Isolation and Immunochemical and Chemical Characterization of Carcinoembryonic Antigen-like Substances in Colon Lavages of Healthy Individuals

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SUMMARY

Material with carcinoembryonic antigen (CEA)-like activity, isolated from colon lavages of healthy individuals, has been chemically characterized. The CEA-like activity in these lavages was purified by gel filtration on Sepharose 6B and Sephadex G-200 and by affinity chromatography on concanavalin A linked to Sepharose. The purified material migrated in polyacrylamide-sodium dodecyl sulfate electrophoresis as a single diffuse band with mobility identical with that of tumor CEA. The material with CEA activity from tumor tissue and from normal colon lavages gave lines of identity in double diffusion experiments and had similar inhibition curves with essentially the same specific activities in a radioimmune assay for tumor CEA. The amino acid and carbohydrate compositions were similar to that observed in CEA isolated from tumor tissue. The authors therefore concluded that CEA was not present in nonmalignant tissue.

INTRODUCTION

CEA\(^4\) was demonstrated by Gold and Freedman (16, 17) in saline extracts of digestive tract tumors and embryonic gut tissue. Antibody against the tumor extract, after absorption with normal tissue extract, retained its ability to precipitate tumor extracts and fetal digestive tract extracts but no longer precipitated extracts of normal tissue. The authors therefore concluded that CEA was not present in nonmalignant adult tissue.

Martin and Martin (26) subsequently found that it was possible to inhibit the precipitation of CEA by anti-CEA with high concentrations of extracts of normal tissues. Their findings raised the question of whether the requirement for large amounts of normal material in order to obtain inhibition was caused by a concentration of CEA present in normal tissue several orders of magnitude lower than that in tumor tissue or whether there was a substance in normal tissue that was weakly cross-reactive with CEA. The initial report by Martin and Martin was followed by other reports that demonstrated that extracts of normal tissue inhibited in radioimmune assays (3, 20, 23, 27, 28) and complement fixation assays (36) for CEA from tumor tissue.

However, in none of these laboratories was sufficient purified material available to analyze structurally the substance from normal tissue. Some reports included the fact that the inhibition curves obtained from radioimmune assays of tumor and normal tissue extracts were parallel (4), although this evidence does not demonstrate chemical identity (12).

NCA [also called normal glycoprotein (25), CCEA2 (42), and CEX (7)] is a substance isolated from normal tissue that cross-reacts with CEA from tumor tissue but has a much lower molecular weight (43). NCA probably is also identical with CCAII (30) and with the \(\beta_2\) antigen of Orjasaeter (31, 32). Immunodiffusion experiments have indicated that CEA and NCA have both unique and shared antigenic determinants. The demonstration of NCA in normal tissue, which could be distinguished from tumor CEA by its size, appeared for a time to resolve the controversy. However, some groups subsequently reported the presence of material with CEA-like activity in normal serum and normal tissue extracts that was similar to CEA in elution from gel filtration columns (4, 21, 35). Unfortunately, the concentration of this substance in normal tissue was so low that sufficient material could not be obtained for chemical characterization.

In 1974 Go et al. (15) measured the secretion in various portions of the gastrointestinal tract of material that inhibited in the assay for CEA. The colon secreted the largest quantity of the material. The inhibitory material eluted from a Sephadex G-200 column in the same position as CEA from tumor tissue and well ahead of the usual elution position of NCA. The presence of large quantities of material with CEA-like activity in the colon lavages of healthy individuals made it possible to isolate sufficient material to be characterized chemically and directly compared with CEA. The isolation and characterization of this material is the subject of this report.

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4 The abbreviations used are: CEA, carcinoembryonic antigen; NCA, normal cross-reacting antigen; Con A, concanavalin A; SDS, sodium dodecyl sulfate.

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**MATERIALS AND METHODS**

**CEA and Anti-CEA.** CEA was obtained from liver metastases of colon adenocarcinomas by treatment of a water homogenate of liver tissue with an equal volume of 2 M perchloric acid to precipitate the proteins, followed by chromatography of the dialyzed supernatant on Sepharose 4B and Sephadex G-200 (5). A final purification on Con A-linked Sepharose was performed (33). The anti-CEA serum used was obtained from a goat (Ace) producing antibodies 4 years after an initial series of injections of 10 μg of CEA for 3 months, followed by booster shots of 1 to 5 μg at 4, 11, 16, 19, and 22 months (11). The bleedings used in the study were taken on the 3rd (Ace 5) and 43rd (Ace 67 to 70) months after initial immunization. These antisera had the same cancer specificity when tested with clinical samples as did other antisera available for clinical testing (W. L. Go, unpublished observations; Ref. 24).

