THE RADIOIMMUNOASSAY OF CIRCULATING CARCINOEMBRYONIC ANTIGEN OF THE HUMAN DIGESTIVE SYSTEM*

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Abstract.—A radioimmunoassay has been developed for determining the serum levels of carcinoembryonic antigen of the human digestive system in patients with cancer of the colon and rectum. The assay is simple to perform and has a high degree of reproducibility and specificity. The test detects a concentration of 2.5 ng of carcinoembryonic antigen per ml of serum and this has provided the first demonstration of a circulating tumor-specific antigen in the sera of cancer patients.

The carcinoembryonic antigen (CEA), first described in this laboratory, has been found exclusively in adenocarcinomata arising from the entodermally-derived digestive system epithelium, and in embryonic and fetal digestive tissues in the first two trimesters of gestation. The CEA has been characterized as a protein-polysaccharide complex which is soluble in 1.0 M perchloric acid and 50 per cent ammonium sulfate. Purified CEA isolated from different metastatic tumors was found to be of consistent amino acid and carbohydrate composition.

Serologic studies revealed that 70 per cent of patients suffering from primary, nonmetastatic cancers of the digestive system, and a comparable proportion of women during pregnancy and the immediate post-partum period contained circulating anti-CEA antibodies in their sera. However, in patients where the tumor had undergone spread from its primary site in the digestive system to other organs, no anti-CEA antibodies could be demonstrated in any case. There are at least two possible explanations for this observation.

First, it may be that a large mass of tumor serves as an antibody “sink,” adsorbing the anti-CEA antibodies as the blood circulates through the tumor tissue.

Experiments have, in fact, shown that the CEA is intimately associated with the tumor cell surface and would, therefore, be readily available to interact in this fashion with its corresponding antibodies.

The second possibility is that the tumor tissue may release CEA directly into the circulation. With a large tumor mass, the corresponding serum concentration of the antigenic material would be relatively high and might lead to CEA-anti-CEA complexes in antigen excess. The anti-CEA antibody constituents would, under these circumstances, no longer be available for participation in subsequent serologic reactions. The purpose of the present study was to develop a radioimmunoassay for the detection of circulating CEA in an attempt to confirm the second hypothesis.

Materials and Methods.—Purified CEA: Purification of the CEA was carried out by a modification of a technique which has been previously described. Homogenates of metastatic cancer tissue which had originated within the digestive system were extracted in 1.0 M perchloric acid at room temperature for 20 min. After removal of the resultant

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precipitate by centrifugation, the supernatant was exhaustively dialyzed against water, lyophilized, and redissolved in a small volume of distilled water. The final, purified CEA fraction was obtained by sequential chromatography on Sepharose 4B and Sephadex G-200, followed by electrophoresis on a Sephadex G-25 block. The purified CEA obtained in this manner was used in all subsequent procedures including immunization for specific antiserum, radioiodination, and as the standard reference material during the radioimmunoassay.

**Radioiodination of CEA:** The purified CEA was radioiodinated by the method of Hunter and Greenwood.6

The final reaction mixture of 500 µl contained 100 µg of Chloramine T, 2-4 mg of CEA, and 4 mCi of 125I (New England Nuclear Corp., Boston, Mass.). The reaction was allowed to proceed for 1 min at room temperature and was then stopped by the addition of 240 µg of sodium metabisulphite. The 125I-CEA was separated from residual unreacted 125I by dialysis against water.

The effects of iodination on the antigenic properties of CEA were determined by two methods: (a) radioimmunoelectrophoresis of 125I-CEA against monospecific goat anti-CEA antiserum; and (b) a comparison, by radioimmunoassay, of the quantitative antigenic activity of standard CEA with that of identical amounts of 125I-CEA prepared in exactly the same fashion as that employed in the preparation of 125I-CEA.

**Preparation of anti-CEA antiserum:** Goats were immunized with purified CEA. After adequate immunization the antisera were harvested, adsorbed to completely ensure specific anti-CEA activity, and tested as previously described.1,2

**Collection and preparation of blood samples:** Blood samples were obtained from 200 patients admitted to hospital suffering from various diseases. Each specimen was coded and handled on the basis of a double-blind design. Determinations were always performed in duplicate.

An equal volume of 2.0 M perchloric acid was added to each 5 ml vol of serum, and the mixture stirred at room temperature for 20 min. The precipitate was removed by centrifugation in a fixed-angle rotor at 9000 g for 10 min at 4°C. The sediment was discarded and the supernatant dialyzed against cold, running tap water for 24 hr, and then against repeated changes of distilled water at 4°C for an additional 24 hr. The dialyzed residue was lyophilized, yielding approximately 7 mg of powdered material in each case.

**Radioimmunoassay of CEA:** During the procedure normal human serum was diluted either 1:10 or 1:100 with borate buffer (M/2 = 0.1, pH 8.4), and these preparations employed as diluents for the anti-CEA antiserum and the 125I-CEA, respectively.

