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

Marianne L. Egan, David G. Pritchard, Charles W. Todd, and Vay Liang W. Go

Division of Immunology, City of Hope National Medical Center, Duarte, California 91010 [M. L. E., D. G. P., C. W. T.,] and Gastroenterology Unit, Mayo Clinic and Mayo Foundation, Rochester, Minnesota 55901 (V. L. G.)

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 those observed in CEA isolated from tumor tissue on whether there was a substance in normal tissue that was similar to CEA in elution from gel filtration columns but had a much lower molecular weight (43). NCA probably is also identical with CCAIII (30) and with the 

INTRODUCTION

CEA 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 CCAIII (30) and with the 

1 This research was supported by USPHS Grants CA 19163 and CA 17491 from the National Large Bowel Cancer Project and by Grants CA 16344 and CA 23854, all of the National Cancer Institute.

2 Present address: Department of Microbiology, University of Alabama in Birmingham, University Station, Birmingham, Ala. 35294.

3 To whom requests for reprints should be addressed, at Department of Microbiology, University of Alabama in Birmingham, University Station, Birmingham, Ala. 35294.

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

Received March 2, 1977; accepted May 6, 1977.

2638 CANCER RESEARCH VOL. 37
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 ho 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). 57Co was used in place of 3HNa 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 a Sephadex G-200 column 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 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 Segret 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 dansylation 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 N 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-amino-ethyl)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 dimethylsulfoninic anion was used to generate the polysaccharide aldehyde before the addition of methyl iodide. The dimethylsulfoninic 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 aldithiol 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 21-402B 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 aldithiol 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% a-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 fractions were 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
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, pro-
line, and threonine were found in material from both sources. Lysine was found to be the single NH₂-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-acetylgalactosamine since previous studies
have suggested that galactose is linked to N-acetylgalacto-
samine (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 ¹²⁵I-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 inhibi-
tion curve with tumor CEA (Chart 4).

**DISSCUSSION**

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 partici-
pated 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 volun-
tees had the same NH₂-terminal amino acid residue as did
CEA from tumor tissue and a very similar amino acid com-
position (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. Fur-
thermore, the sequence of the 1st 24 amino-terminal resi-
dues was identical with that reported for tumor CEA prepa-
ations (40),

**Table 1**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Tumor tissue*</th>
<th>Normal colon washings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>5.0</td>
<td>5.4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.7</td>
<td>3.0</td>
</tr>
<tr>
<td>Trytophan</td>
<td>1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.9</td>
<td>3.2</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Arginme</td>
<td>3.7</td>
<td>3.1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>14.9</td>
<td>13.4</td>
</tr>
<tr>
<td>Threonine</td>
<td>8.4</td>
<td>8.0</td>
</tr>
<tr>
<td>Serine</td>
<td>9.9</td>
<td>10.2</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>9.8</td>
<td>9.6</td>
</tr>
<tr>
<td>Proline</td>
<td>8.1</td>
<td>9.4</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.4</td>
<td>5.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.6</td>
<td>5.5</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>1.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Valine</td>
<td>6.4</td>
<td>6.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.1</td>
<td>4.3</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.5</td>
<td>8.4</td>
</tr>
</tbody>
</table>

* Average values of CEA preparations from 3 different tumors.

---


---

**Table 1**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Moles/100 moles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>5.0 (Tumor)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.7 (Normal)</td>
</tr>
<tr>
<td>Trytophan</td>
<td>1.2 (Tumor)</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.9 (Normal)</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.9 (Normal)</td>
</tr>
<tr>
<td>Arginme</td>
<td>3.7 (Normal)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>14.9 (Tumor)</td>
</tr>
<tr>
<td>Threonine</td>
<td>8.4 (Normal)</td>
</tr>
<tr>
<td>Serine</td>
<td>9.9 (Tumor)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>9.8 (Normal)</td>
</tr>
<tr>
<td>Proline</td>
<td>8.1 (Normal)</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.4 (Normal)</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.6 (Normal)</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>1.3 (Normal)</td>
</tr>
<tr>
<td>Valine</td>
<td>6.4 (Normal)</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.0 (Normal)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.1 (Normal)</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.5 (Normal)</td>
</tr>
</tbody>
</table>

---

* Amino acid composition of CEA from tumor tissue and normal colon washings.
Table 2
Structural units of the polysaccharide portion of CEA from tumor tissue and normal colon washings

<table>
<thead>
<tr>
<th>Structural unit</th>
<th>Glycosidic linkage</th>
<th>CEA, Moles/10^5 g of material</th>
<th>CEA2</th>
<th>CEA3</th>
<th>CEA4</th>
<th>NCW</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetylgalactosamine</td>
<td>Terminal</td>
<td>13</td>
<td>6</td>
<td>19</td>
<td>7</td>
<td>NA</td>
</tr>
<tr>
<td>Fucose</td>
<td>Terminal</td>
<td>57</td>
<td>57</td>
<td>58</td>
<td>53</td>
<td>49</td>
</tr>
<tr>
<td>Mannose</td>
<td>Terminal</td>
<td>5</td>
<td>8</td>
<td>5</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>2, 4</td>
<td>11</td>
<td>10</td>
<td>12</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2, 6</td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>3, 6</td>
<td>13</td>
<td>14</td>
<td>6</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>43</td>
<td>44</td>
<td>39</td>
<td>40</td>
<td>46</td>
</tr>
<tr>
<td>Galactose</td>
<td>Terminal</td>
<td>24</td>
<td>40</td>
<td>32</td>
<td>35</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>34</td>
<td>12</td>
<td>25</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>70</td>
<td>64</td>
<td>74</td>
<td>54</td>
<td>49</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>Terminal</td>
<td>Trace</td>
<td>9</td>
<td>18</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>51</td>
<td>52</td>
<td>49</td>
<td>61</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>3, 4</td>
<td>35</td>
<td>42</td>
<td>32</td>
<td>46</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>86</td>
<td>103</td>
<td>99</td>
<td>118</td>
<td>103</td>
</tr>
</tbody>
</table>

* Subscripts 1 to 4 indicate CEA purified from different tumors. All tumors were liver metastases that had originated from the colon.  
* NCW, normal colon washings; NA, not analyzed.

Acknowledgments

We thank Ronald Card and Nancy Buker for their excellent technical assistance. We thank William S. Schnute, Jr., for determining the mass spectra, and David Bills, for amino acid analysis.

References

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