**Radioimmune Assay for CEA.** CEA was measured by the triple-isotope, double-antibody radioimmune assay for CEA (6, 13). $^{57}$Co was used in place of $^{38}$Na as a volume marker (14).

**Lavage of the Colon of Healthy Individuals.** The colonic saline lavage was obtained from 14 healthy subjects (age 30 to 56 years, 1 female and 13 males) whose sera CEA assay values were less than 2.5 ng/ml. Appropriate informed consent was obtained for all volunteers prior to study. Colonic saline lavage was collected either by saline enema in 6 subjects or by colonic perfusion technique (15) in 8 subjects. After the colon had been cleansed with 0.9% NaCl solution, an additional 1000 to 1200 ml were obtained from each individual for the study. Care was taken to keep the samples cold at all times. Individual samples were centrifuged for 15 min in a Sorvall GLC-2B centrifuge to remove particulate matter, filtered through a Millipore cellulose pad, and concentrated in an Amicon DC-2 concentrator-dialyzer to 200 ml.

**Isolation of Material with CEA-like Antigenic Determinants from Colon Lavages.** The centrifuged and concentrated lavage was treated at 4°C with an equal volume of 2 M perchloric acid, centrifuged to remove the precipitated proteins, dialyzed against running deionized water, further dialyzed and concentrated using an Amicon PM 10 membrane (Amicon, Lexington, Mass.), and lyophilized. The lyophilized samples from the 14 individuals were pooled. A total of 2.4 g of perchloric acid extract was obtained. The pool was chromatographed on a Sepharose 6B column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) that had been equilibrated with 0.05 M phosphate buffer, pH 5.5, in 0.1 M NaCl. Fractions from the Sepharose 6B column containing the CEA activity as measured by radioimmune assay were pooled, dialyzed, lyophilized, and applied to Sephadex G-200 columns equilibrated with the same phosphate buffer as above. Fractions containing CEA antigenic activity were pooled, lyophilized, and chromatographed on Con A-Sepharose (Pharmacia).

**Chromatography on Con A-Sepharose.** The sample was dissolved in Con A buffer (1 M NaCl/0.1 M acetate, pH 5.5; and 10 mM concentrations each of MgCl₂, MnCl₂, and CaCl₂) and applied to a column of Con A-Sepharose equilibrated at 4°C with the same buffer. After elution of the nonbound fraction with Con A buffer, the column temperature was raised to 37°C and the bound fraction was eluted with 20% α-methyl-D-mannoside (Grade III; Sigma Chemical Co., St. Louis, Mo.) in Con A buffer. This was dialyzed against 6 additional changes of distilled water, after no free α-methyl-D-mannoside had been detected, and lyophilized.

**SDS-Polyacrylamide Electrophoresis.** Electrophoresis was performed in 6% polyacrylamide gels containing 0.1% SDS in 0.1 M Tris-glycine buffer, pH 8.1. Samples were subjected to electrophoresis for 1.5 hr at 8 ma/gel. Gels were stained with 0.5% Coomassie blue in 25% isopropyl alcohol and 10% acetic acid and destained with 10% acetic acid. Duplicate gels were stained for carbohydrate by a modification (29) of the periodic acid-Schiff method of Segrest and Jackson (38). For determination of the position of CEA activity, an unstained gel was cut into slices approximately 1 mm thick. Each slice was shaken overnight in 0.5 ml of a solution of 1 mg gelatin per ml in 0.075 M NaCl buffered with 0.075 M sodium phosphate to pH 7.2. An aliquot of the supernatant was tested in the radioimmune assay for CEA.

**Amino Terminal Analysis.** Amino terminals were identified by a modified dansylsine procedure of Hartley (19). To the sample (200 μg) dissolved in 50 μl of water were added 50 μl of 0.2 M NaHCO₃ and 50 μl of 0.36% dansyl chloride in acetone (w/v). The sample was incubated for 1 hr at 37°C, extracted 3 times with toluene, dried under N₂, and extracted with 300 μl of acetone/1 N HCl (v/v). The dansylated protein was dissolved in 0.01 M acetic acid and applied to a Dowex 50, H⁺ form column equilibrated with 0.01 M acetic acid. The column was washed with 5 to 10 ml of 0.1 N acetic acid, and the sample eluted with H₂O/acetone/concentrated NH₄OH, 80/20/4, v/v/v. After evaporation to dryness the sample was hydrolyzed in 6 N HCl for 12 hr at 110°C. The HCl was evaporated off under N₂ and the sample was dissolved in 100 μl of ethyl acetate saturated with water. The ethyl acetate extract was dried under N₂ and dissolved in 10 μl of 50% aqueous pyridine. Dansylated amino acids were identified on polyamide plates.