The radioimmunoassay was based on the modification of the coprecipitation-inhibition technique described by Farr.7 The standard inhibition curve was obtained as follows: Doubling quantities of standard CEA were added to a series of tubes, each of which contained the powdered extract of 5 ml of normal human serum. A 500 µl vol of diluted anti-CEA antiserum was then added to each tube. The resulting solutions were incubated at 4°C for 8 hr, following which 500 µl of 125I-CEA was added to each of the tubes. The incubation was continued under the same conditions for an additional 18 hr. At the end of this period, 1.0 ml of cold, saturated ammonium sulfate was added to each of the tubes. In the resulting 50%-saturated solutions of ammonium sulfate, the antibody-bound CEA underwent coprecipitation, while the free CEA remained in solution. After a further wash in 3.0 ml of 50%-saturated ammonium sulfate, at 4°C, the 125I-CEA content of the precipitate was determined in a Nuclear-Chicago Dual Channel Automated Scintillation Gamma-Ray analyzer.

The powdered extracts of serum obtained from the 200 patients were processed in an identical fashion, with the obvious exception that no standard CEA was added to any of the specimens.

**Results.—Sensitivity of the radioimmunoassay:** The conditions employed in the conjugation of 125I to CEA by the Chloramine T technique resulted in a radioiodination efficiency of 14 per cent. The final preparation of 125I-CEA,
therefore, had a rather low specific activity of 0.14 $\mu$Ci/$\mu$g. In order to have a sufficient amount of radioactivity in each tube, a $^{125}$I-CEA concentration of approximately 200 ng/ml was employed during the series of experiments. The standard inhibition curve was obtained by using this concentration of $^{125}$I-CEA and an anti-CEA dilution of 1:4000 (Fig. 1). Under these conditions, a maximum of 45–50 per cent of the radioactive material could be coprecipitated from the reaction solution by 50 per cent ammonium sulfate. A quantity of 2.5 ng of CEA/ml of serum could be reproducibly detected.

The effect of radioiodination on the antigenicity of CEA: The radioimmunoelectrophoretic pattern of $^{125}$I-CEA against monospecific anti-CEA is shown in Figure 2. A single band developed upon autoradiography of the radioimmunoelectrophoretic pattern. Furthermore, the shape and position of the band was identical to those obtained upon ordinary immunoelectrophoresis of standard CEA and $^{125}$I-CEA against anti-CEA antiserum (Fig. 2).

When the same quantities of either standard CEA or $^{127}$I-CEA were employed as inhibitors in the radioimmunoassay, identical inhibition curves were obtained (Fig. 3). Iodination of CEA would, therefore, appear to have no demonstrable adverse effects upon either the qualitative or quantitative aspects of its antigenicity.

![Fig. 1.-Standard inhibition curve obtained using an anti-CEA antiserum concentration of 1:4000 and a $^{125}$I-CEA concentration of 200 ng/ml. The reaction, at each concentration of standard CEA, was carried out in the extract of 5 ml of normal serum and the values on the abscissa are expressed as ng of CEA/ml of serum.](image1)

![Fig. 2.—(A) Autoradiograph of radioimmunoelectrophoretic pattern of $^{125}$I-CEA against anti-CEA antiserum. (B) Immunoelectrophoretic patterns of $^{125}$I-CEA and standard CEA against anti-CEA antiserum. In all 3 cases single bands of the same shape and position were obtained.](image2)
The effect of the serum extraction procedure on the recovery of CEA activity: To assess the validity of the technique of serum extraction, it was important to determine whether the method employed would lead to quantitative CEA recovery. In order to do this, known amounts of standard CEA were added to 5-ml aliquots of normal human serum which were then subjected to the extraction procedure. The resultant powdered extracts were examined for quantitative CEA content by the radioimmunoassay. The recovery of CEA exceeded 95 per cent in every case.

The detection of circulating CEA in human sera: The types of patients studied and the levels of circulating CEA determined are shown in Figure 4. Circulating CEA could not be detected in any serum obtained from normal subjects, pregnant women, patients with cancerous diseases of nongastrointestinal system organs, patients with nonmalignant diseases of the digestive organs, or patients with nonenteric, nonneoplastic conditions.

In 36 patients with adenocarcinomata of either the colon or rectum, studied at a

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Fig. 3.—Comparison of standard CEA and 125I-CEA as inhibitors in the radioimmunoassay. Identical results were obtained with each preparation. The reactions were carried out in the absence of serum extracts, and the values on the abscissa are expressed as ng of CEA/ml of reaction mixture. O = Standard CEA and • = 125I-CEA.

Fig. 4.—Groups of patients studied and the circulating CEA concentrations detected in each case. No patient is represented in any group more than once. ▼ Represents the sera of patients with disseminated gastric cancers and • represents the serum of a patient with a disseminated pancreatic cancer which gave detectable circulating CEA values. Values on the ordinate are expressed in ng of CEA/ml of serum.
time when tumor tissue was known to be present within the body, all but one serum was found to have detectable levels of circulating CEA. The only known false negative result recorded to date was in a patient with a localized, invasive polypoid adenocarcinoma of the transverse colon. At operation, this patient was found to have a volvulus of the large bowel with circulatory impairment.

