Carcinoembryonic antigen: Evidence for multiple antigentic determinants and isoantigens

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ABSTRACT Carcinoembryonic antigen (CEA) preparations, from various sources were compared by radioimmunoassay. The preparations studied included four CEA standards (CEA-Roche, CEA-Montreal, CEA-City of Hope, and CEA-British) and CEA from serum and liver metastases of a patient with cancer of the colon who had an extremely high concentration of serum CEA (more than 26,000 ng/ml). The data indicate that the CEA-Roche standard differs significantly from the other three CEA standards tested, and that the serum CEA from the patient was antigenically different from the three CEA standards as well as from the CEA obtained from the patient’s own liver metastases. These antigenic differences were reflected in radioimmunoassay inhibition curves that were different and that were not affected by pericholic acid extraction of CEA. Because of the antigenic variation in the serum CEA, markedly different CEA concentrations (varying by three orders of magnitude) were measured by two different antisera (Roche and Montreal). All the various CEA standards and samples chromatographed on columns of Sepharose-6B, despite the large antigenic variation.

We postulate that CEA consists of a family of “isoantigens” with multiple antigenic determinants. We identified a serum CEA isoantigen that was different from the currently available standards. Consequently, we believe that results of radioimmunoassays currently used for CEA measurement may not represent absolute concentrations of serum “CEA”, but may reflect the binding affinity of different isoantigens to a particular polyvalent CEA antiserum.

After the demonstration of a tumor-specific antigen (carcinoembryonic antigen, CEA) in adenocarcinomas of the gastrointestinal tract by Gold and Freedman (1), it was shown that immunoreactive CEA was present in the plasma of almost all patients with colon carcinoma. This suggested that serologic measurement of this antigen could be a useful diagnostic test for this malignancy (2). However, the availability of a well characterized CEA standard is an important prerequisite for a valid, generally applicable CEA assay.

Coligan et al. (3) isolated CEA from several gastrointestinal tumors and found that their CEA preparation (CEA-City of Hope) was immunologically indistinguishable from CEA prepared by Gold and Freedman (CEA-Montreal) (4). The “First British CEA Standard” (CEA-British) was prepared from liver metastases arising from colon carcinoma by methods similar to those used by Gold and Freedman (4) and Coligan et al. (3), and was found (by the National Institute of Biological Standards and Control, London) to be similar to the CEA-Montreal and CEA-City of Hope by immunodiffusion and polyacrylamide gel electrophoresis, as well as in amino-acid and carbohydrate composition. Hoffmann-La-Roche (Nutley, N.J.) prepared their own CEA (CEA-Roche) and developed a standardized method and kit for routine clinical assays (5, 6). Although studies in which both Roche and Montreal reagents were used showed a high degree of correlation of positive CEA tests in sera from cancer patients (7, 8), a comparative immunochemical analysis of all four standards has not been reported.

We previously observed marked variation (of more than two orders of magnitude) in the assay of CEA in sera of some cancer patients when two different anti-CEA antisera were used (9, 10). This antigenically different serum CEA was demonstrable in all sera tested which contained very high levels (more than 500 ng/ml) of CEA, as measured by Roche reagents (9, 10). These observations led us to re-examine the four presently available CEA standards by radioimmunoassay (RIA) and to compare these with CEA in the serum and tumor of a patient with colon cancer who had extremely high serum CEA levels. Our results indicate that the various CEA standards are not immunologically identical, and suggest that CEA consists of a family or series of isoantigens with various combinations of antigenic determinants that react quite differently with different anti-CEA antisera.

MATERIAL AND METHODS

CEA Standards. CEA-Roche (purchased from Hoffmann-LaRoche, Nutley, N.J.) was reported to contain 125 ng of CEA activity per ml. CEA-Montreal was generously supplied by Dr. P. Gold (McGill University Medical School, Montreal); CEA-City of Hope was provided by Dr. C. W. Todd (City of Hope National Medical Center, Duarte, Calif.); and CEA-British (73/601-2/22J) was obtained from the Medical Research Council of U.K. The source material for the latter three standards were liver metastases arising from primary carcinomas of the colon, and were reported to be prepared essentially by the method of Krupey et al. (4).

Anti-CEA Antisera. Both commercially available goat antiserum against CEA (Roche anti-CEA) as well as goat antiserum against CEA kindly provided by Dr. P. Gold were used in these studies. The latter antiserum was absorbed with 50 mg/ml of dialyzed and lyophilized (1.0 M) pericholic acid extracts of normal human plasma, colon, liver, and lung.

