The Isolation of Carcinoembryonic Antigen from Tumor Tissue at Neutral pH

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SUMMARY

Carcinoembryonic antigen (CEA) was purified from tumor tissue under mild conditions at neutral pH by a procedure that utilized affinity chromatography on concanavalin A. Further purification by gel filtration provided CEA in 10 to 20% yield and 10% purity. Antibody to this preparation was rendered specific for CEA by adsorption on a column of normal liver proteins bound to Sepharose. On reaction by immunodiffusion against a crude tumor extract, the adsorbed antibody produced two precipitin lines, of which one was relatively weak. These two precipitin lines fused completely with the two respective lines produced by antibody to perchloric acid-treated CEA. The major antigen found in crude tumor extracts and in CEA preparations purified at neutral pH was nearly undetectable in perchloric acid extracts of tumor homogenates. Further investigations showed that 60 to 70% of the CEA in crude tumor extracts and in preparations isolated at neutral pH is destroyed and/or becomes insoluble under acidic conditions.

INTRODUCTION

CEA is a tumor-associated antigen first identified in adenocarcinoma of the colon by Gold and Freedman (11). Later, it was also found in adenocarcinomas of the pancreas and liver (12). As more sensitive immunological techniques were developed, relatively low levels were detected in normal tissues (2, 21, 22).

Originally, CEA was isolated from tumor homogenates by extraction with M perchloric acid which precipitates most of the tissue proteins while a few, including CEA, remain in solution (17). The soluble proteins were fractionated further by gel filtration and preparative electrophoresis. All purification procedures developed later used perchloric acid extraction (7, 16, 18). CEA is generally considered to be stable in this acid because it is soluble and its immunological properties are not destroyed (17).

CEA appears to be a mixture of structurally related glycoproteins (6). Isoelectric focusing separated a CEA preparation into several components with the major ones being isoelectric between pH 1 and 3 (6). These components had different amino acid and sugar compositions.

Antiserum raised to CEA preparations usually contains antibody to antigens found in extracts of noncancerous tissues. The antiserum is typically rendered monospecific for CEA by adsorption with proteins from normal tissues (11, 12, 15, 25). Some adsorbed antisera gave 2 precipitin lines by immunodiffusion with perchloric acid extracts of tumor tissue (11, 15, 22). Further fractionation of the CEA preparations by gel filtration and electrophoresis failed to separate the 2 antigens (15).

Strong acid can hydrolyze glycosidic bonds in the carbohydrate moiety of CEA and can denature the polypeptide portion. Purification of CEA without acid conditions could yield higher quality antigen and, consequently, better antiserum. Binding of CEA to Con A has been reported (4, 5).

We have found that affinity chromatography on immobilized Con A is a useful step for isolation of CEA. Some properties of CEA isolated at neutral pH are compared with the antigen prepared by extraction with perchloric acid.

MATERIALS AND METHODS

Purification of CEA. Liver metastases of carcinoma of the colon were dissected from normal tissue, and 150 g were homogenized with 450 ml distilled water for 10 min at 0° in a Sorvall Omnimixer (Ivan Sorvall Co., Newton, Conn.). The homogenate was treated sonically for 10 min at 0° with a Branson Model W140D sonic oscillator (Branson Sonic Power Co., Plainview, N. Y.) operated at 70% of full power. Cell fragments were separated by centrifugation at 9000 × g for 20 min. The supernatant was dialyzed against Tris-acetate buffer, pH 6.9, ι/2 0.02, for 40 hr at 5°. CEA was measured by radioimmunoassay, were pooled and passed through Tris-acetate buffer, pH 6.9, ι/2 0.02, and 2 liters of this buffer containing 0.5 M NaCl. Fractions (22 ml) were collected, and those containing more than 2 µg CEA per ml, measured by radioimmunoassay, were pooled and passed at a flow rate of 50 ml/hr through a 2.5- × 29-cm column of DEAE-cellulose previously equilibrated with the Tris-acetate buffer. The chromatogram was developed at room temperature with a linear salt gradient generated with 2 liters, Tris-acetate buffer, pH 6.9, ι/2 0.02, and 2 liters of this buffer containing 0.5 M NaCl. Fractions (22 ml) were collected, and those containing more than 2 µg CEA per ml, measured by radioimmunoassay, were pooled and passed at a flow rate of 50 ml/hr through a 2.5- × 29-cm column of Con A bound to Sepharose (Pharmacia Fine Chemicals, Piscataway, N. J.); 8 mg Con A bound per ml Sepharose. Then the column was washed with 0.2 M sodium citrate buffer, pH 6.5, containing 0.5 M NaCl, 50 mM MgCl₂, 50 mM MnCl₂, and 50 mM CaCl₂. When the absorbance at 280 nm of the effluent decreased to 0.1, 120 ml of 0.1 M α-methyl-d-mannoside in the citrate buffer was passed into the column. Elution with sugar was repeated 20 and 44 hr later. The