**Amino Acid and Amino Sugar Composition.** Samples were hydrolyzed under vacuum for 24 and 48 hr in 0.5 ml 3 N p-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (22). The amino sugars and basic amino acids were eluted from a PA 35 (Beckman Instruments, Inc., Fullerton, Calif.) column using sodium citrate buffers (0.4 N sodium citrate), pH 5.27 and 5.87. The acidic and neutral amino acids were eluted from an AA15 resin (Beckman) with sodium citrate buffers (0.2 N sodium citrate), pH 3.27 and 4.55.

**Carbohydrate Analysis.** Neutral sugars were analyzed by gas chromatography of the trimethylsilyl derivatives of the methyl glycosides according to the procedure of Pritchard and Todd (34).

**Methylation Analysis.** Samples were methylated by the method of Hakomori (18), in which the dimethylsulfonfinyl anion was used to generate the polysaccharide alkoxide before the addition of methyl iodide. The dimethylsulfonfinyl anion was prepared as described by Sandford and Conrad (37).
Acetylation, hydrolysis, reduction, and acetylation of the permethylated polysaccharides were performed using the procedures described by Stellner et al. (39).

Identification of the partially methylated aldol acetates was carried out according to the method of Bjornsdal et al. (1) for the neutral sugar derivatives and according to the method of Stellner et al. (39) for the amino sugar derivatives. A Varian Model 2740 gas chromatograph equipped with a flame ionization detector connected to a Dupont Model 21-094B double-focusing mass spectrometer was used for these analyses. A 6-foot column (2 mm inside diameter) of 3% ECNSS-M coated on Gas Chrom Q (Applied Science Laboratories, Inc., State College, Pa.) was used to separate the aldol acetates derived from the methylated sugars. Isothermal runs were made at 160° for the neutral sugars and at 180° for the amino sugars. Mass spectra of the carbohydrate derivatives were taken over a mass range of 35 to 600 atomic mass units using an ionizing potential of 70 eV. Scans were taken every 8.47 sec, and the spectra were processed using a Dupont 21-094B disc-based data system.

Mucopolysaccharide Analysis. Electrophoresis of samples was carried out on a Beckman Model R-101 Microzone Cell as described previously (33). Mucopolysaccharides were stained with 1% Alcian blue (Eastman Kodak Co., Rochester, N. Y.) in 5% acetic acid.

RESULTS

Purification. After treatment with perchloric acid to precipitate the protein, the samples were applied to a column of Sepharose 6B (Chart 1). The material with CEA activity as measured by radioimmune assay eluted from 42 to 62% column volume, in a pattern identical with that obtained with tumor extracts. Since the activity in the fractions eluting after 55% column volume represented a very small percentage of the total CEA-like activity, these fractions were not further purified. The fractions from 45 to 55% of column volume were pooled for further purification on a Sephadex G-200 column. The pattern was identical with that obtained from tumor preparations (Chart 2).

Material from the G-200 column that eluted from 34 to 45% column volume was pooled, dialyzed, lyophilized, and applied to a column of Con A linked to Sepharose. It has previously been demonstrated in preparations of tumor tissue that contaminating mucopolysaccharides and substances with blood group activity will not adsorb to Con A whereas most of the material with CEA activity will adsorb. This activity can be eluted with 20% α-methyl-D-mannopyranoside after the column temperature has been raised to 37° (33). Similar results were obtained with normal colon washings (Chart 3). The material that did not bind to Con A contained mucopolysaccharides and a substance that inhibited the agglutination of human O cells by H substance. The unbound fractions also contained a small amount of CEA-like activity, but 1000-fold less than that which was bound to Con A. The peak that did not bind to Con A was not due to overloading of the column since incubation with a fresh batch of Con A did not absorb the activity.

Almost 100% of the material that inhibited in a radioimmune assay for CEA was found in the Con A-adsorbed fraction. As is usually observed with CEA from tumor tissue, the CEA activity eluted as a sharp peak with a slight shoulder on the descending side and slightly ahead of the peak of absorbance at 220 and 280 nm. The material in Fractions 148 to 160 was pooled and characterized chemically and immunochemically.

Acrylamide Electrophoresis. The Con A-purified fraction was analyzed by electrophoresis in 6% acrylamide/0.1% SDS. A diffuse, single band, 0.5 to 2 cm from the origin, was obtained when the gels were stained with Coomassie blue or periodic acid-Schiff stain. The mobility of the band was identical with a standard of CEA prepared from tumor tissue. The CEA activity measured by radioimmune

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assay was located in the position in the gel that stained with Coomassie blue and periodic acid-Schiff stain.