Serum and Tissue Specimens. The serum chosen for study was from a patient (R. K.) who died of carcinoma of the colon with widespread liver metastases. At the time of death, his serum concentration of CEA exceeded 25,000 ng/ml, as measured by the standard Roche CEA assay kit (6). To our knowledge, this is the highest serum CEA value reported to date. The serum of this patient was tested by RIA directly. In some experiments, a dialyzed and lyophilized pericholic acid extract of the serum was prepared as described elsewhere (2). Liver tissue was obtained from the same patient (R. K.) at post mortem. Metastatic cancer was dissected out and homogenized in 4 volumes of phosphate-

Abbreviations: CEA, carcinoembryonic antigen; RIA, radioimmunoassay.
buffered saline. A high-speed supernatant (48,000 × g for 30 min) of this homogenate was tested by RIA.

Gel Filtration Chromatography. Sepharose-6B was suspended in about 10 volumes of eluent (0.05 M KH₂PO₄, pH 7.5) and a column (2.5 × 100 cm), was prepared and calibrated (11).

Radioimmunoassays of CEA. Commercially available Roche anti-CEA antisera (25 µl) was dissolved in 475 µl of borate buffer (0.05 M, pH 8.5) containing 1.60 vol/vol serum from normal blood donors with blood group AB (AB-borate) and incubated for 18 hr at 37° with 10–200 µl of either CEA-standards, serum samples, or liver extract. (The volume was adjusted to 700 µl with borate buffer before incubation.) Then 300 µl of borate buffer containing 25 µl of ¹²⁵I-labeled CEA Roche (about 0.5 ng of CEA, 1.0 to 1.2 × 10⁶ cpm) was added. After incubation at 37° for 4 hr, 1 ml of saturated (NH₄)₂SO₄ was added and the incubation continued overnight at 4°. After centrifugation for 20 min at 30,000 rpm, the supernatant was decanted and the radioactivity was measured in the supernatant. Under these conditions, approximately 40–50% of ¹²⁵I-labeled CEA was specifically precipitated.

When absorbed Montreal anti-CEA antiserum was used, a dilution of 1:16,000 vol/vol in AB-borate was chosen on the basis of an antibody titration curve (10), and 500 µl of this solution was used in the assay. This dilution of antibody resulted in maximum inhibition in RIA with all CEA samples used in this study. About 30% of the added ¹²⁵I-labeled CEA was precipitated under these conditions.

The CEA standards as well as all other samples were carried through the analytical procedure simultaneously and in quadruplicate. Each set of test tubes containing the samples was "sandwiched" between two sets of test tubes containing standard CEA solutions. In each separate experiment the tested substances were compared only with the standards analyzed simultaneously with the samples. Standard error of quadruplicate determinations was less than ±5%.

**RESULTS**

**Comparison of CEA standards by RIA**

Four different CEA standards (Roche, City of Hope, Montreal, and British) were compared simultaneously by RIA in which two different anti-CEA antisera were used. Fig. 1A shows that RIA inhibition curves were similar with City of Hope, Montreal, and British CEA standards when Roche anti-CEA was used, whereas the inhibition curve of the CEA-Roche standard was clearly different. When three of the CEA standards were tested by the identical RIA method but with Montreal instead of Roche anti-CEA (see Fig. 2A), the British, Montreal, and City of Hope CEA standards resulted in inhibition curves almost identical to those obtained with Roche anti-CEA, but the Roche CEA standard was always different, both quantitatively and qualitatively, from the other CEA standards. The amount of CEA producing 50% inhibition was one to two orders of magnitude smaller with CEA-Roche than that measured with the other three standards (compare Figs. 1A, 2A, and 3A).
Comparison of CEA in serum (R. K.) with CEA standards
Serum R. K. contained approximately 25,000 ng/ml of CEA when assayed by the standard Roche-CEA assay kit as directed (kindly performed by Drs. H. Z. Kupchik and N. Zamcheck, Boston City Hospital). The same serum contained 26,200 ng/ml of CEA when assayed by the Farr method when reagents from the commercial Roche CEA-assay kit were used. The assay results were identical (SEM < 5%) when any serum aliquot used was within the linear range of the inhibition curve of CEA-Roche standard assayed simultaneously. However, when this serum was analyzed under identical conditions, except for replacing the Roche anti-CEA antiserum with absorbed Montreal anti-CEA antiserum, markedly different results were obtained with this serum although similar results were obtained with CEA standards.