1 The abbreviations used are: CEA, carcinoembryonic antigen; Con A, concanavalin A.

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sugar eluates were combined and concentrated to 5 ml by pressure dialysis.

The Con A column was regenerated by washing with 2 bed volumes of the sodium citrate buffer each day for 7 to 10 days. With this treatment, 1 column was used for 12 CEA preparations without noticeable change in binding efficiency.

The concentrated sugar eluate was chromatographed at 7° on a 2.5- x 90-cm column of Sepharose 4B equilibrated with sodium acetate-acetic acid buffer, pH 6.5, I'2 0.05, containing 0.15 M NaCl. Fractions (15 ml) with more than 5 μg CEA per ml were pooled. The protein was concentrated to 5 ml by pressure dialysis and fractionated further by gel filtration on 2.5- x 90-cm column of Sephadex G-200 equilibrated with the sodium acetate-acetic acid buffer. Again, 15-ml fractions were collected, and assays for CEA revealed 2 components, as described in “Results.” The main peak and shoulder were collected into separate pools. They were concentrated to 2 to 5 ml each and stored at −15° until needed.

Isolation of CEA by Extraction with Perchloric Acid. Tumor tissue homogenates were extracted with perchloric acid by the method of Krupay et al. (18). After dialysis against distilled water, the acid-soluble materials were dialyzed into Tris-acetate buffer, pH 6.9, I'2 0.02. Then the protein was concentrated by pressure dialysis and used for immunodiffusion measurements.

Crude Extracts of Normal and Tumor Tissues. Normal liver and colon were autopsy specimens without known neoplastic disease. The tissues were homogenized as described above for tumor tissue. Particulate material was separated by centrifugation and the supernatants were dialyzed against distilled water. The dialysate was concentrated by pressure dialysis.

Crude extracts of tumor tissue were prepared by the same procedure for use in immunodiffusion studies.

Binding of Protein to Sepharose. The method of Axen and Ernback (1) was used to bind proteins to cyanogen bromide-activated Sepharose. Sepharose-4B, 20 ml packed, was washed with 250 ml distilled water on a scintered glass funnel and transferred to a beaker with 40 ml water. The pH was adjusted to 11 with 5 N NaOH, and 7 g cyanogen bromide dissolved in 30 ml water were added. The pH was maintained near 11 by the addition of NaOH, and the temperature was kept between 20° and 25° by the addition of ice. When the reaction had proceeded for 12 min, the activated Sepharose was washed with 250 ml cold (0°) 0.2 M sodium citrate buffer, pH 6.5, on a scintered glass funnel and was transferred into a flask with 25 ml cold sodium citrate. The crude extract from 10 g normal liver, prepared as described above, was added and the suspension was stirred at 7° overnight. Then the protein-Sepharose conjugate was washed with 250 ml sodium citrate buffer, and unreacted sites were blocked by stirring for 2 hr with 40 ml 1 M ethanolamine in the sodium citrate buffer adjusted to final pH 8.0. The conjugated Sepharose was washed with 250 ml phosphate-buffered saline (0.04 sodium phosphate, pH 7.4, containing 0.12 M NaCl), and poured into a column for use as an immunoadsorbent.