**Amino Acid and Amino Terminal Analysis.** The amino acid compositions of material with CEA-like activity from tumor tissue and normal colon washings are very similar (Table 1). Aspartic acid (including asparagine) was the most abundant amino acid in both tumor and normal preparations. Relatively high levels of serine, glutamic acid, proline, and threonine were found in material from both sources. Lysine was found to be the single NH$_2$-terminal amino acid in the material from normal colon washings.

**Carbohydrate Composition and Methylation Analysis.** Carbohydrate compositions of CEA samples isolated from different tumors vary slightly. The material isolated from normal colon washings has a carbohydrate composition very similar to that of CEA preparations (Table 2). All of the carbohydrate structural units found in CEA were also found in the material isolated from normal colon washings. The relative amounts of the various linkages were very similar to those observed in CEA (Table 2).

The relatively lower amount of total galactose in colon washings may be related to the relatively higher percentage of terminal N-acetylglucosamine since previous studies have suggested that galactose is linked to N-acetylgulosamine (6, 10).

**Immunological Characterization.** The material from normal colon washings and tumor CEA gave lines of identity in double diffusion analysis against goat anti-CEA (Ace 5 and Ace 67 to 70). When tested in the radioimmune assay with $^{125}$I-labeled CEA from tumor tissue, the sample from normal colon washings gave an inhibition curve that was similar to, although not absolutely identical with, the inhibition curve with tumor CEA (Chart 4).

**DISCUSSION**

The results reported here strongly support the view that CEA is not restricted to fetal and neoplastic tissue but is also present in healthy tissue. All volunteers who participated in this study remain apparently healthy to date. It is possible that the presence of a small quantity of CEA in the normal adult is analogous to the small quantity of fetal hemoglobin that continues to be synthesized in the adult (2, 45, 46).

The CEA-like material obtained from these normal volunteers had the same NH$_2$-terminal amino acid residue as did CEA from tumor tissue and a very similar amino acid composition (9, 41). The carbohydrate composition and the linkage positions of the carbohydrate residues were also very similar to those found in tumor CEA (6, 41). The electrophoretic mobility of the material in SDS-polyacrylamide electrophoresis was identical with that of tumor CEA. Furthermore, the sequence of the 1st 24 amino-terminal residues was identical with that reported for tumor CEA preparations (40).$^5$

Immunological results are consistent with the structural identity of the substances from normal and tumor tissue since a line of identity was obtained with the 2 substances in double diffusion analysis. Although minor differences were observed by radioimmune assay, the variation in the inhibition curve was no greater than has been observed by us (M. L. Egan, unpublished observations) and others (44) for different preparations of CEA from tumor tissue or serum samples.

The collective chemical and immunological evidence obtained in this study strongly suggests that the material isolated from the colon of healthy individuals is very similar to, and perhaps identical with, the CEA of tumor tissue.


**Table 1**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Tumor tissue*</th>
<th>Normal colon washings</th>
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<tr>
<td>Tyrosine</td>
<td>5.0</td>
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<td>Phenylalanine</td>
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<td>0.9</td>
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<td>Aspartic acid</td>
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<td>Serine</td>
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<td>Glutamic acid</td>
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<tr>
<td>Proline</td>
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<td>9.4</td>
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<tr>
<td>Glycine</td>
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<td>5.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.6</td>
<td>5.5</td>
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<tr>
<td>Half-cystine</td>
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<td>1.7</td>
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<tr>
<td>Valine</td>
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<td>6.3</td>
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<tr>
<td>Methionine</td>
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<td>0.9</td>
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<td>Isoleucine</td>
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<td>Leucine</td>
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<td>8.4</td>
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* Average values of CEA preparations from 3 different tumors.
Structural units of the polysaccharide portion of CEA from tumor tissue and normal colon washings

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<tr>
<th>Structural unit</th>
<th>Glycosidic linkage</th>
<th>CEA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CEA&lt;sub&gt;2&lt;/sub&gt;</th>
<th>CEA&lt;sub&gt;3&lt;/sub&gt;</th>
<th>CEA&lt;sub&gt;4&lt;/sub&gt;</th>
<th>NCW&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>74</td>
<td>54</td>
<td>49</td>
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<tr>
<td>N-Acetylglucosamine</td>
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<td>103</td>
<td>99</td>
<td>118</td>
<td>103</td>
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<sup>a</sup> Subscripts 1 to 4 indicate CEA purified from different tumors. All tumors were liver metastases that had originated from the colon.

<sup>b</sup> NCW, normal colon washings; NA, not analyzed.

Chart 4. Radioimmune assay inhibition curves obtained with 125I-labeled CEA from tumor tissue, 1:4000 dilution of Ace(67 to 70) goat anti-CEA, and Con A-purified CEA from tumor tissue (C) or from normal colon washings (O).

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