The inhibition curve in Fig. 1B (Roche anti-CEA) shows that the inhibition increases with increasing amounts of serum until a maximum of 90% inhibition is reached. As little as 0.5 µl of this serum resulted in maximal inhibition.

Fig. 2. Inhibition curves of CEA standards (A), cancer serum R. K. (B), and normal serum (C) in RIA with absorbed Montreal anti-CEA antiserum.

Fig. 3. Inhibition curves of CEA standards (A) and a dialyzed and lyophilized perchloric acid extract of a cancer serum R. K. (B) with Montreal anti-CEA antiserum. Serum R. K. was extracted with dilute perchloric acid and dialyzed, lyophilized, and reconstituted to its original volume with 0.9% (wt/vol) NaCl.

Fig. 4. Inhibition curves of CEA standards (A) and the extract (B) of the liver metastases from the same patient (R. K.) when Montreal anti-CEA antiserum was used. Tissue (67.7 g) was homogenized twice in 4 volumes of phosphate-buffered saline, and supernatants were centrifuged at 48,000 X g for 30 min.
Furthermore, the addition of 3.125 ng of CEA-Roche was easily measurable in the presence of 0.05 μl but was not measurable in the presence of 0.5 μl of the serum, as would be expected if CEA-Roche and the serum CEA were immunologically identical (Fig. 1B).

The use of Montreal anti-CEA antisera under identical conditions resulted in inhibition curves for the CEA-standards (Fig. 2A) which were virtually identical to those obtained with Roche anti-CEA antisera. However, increasing amounts of serum R. K. resulted in an inhibition curve with a distinctly different slope from all the standards, and the maximum inhibition reached only 40% (Fig. 2B). Furthermore, the addition of 6.25 ng of CEA-Roche resulted in significant additional inhibition in the presence of from 0.4 to 40.0 μl of serum R. K., which contained between 10.5 and 1,050 ng of CEA when assayed with the Roche CEA kit. These results indicate that this antisera reacted with antigenic determinants on the serum CEA which are distinctly different from the CEA-Roche standard.

The addition of increasing amounts of normal serum (up to 50 μl) did not significantly inhibit in the radioimmunoassay (Fig. 2C). Furthermore, the identical inhibition caused by 6.25 ng of CEA-Roche, when added to increasing aliquots of normal serum (Fig. 2C), demonstrates a lack of inhibition by normal serum in these aliquots when the "direct" CEA radioimmunoassay was used.

**Effect of perchloric acid extraction upon serum CEA**

Serum R. K. was extracted with dilute perchloric acid (2). The resultant supernatant was dialyzed against large volumes of distilled water and lyophilized, and the residue reconstituted to the original serum volume with 0.9% NaCl. Increasing aliquots of this perchloric acid extract resulted in increasing inhibition to a maximum greater than that seen with unextracted serum under the same conditions with the same reagents (Fig. 3A). However, the addition of 6.25 ng of CEA-Roche still produced significant additional inhibition in the presence of from 0.1 to 40 μl of this cancer serum extract (Fig. 3B). Thus, the antigenic difference between serum CEA and the CEA-Roche noted in Fig. 2B was not abolished or significantly altered by perchloric acid extraction. This observation is of importance because all presently available CEA standards are prepared from perchloric acid extracts of tumors.

**Differences between serum and liver CEA**

CEA standards and antigen preparations used for raising anti-CEA antisera are usually prepared from liver metastases (1–5). Therefore, we compared the CEA in the serum of this patient to the CEA extracted from his own liver metastases. Increasing amounts of the liver metastases extract resulted in increasing inhibition until a maximum of 65% was reached when Montreal antisera was used (see Fig. 4B). The slope and maximum inhibition caused by the liver extract was distinctly different from either Roche or Montreal standards shown in Fig. 4A. Furthermore, the addition of 6.25 ng of CEA-Roche resulted in no additional detectable inhibition when it was added to 10 μl or more of the tissue extract. The convergence of the inhibition curves when CEA was added to the tissue extract indicates that the tissue CEA may share some antigenic determinants with the CEA-Roche standard that it does not share with the antigenically different CEA circulating in the patient's serum (see Figs. 2B and 3B).

