Adenocarcinoma, endometrium	35.0
Adenocarcinoma, breast	1.1
Duct cell carcinoma, breast	1.2
Squamous cell carcinoma, lung	2.6
Undifferentiated adenocarcinoma, primary unknown	1.4

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