Preparation of Antiserum. For the initial immunization, 20 to 40 μg of purified CEA in complete Freund's adjuvant were injected s.c. into goats, near the footpads. Booster injections in incomplete Freund's adjuvant were administered s.c. in the back at 3-week intervals. Three months after the initial injection, the antiserum had developed sufficiently high titers for use in immunodiffusion measurements. Antiserum to CEA purified by perchloric acid extraction was kindly provided by Dr. C. W. Todd (7).

Adsorption of Antiserum. Antiserum to CEA purified at neutral pH was rendered monospecific for CEA by adsorption on a column of normal liver proteins bound to Sepharose. The immunoadsorbent described above was equilibrated with phosphate-buffered saline, pH 7.4, at room temperature, and 2 ml antiserum were washed through at a flow rate such that unbound proteins eluted in 10 to 15 hr. The absorbance at 280 nm of the effluent was monitored, and serum proteins that eluted were concentrated to 2 ml by pressure dialysis.

Immunodiffusion Measurements. A suspension of 1% Nobel agar (Difco Laboratories, Detroit, Mich.) in 1 M glycine, 0.8 mM sodium azide, and 0.15 M NaCl was adjusted to pH 7.0 to 7.5 with NaOH and heated at 95° for 5 min. This suspension was poured into 1.5-mm thick layers on glass plates. Immunodiffusion was conducted for 24 to 48 hr in a humid chamber at room temperature.

Immunoelectrophoresis was conducted in 1% agarose by the method of Scheidegger (24).

Radioimmunoassay. CEA and antibody to CEA for the radioimmunoassay were provided by Dr. C. W. Todd (7). The CEA was radiolabeled with 125I by the method of Egan et al. (10). Assays were conducted in phosphate-buffered saline, pH 7.4, containing 1% bovine serum albumin. Antibody and antigens were incubated at 5° in a 0.5-ml reaction overnight, and then rabbit anti-goat IgG was added. This 2nd antibody was allowed to react at room temperature for 2 hr. The antigen-antibody precipitate was collected by centrifugation and washed twice with 1 ml phosphate-buffered saline containing 1% bovine serum albumin. The washed precipitate was dissolved in 1 ml 0.1 N NaOH for counting 125I decay.

Protein Assay. The method of Lowry et al. (20) was used to measure total protein during purification of CEA. Bovine serum albumin was used as the standard.

Electrophoresis. Isoelectric focusing was conducted in a 110-ml column (LKB Products, Stockholm, Sweden) with 1% (w/v) ampholytes. The protein was included in the dense solution used to develop the sucrose gradient. The column was maintained at 10° and a potential of 500 V was applied for 60 hr. Then the absorbance at 280 nm of the column effluent was monitored and 2-ml fractions were collected. The pH of the fractions was measured and they were assayed for CEA by the radioimmunoassay method.

Disc electrophoresis was conducted in 7% polyacrylamide gels at pH 8.9 by the method described by Davis (8).

RESULTS

Extraction of Tumor Tissue. CEA is associated with the tumor cell surface (13) and is partially released by homoge-
nization in distilled water (Table 1). To determine the most effective conditions for extraction, aliquots of homogenate were treated by the methods listed in Table 1. Particulate matter was removed by centrifugation, and the supernatants were dialyzed against Tris-acetate buffer, pH 6.9, Γ/2 0.02, for 40 hr and then assayed for CEA. The results shown in Table 1 indicate that the highest yield of CEA was obtained by sonic disruption, and the lowest by perchloric acid extraction. The detergents, sodium dodecyl sulfate and Tween 20, gave essentially the same yield as water alone.

**DEAE-Cellulose Chromatography.** Chromatography of dialyzed tumor extracts on DEAE-cellulose removed protein and lipid material that interfered with the subsequent affinity chromatography on Con A. Some heterogeneity of the CEA is evident from the elution profile in Chart 1. Since the components were poorly resolved, fractions containing more than 2 μg CEA/ml were collected into 1 pool. The recoveries of CEA varied from 25 to 50% (Table 2).