The results of gel filtration of the various CEA preparations are shown in Fig. 5. CEA in the tumor extract, whole serum, and perchloric acid extracted serum all chromatographed with iodinated CEA-Roche. In additional experiments we noted that CEA-Montreal also chromatographed with 125I-labeled CEA-Roche.

**DISCUSSION**

Our data demonstrate that CEA-Roche, which is currently used extensively in clinical laboratories for CEA assays, was different from the other three CEA standards tested in this study (CEA-Montreal, CEA-City of Hope, and CEA-British). Under the conditions of the radioimmunoassay used, the latter three standards resulted in very similar inhibition curves, but all three were clearly different from CEA-Roche when either Montreal or Roche anti-CEA antisera was used.

Furthermore, a serum CEA was shown to be immunologically different from all four CEA standards; this has important implications for CEA assays. The difference between the inhibition curves of the standards and the early plateau of maximum inhibition seen in a serum CEA, when Montreal anti-CEA was used, resulted in a calculated CEA concentration which varied with the amount assayed. For example, 1 μl of serum R. K. resulted in 32.9% inhibition as measured.
by RIA (Fig. 2B). Based on simultaneously prepared standard curves (Fig. 2A), the serum was calculated to contain 8 ng of CEA/μl when CEA-Roche was used as the standard, and 130 or 168 ng of CEA/μl, respectively, when Montreal or British CEA standards was used. In addition, the slope of the inhibition curve of the serum CEA assayed directly (i.e., without prior perchloric acid extraction) differed from that of the standards and was nonlinear. When the amount of the serum analyzed was increased, the final calculated concentration (expressed as ng of CEA/ml of serum) decreased. If a perchloric acid extract from 5 ml of this serum were to be used in this RIA, as originally suggested by Thomson et al. (2), the calculated CEA concentration would yield a value of less than 50 ng/ml, in contrast to the value of more than 25,000 ng/ml obtained with the Roche assay kit. Therefore, the calculated serum CEA concentration can vary by three orders of magnitude depending on the CEA standard used, the amount of serum actually tested, and the particular anti-CEA antiserum used in RIA.

CEA can be separated into multiple molecular forms, by ion exchange chromatography and isoelectric focusing (12–14). These forms with different net charges contained a similar amino-acid composition and apparently differed only in carbohydrate content. However, the different CEA forms were said to be antigenically identical by Ouchterlony diffusion (12). Our results with radioimmunoassay suggest that CEA must contain a number of different antigenic determinants.

Normal crossreacting glycoproteins such as NCA (normal colon antigen) described by von Kleist and others (15), cannot explain our results. The serum CEA was similar in molecular weight to the CEA standards. Furthermore, the Montreal antiserum was heavily absorbed with perchloric acid extracts of normal lung, liver, colon, and plasma, as described by Thomson et al. (2).

To explain the results of our experiment, we must assume that CEA is a family of “isoantigens” which have multiple antigenic determinants. Some of these antigenic determinants may be common to all the isoantigens, whereas others may be antigenically distinct by RIA. Furthermore, different anti-CEA antiseras may consist of antibody species that recognize different CEA determinants on the different CEA isoantigens. Thus, the results of radioimmunoassay may only reflect the binding affinity of a polyvalent anti-CEA antibodies to a mixture of different CEA isoantigens.

Attempts to determine the chemical nature of the immunodominant antigenic determinants of CEA have so far been inconclusive (16–21). Blood group A and nonspecific crossreacting antigens such as NCA may share one or more antigenic determinants with CEA, but are chemically distinguishable from CEA (15). Our experiments suggest that there are a number of CEA isoantigens not recognizable by some presently available anti-CEA antiseras which are presumed to be nonspecific. The concept of multiple CEA isoantigens may explain why the sera of many patients with nonmalignant conditions (e.g., chronic inflammatory bowel disease without cancer) contain immunoreactive substances which mimic CEA by radioimmunoassay (22).

The recent isolation of CEA-S, a fraction separated from a purified CEA preparation, but antigenically distinguishable from CEA (23, 24), is also consistent with the assumption that CEA is a family of isoantigens. Production of antiseras with restricted specificity to the different CEA isoantigens appears to offer a new approach for the detection of a more tumor-specific form of CEA and a more reliable serologic diagnosis of malignancy.

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