Evidence for Common and Distinct Determinants of Colon Carcinoembryonic Antigen, Colon Carcinoma Antigen-Ill, and Molecules with Carcinoembryonic Antigen Activity Isolated from Breast and Ovarian Cancer

Stanley E. Chism, Noel L. Warner, J. Vivian Wells, Pauline Crewther, Susan Hunt, John J. Marchalonis, and H. Hugh Fudenberg

SUMMARY

This study was designed to answer the question, do molecules with carcinoembryonic antigen (CEA) activity from colon, breast, and ovarian cancer differ? Extracts of two breast and three ovarian cancers with CEA activity were compared to three colon cancer CEA preparations and to the related antigen, colon carcinoma antigen-Ill, in terms of lectin- and antiserum-binding properties. With the use of Farr-type radioimmunoassays with the lectins, concanavalin A and wheat germ agglutinin, the iodinated colon CEA and CEA-like preparations from breast and ovarian cancer all showed distinctly different patterns of binding. Specificity of binding was confirmed by inhibition studies with the relevant monosaccharides. Similarly, with antisera prepared against colon CEA, colon carcinoma antigen-Ill, or breast CEA, it was shown that, although all preparations shared some antigens, unique antigenic determinants were also present on all preparations. These data are consistent with the concept of a series of closely related CEA and CEA-like molecules with distinct characteristics for each tissue source of CEA.

INTRODUCTION

CEA from colonic adenocarcinoma has been extensively...
(37). However, it is probable that, on a large glycoprotein such as CEA, many immunogenic sites may exist in addition to the dominant CEA site (24), and therefore an antiserum raised against even highly purified CEA preparations would contain several populations of antibodies.

Accordingly, it is quite possible that the CEA-like molecules derived from various tumor sources may each have unique as well as shared specificities. In attempting to probe this question, we describe in this paper an analysis of breast and ovarian antigen extracts, which share at least 1 determinant with colon CEA but appear also to possess nonshared (unique?) determinants. These substances are compared to colon cancer-derived CEA and CCA-III in terms of antiserum- and lectin-binding site characteristics.

MATERIALS AND METHODS

Antigen Preparation. The CEA preparations from breast and ovarian cancer were isolated following the technique described by Krupey et al. (18). Tissue was weighed, diced, and homogenized in a blender to which a volume of 0.15 M NaCl had been added (volume of 0.15 M NaCl in ml, 4 times the weight of tissue in g). The homogenate was extracted for 15 min with an equal volume of 2 m perchloric acid and neutralized with KOH; the supernatant was separated from insoluble potassium perchlorate, exhaustively dialyzed against water, and freeze dried. The material was then sequentially chromatographed on Sepharose 4B and Sephadex G-200 columns, and fractions containing CEA activity were determined by their inhibitory activity of the G-21 colon CEA radioimmunoassay described below. Protein content of the fractions was monitored by absorbance at A 280. Additional purification was then achieved with block electrophoresis in Sephadex G-25. Reference colon CEA preparations and antisera were kindly provided by Dr. P. Gold, Montreal, Canada; Dr. H. Hansen, Nutley, N. J.; and Dr. C. Todd, Duarte, Calif. Dr. H. Hansen of Hoffman-La Roche Inc., Nutley, N. J., also generously donated the related antigen CCA-III.

The procedure of isolating CEA-like molecules from non-cancer tissues selects molecules that are at least antigenically cross-reactive with colon cancer CEA but that may not be identical. Our purification procedure, therefore, was not designed to identify new antigens or other CEA-like antigens that may have been present but only those that were cross-reactive with colon-derived CEA. In all column procedures, material was selected for further processing on the basis of 2 criteria, whether it possessed CEA inhibitory activity but was relatively free of other proteins in terms of A 280 absorbance measurements. All material selected was taken from column fractions in the region of 200,000 to 300,000 daltons.

Antisera Preparation. Antigens prepared as above were used to immunize rabbits. The antigen was emulsified in incomplete Freund’s adjuvant and injected 3 times into 4 s.c. sites at 7- to 14-day intervals. Antibody titer was monitored by determining the serial dilution that would precipitate approximately 50% of the given antigen preparation. Ten µl of inhibitor dilution were then added to 50 µl of 125I-labeled antigen, and 50 µl of the appropriate antiserum (or lectin) dilution were added. The rest of the assay was as described above. Duplicate control tubes included 125I-labeled antigen alone and 125I-labeled antigen with antiserum or lectin. In all inhibition assays, serial dilutions of inhibitors (tumor extracts or monosaccharide solutions) were tested to establish dose-response relationships.