**Affinity Chromatography on Con A.** The immobilized Con A bound over 90% of the CEA pooled from the DEAE-cellulose chromatogram, while a large portion of the protein impurities passed directly through the column (Chart 2). Most of the protein bound to Con A was eluted immediately with α-methyl-d-mannoside; however, after the column stood overnight, additional protein was recovered. Affinity chromatography increased the purity of the CEA 8-fold (Table 2).

**Gel Filtration.** The CEA eluted from Con A was separated from higher-molecular-weight contaminants by chromatography on Sepharose-4B (Chart 3A). The antigen eluted as an asymmetrical peak between 75 and 100% of the column bed volume. Further gel filtration on Sephadex G-200 separated CEA from some of the lower-molecular-weight proteins (Chart 3B). About 70% of the antigen eluted as a symmetrical peak between 35 and 50% of the column bed volume. The remaining 30% appeared as a shoulder on the main peak (Chart 3B). Approximately 10% of the protein in the main peak was CEA, and this represented 10 to 20% of the antigen originally extracted from the tumor tissue (Table 2).

**Isoelectric Focusing.** Further purification of CEA was attempted by isoelectric focusing with pH 3 to 6 ampholytes. The protein remained in solution during the run, and CEA levels measured in the column effluent revealed an elution profile very similar to that reported by Coligan et al. (6). Much different results were obtained when isoelectric focusing was conducted with pH 2.5 to 4.0 ampholytes. Between 30 and 40% of the CEA was recovered in solution in the column effluent. Most of this was in fractions with pH above 5.5. Fractions with pH 3.5 to 4.5 contained precipitated protein which was separated by centrifugation and extracted with 0.25 M Tris-HCl buffer, pH 8.6. CEA recovered in the extract represented 10% of the original antigen. By mass balance, approximately 50% of the original CEA precipitated during isoelectric focusing and would not dissolve at pH 8.6.

**Solubility of CEA under Acidic Conditions.** The solubility of CEA at acid pH was examined further by dialyzing a purified preparation containing 85 μg CEA per ml against sodium acetate-acetic acid buffer, pH 4.3, Γ/2 0.001, for 23 hr.

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**Table 1**

<table>
<thead>
<tr>
<th>Extraction method*</th>
<th>Yield (μg CEA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>14</td>
</tr>
<tr>
<td>1% NaCl</td>
<td>10</td>
</tr>
<tr>
<td>1% Tween 20</td>
<td>17</td>
</tr>
<tr>
<td>1% Sodium dodecyl sulfate</td>
<td>13</td>
</tr>
<tr>
<td>1-Butanol*</td>
<td>11</td>
</tr>
<tr>
<td>1% HClO₄</td>
<td>6.3</td>
</tr>
<tr>
<td>Sonic disruption*</td>
<td>23</td>
</tr>
</tbody>
</table>

* Aliquots of a tumor homogenate (2 ml) in distilled water were mixed with 2 ml of the appropriate solutions to give the designated final composition. These mixtures were stirred at room temperature for 30 min and then processed as described in "Results."

* The homogenate was stirred with an equal volume of 1-butanol for 30 min. The aqueous phase was then dialyzed and CEA analyzed.

* Sonic disruption was conducted as described in "Materials and Methods."

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**Table 2**

<table>
<thead>
<tr>
<th>Total CEA (mg)</th>
<th>Total protein (mg)</th>
<th>Recovery of CEA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue extract</td>
<td>10.3</td>
<td>5900</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>2.9</td>
<td>1600</td>
</tr>
<tr>
<td>Affinity chromatography on Con A</td>
<td>2.6</td>
<td>170</td>
</tr>
<tr>
<td>Sephadex chromatography</td>
<td>2.1</td>
<td>108</td>
</tr>
<tr>
<td>Peak 1</td>
<td>1.3</td>
<td>11.5</td>
</tr>
<tr>
<td>Peak 2</td>
<td>0.6</td>
<td>19</td>
</tr>
</tbody>
</table>