The lowest positive value thus far recorded in the serum of a patient with a malignant large bowel tumor was 3 ng/ml in a woman with a malignant colonic polyp with mucosal invasion. No false positive results have been obtained to date.

Patients who had undergone surgery for colonic or rectal cancer and who showed no evidence of residual or recurrent tumor growth did not have detectable circulating CEA levels (Fig. 4). In seven cases where both preoperative and postoperative blood samples were obtained, the postoperative values of serum CEA remained elevated only in the one patient with evidence of tumor dissemination at operation (Fig. 5).

Sera were obtained from 32 patients with cancerous disease of digestive organs other than the large bowel and rectum. The lesions involved in this series of cases included a primary hepatoma and adenocarcinomata of the stomach, pancreas, small bowel, ampulla of Vater, biliary tract, and esophagus. In only two patients with metastatic gastric cancer and in one patient with metastatic adenocarcinoma of the pancreas were circulating levels of CEA detected (Fig. 4).

The specificity of the radioimmunoassay for CEA in human serum: In a patient with metastatic adenocarcinoma of the large bowel, a very high level of circulating CEA was detected. Serum specimens from this patient were serially diluted in normal human serum and each dilution processed for its content of CEA by radioimmunoassay. The results obtained are shown in Figure 6. The inhibition curve obtained with dilutions of this patient’s serum is compared with an inhibition curve prepared employing standard CEA as inhibitor. It will be observed that the slopes of the curves are parallel. This indicates that the levels of circulating CEA can be expressed in terms of known quantities of purified antigen, and that the coprecipitation-inhibition observed was not due to nonspecific interactions.
Discussion.—Since the description of the radioimmunoassay for insulin by Yalow and Berson in 1960, this technique has been primarily applied to a variety of protein and polypeptide hormones. The present study deals with the development of a highly specific radioimmunoassay for the detection of circulating carcinoembryonic antigen of the human digestive system in the sera of patients. It, therefore, represents the first successful attempt to determine the existence of human tumor growth in situ by the demonstration of a circulating tumor-specific antigen.

The sensitivity of the radioimmunoassay for CEA, at present, allows the detection of 2.5 ng of CEA/ml of serum. The major limiting factor in the sensitivity of the assay is the relatively low specific activity of the 125I-CEA obtained as compared, for example, with the specific activities of the radiolabeled materials employed in the hormone assays. It must be remembered, however, that in most of the hormone systems the material being assayed is of relatively low molecular weight and is a pure protein. On the other hand, CEA has a molecular weight of about 200,000 and the bulk of the molecule is composed of carbohydrate. Furthermore, the concentrations of tyrosine and histidine are 0.057 and 0.072 μmoles/mg of CEA, respectively. The available sites for 125I binding in the CEA molecule are, therefore, extremely limited. Consequently, the efficiency of labeling and the resulting specific activity of the 125I-CEA are relatively low.

In attempting to overcome the limitation on sensitivity imposed by this situation, two steps were taken. First, it was established that virtually all of the CEA present in a 5-ml volume of serum could be extracted by the procedure employed. Hence, a 5-ml volume of serum was extracted for each CEA determination. Secondly, the incubation of the anti-CEA antiserum with the patient’s serum extract prior to the addition of 125I-CEA would favor the binding of any CEA in the serum extract to the anti-CEA in preference to the binding of the 125I-CEA to the antiserum preparation. The coprecipitation-inhibition of 125I-CEA was therefore augmented, increasing the sensitivity of the assay.

Although CEA is known to be present in all adenocarcinomata of the digestive system, circulating levels of this material in detectable quantities were found con-
sitionally only in patients with adenocarcinomata of the colon and rectum. This finding is, however, not unexpected in that it has been shown that the concentration of CEA is higher in malignant tumors of the colon and rectum than it is in comparable lesions in other portions of the digestive tract. A similar explanation may be given for the failure to detect circulating CEA in the sera of pregnant women. Whether or not this is the only explanation for the phenomenon is presently unknown.

From the clinical standpoint, the demonstration of circulating CEA in the sera of patients with large bowel cancer may have both diagnostic and prognostic significance. With regard to diagnosis, 35 of 36 patients who were known to have tumor tissue of colonic or rectal origin at some site in their bodies had detectable levels of circulating CEA in their sera. As concerns prognosis, it seems reasonable to predict that in a case where the serum level of CEA falls to an undetectable concentration following operation, the recurrence of demonstrable antigen in the serum would indicate the recurrence of tumor growth.

From both the theoretical and practical points of view, consideration must be given to the state in which the CEA exists in the circulation of patients with large bowel cancer. The use of perchloric acid in the initial step of serum extraction lowers the pH to a level capable of dissociating preexisting CEA-anti-CEA complexes in the patient's serum. Hence, the levels of CEA measured in the serum of any given individual may well be composed of both a freely circulating as well as an antibody-complexed CEA.

Definition of symbols for weights employed throughout the manuscript: mg = milligrams (10^{-3} gm), μg = micrograms (10^{-6} gm), ng = nanograms (10^{-9} gm).

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