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Chart 1. DEAE-cellulose chromatography of a tumor tissue extract. The proteins extracted from 150 g of tumor tissue were dialyzed against Tris acetate buffer, pH 6.9, Γ/2 0.02, and passed into a 5- x 35-cm column of DEAE-cellulose previously equilibrated with the Tris-acetate buffer. The chromatogram was developed with a linear salt gradient as described in "Materials and Methods." The absorbance at 280 (solid line) of the column effluent was monitored and 22-ml fractions were collected; broken line, CEA levels measured by radioimmunoassay.
Precipitate that formed was separated by centrifugation, and the supernatant contained 30 μg CEA per ml which was 35% of the original material. Only 3% of the original antigen was extracted from the precipitate with 0.1 M Tris buffer, pH 8.0. Therefore, precipitation of CEA in isoelectric-focusing experiments appears to be an effect of pH and is not dependent on the ampholytes or sucrose.

**Immunological Studies.** Immunodiffusion patterns obtained with unadsorbed antiserum to purified CEA showed antibody activity to several proteins in extracts of normal liver and colon (Fig. 1). Adsorption of the antiserum on a column of normal liver proteins bound to Sepharose removed the antibodies to normal tissue antigens (Fig. 1). This demonstrates that a complex mixture of tissue proteins can be immobilized and used effectively as an immunoadsorbent.

The adsorbed antibody reacted with tumor extracts to produce the strong precipitin line seen in Fig. 1 and also a weak line which is evident in Fig. 2. Reactions of identity were obtained when the adsorbed antibody was compared with the antibody to perchloric acid-treated CEA (Fig. 2). Although this conclusion is not demonstrated clearly in Fig. 2 for the weak precipitin lines, they appeared to fuse completely when the immunodiffusion plate was viewed directly.

**Further Characterization of the Purified CEA.** On examination by immunoelectrophoresis, the purified CEA developed at least 3 precipitin lines with the unadsorbed antibody. With the adsorbed antibody, 1 precipitin line developed in the β- and γ-globulin regions.

Disc electrophoresis was conducted in 7% acrylamide gels. After staining with Coomassie blue, 2 major bands were located near the origin of the separating gel, and these were also seen in gels treated with a periodic acid-Schiff stain for oxidizable sugars (26). Several minor bands that migrated ahead of the major ones were also visible with the Coomassie blue stain. An unstained gel was cut in half along the longitudinal axis and placed on an agar plate parallel to a strip of filter paper saturated with the adsorbed antibody to CEA. The precipitin line that developed in the agar indicated that during electrophoresis the antigen had migrated to the region of the stained bands.

The immunological and electrophoretic measurements indicated that our purified preparations contain several components in addition to CEA. The major components appear to be glycoproteins because they were stained by both Coomassie blue and the periodic acid-Schiff reagent. The preparations have an optical absorption maximum at
278 nm which is further evidence for the presence of protein. Detailed chemical characterization of the CEA isolated by the procedure presented here must await further purification.

**DISCUSSION**

CEA is usually purified from tissue homogenates by procedures that use perchloric acid extraction (7, 16). Although this glycoprotein is considered to be stable in acid, we have found that 70% of the CEA in tumor homogenates is lost in M perchloric acid (Table 1). Loss of the antigen from solution was also a problem under relatively mild conditions at neutral pH. For example, only 25 to 50% of the CEA in tumor extracts was recovered during chromatography on DEAE-cellulose at pH 6.9 (Table 2). Furthermore, in preliminary investigations we found that some purification methods, such as ammonium sulfate fractionation, gave very low recoveries.

Chu et al. (4, 5) have reported that 60% of the 125I-CEA in their preparation was bound by Con A and the binding was reversed by mannose. We used affinity chromatography to purify CEA from crude tumor extracts and found that 90% of the CEA was bound by immobilized Con A (Chart 2, Table 2). Further fractionation of the glycoproteins by gel filtration provided CEA that was about 10% pure. This procedure was used successfully to isolate several preparations of CEA from 3 specimens of liver metastases.

Isoelectric focusing has been used to purify CEA and separate different chemical species (6, 9, 23). When we attempted to purify our preparation by this method with pH 3 to 6 ampholytes, a major component of the CEA was found in fractions with pH 1 to 3. Similar elution profiles were reported by Coligan et al. (6) and are not very satisfactory because the pH gradient was very steep in the region of interest. When we used pH 2.5 to 4.0 ampholytes in order to cover the low pH range more effectively, precipitate formed in the region with pH 3.5 to 4.5, and the soluble CEA, representing 30 to 40% of that applied to the column, was mostly located above pH 5.5. Apparently, the CEA precipitated in the acidic region, floated up the sucrose gradient, and dissolved in the strongly alkaline region. Possibly, previous workers did not observe precipitation of CEA during isoelectric focusing because their preparations, which had been exposed to perchloric acid, contained only acid-soluble material.

Antiserum raised with the purified CEA was rendered monospecific for CEA by adsorption with normal liver proteins bound to Sepharose (Fig. 1). Adsorption was conducted with immobilized rather than soluble proteins because the latter method produces soluble antigen-antibody complexes that remain in the antibody preparation (3). These complexes can interfere with subsequent immunological measurements, especially in radioimmunoassays where the complexed antigens can exchange with radiolabeled antigens (3). In some cases, such exchange could explain observations of cross-reacting antigens when extracts of normal tissues were examined (19, 23).

Both the adsorbed antibody to CEA purified at neutral pH and the antibody to perchloric acid-treated CEA produced 2 precipitin lines with crude extracts of tumor tissue (Fig. 2). Occurrence of 2 immunochemically distinct antigens has been reported earlier (15, 22, 25). The relative levels of the 2 antigens in the CEA purified at neutral pH were very similar to those in the crude extract (Fig. 2). The
major antigen, however, was nearly undetectable in the perchloric acid extract. Evidently, this antigen is nearly insoluble, possibly as a result of denaturation, in M perchloric acid. The low solubility accounts, at least in part, for the low recovery of CEA during acid extraction of tumor homogenates (Table 1).

Precipitin lines produced by antibody to CEA isolated at neutral pH fused completely with those produced by antibody to CEA purified by perchloric acid extraction (Fig. 2). The antigen observed in the perchloric acid extract appeared to be identical to the corresponding antigen isolated at neutral pH (Fig. 2). Results of previous studies have provided similar evidence that the immunological properties of CEA are not altered by perchloric acid (2, 12), but a detailed comparison of both antibodies and antigens has not been reported.

In their initial studies, Gold and Freedman (11) identified CEA on the basis of its immunological properties. Later, the antigen was purified by extraction with perchloric acid and its solubility in this medium was used as a further criterion for identification (16-18). Our experimental results indicate that the immunological criterion is more satisfactory than a detailed comparison of both antibodies and antigens for identification (16-18). Our experimental results indicate that the immunological criterion is more satisfactory because tumor extracts contain material(s) which is lost during exposure to perchloric acid but which reacts with antibody to CEA purified by extraction with this acid. Several properties of the CEA isolated at neutral pH are similar to those reported for the antigen isolated by acid extraction. For instance, CEA isolated at neutral pH appears to contain carbohydrate since it binds specifically to Con A (Chart 2). The antigen elutes from gel filtration columns at approximately the same position as reported previously for preparations purified by extraction with perchloric acid (Chart 3; Refs. 7 and 18). Finally, the mobility on immunoelectrophoresis was similar to that reported for CEA by other investigators (7, 16, 18).

The clinical utility of CEA for detection of neoplastic disease depends on accurate quantitative measurement of the antigen in blood. Some assays are conducted with perchloric acid extracts while others are carried out without special treatment of the serum or plasma (10, 14, 25). The low solubility of CEA in acid would be expected to decrease the levels measured following acid extraction. Furthermore, perchloric acid appears to extract some species of CEA more effectively than others. These two points must be taken into consideration in comparing results of clinical measurements carried out by different methods.

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