Lectin Immunoabsorbent Column Binding. Con A-Sepharose or WGA-Sepharose was packed in a small 10-ml column with 0.005 M phosphate-buffered saline, pH 7.4, containing 0.1% Nonidet P-40 and run at a low flow rate of about 10 ml/hr. Labeled samples of CEA preparations containing a known amount of radioactivity were applied to the column and washed through with buffer, and the fractions were collected. When buffer failed to elute additional counts, a 2% solution of monosaccharide solution was passed through the column. MMP was applied to Con A-coupled Sepharose was packed in a small 10-ml column containing 0.1% Nonidet P-40 and run at a flow rate of 10 ml/hr. Labeled samples of CEA preparations containing a known amount of radioactivity were applied to the column and washed through with buffer, and the fractions were collected. When buffer failed to elute additional counts, a 2% solution of monosaccharide solution was passed through the column. MMP was applied to Con A-Sepharose columns, and d-GlcNAc was applied to WGA columns. When additional sugar failed to displace any further 125I-labeled antigen, 3.5 µl of the gel was removed from the column and the amount of retained radioactivity was determined.
Molecular Weight Estimations. Molecular weight estimations of iodinated CEA and CEA-like antigens were made by calibrating Sephadex G-200 columns with substances of known molecular weight: HGG (160,000 daltons), BSA (68,000 daltons), and equine cytochrome c (13,400 daltons). The void volume was determined by blue dextran exclusion. Inclusion volume was determined with free sodium $[^{125}]$iodide.

In addition, antigen preparations were run on 4 and 7% sodium dodecyl sulfate-polyacrylamide gels under nonreduced conditions and stained with Coomassie blue. Mobility was compared to HGG and BSA standards.

RESULTS

G-200 Elution Profiles of Radiiodinated Colon, Breast, and Ovarian Cancer CEA and CCA-III. For assessment of possible molecular weight heterogeneity in iodinated CEA preparations, samples were applied to a 1.6- x 60-cm G-200 column and the profile of eluted radioactivity was determined. Colon cancer-derived CEA, iodinated by either the chloramine T or lactoperoxidase method, consistently showed 3 peaks of radioactivity. Three preparations (CEA-R, CEA-G, and CEA-T) are shown in Chart 1. The material from Peak 1 with a molecular weight of approximately 200,000 daltons will be called hereafter CEA-H, while Peak 2 (CEA-L) has a molecular weight of about 60,000 daltons (Chart 1). The 200,000- and 60,000-dalton components were present in all 3 colon CEA preparations. The 3rd peak elutes in the inclusion volume, and its proportion relative to Peaks 1 and 2 increases with time after iodination, presumably indicating that it is associated with radiation-induced molecular degradation (Chart 2).

Relative to colon CEA, tissue extracts from breast and ovarian cancer and CCA-III have distinctly different elution profiles, as shown in Chart 1. All of these latter materials have a predominant peak of about 60,000 daltons despite the fact that the material iodinated, in the case of breast and ovarian CEA, had been isolated from G-200 preparative gel filtration as a material with a molecular weight of at least 200,000. Identical chromatographic profiles of breast and ovarian CEA were found following both the chloramine T and the lactoperoxidase techniques of iodination. The 60,000-dalton material, therefore, was not a product resulting from oxidation or reduction due to the chloramine T method, because it also occurs with the more gentle lactoperoxidase method. Molecular weight determinations of the noniodinated CEA molecules from breast and ovarian cancer were made by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and revealed a molecular size of 60,000 daltons. These findings suggest that the 60,000-dalton material is not a fragment of a large molecule degraded by iodination, but that it may be loosely associated with another material in the original isolation procedure, perhaps in a manner analogous to that described by Hughes (17).

Relative Antisera-binding Capacity of CEA-related Antigens. The reference throughout all of these studies has been G-21, which has been absorbed with normal human serum and perchloric acid extracts of liver and bowel (3). Precipitation of different iodinated antigens by the various antisera is shown in Chart 3. G-21 precipitated all 3 colon CEA-H preparations and CCA-III. The colon CEA-L, breast CEA, and ovarian CEA (data not shown) lacked G-21 binding sites in sufficient concentration to effect 50% precipitation at a 1:500 dilution. This antiserum was not used at a higher concentration because the usual dilution required to precipitate 50% of colon CEA-H is 1:200,000. G-80 (unabsorbed goat anti-CCA-III) precipitated CCA-III and colon CEA-H at similar concentrations but required a much higher antiserum concentration to precipitate breast and ovarian CEA. Antiseras raised against CEA preparations from breast (R-153, W-10) and ovarian (R-154, R-150, R-149) cancers precipitated the colon CEA-H, CCA-III, breast and ovarian preparation but not colon CEA-L. The data shown for R-153 are representative of all these sera.

From these various binding curves, it is evident that all of the preparations except colon CEA-L share at least 1 antigenic determinant and that at least several of the sera have
Heterogeneity of CEA from Different Tumor Sources

The possibility of an epitope shared between CEA-H and CCA-III was further evaluated with several anti-CEA antisera reported in the literature. CCA-III tested in the zirconyl phosphate gel radioimmunoassay with G-23 antiserum did not interfere with the detection of colon cancer CEA (28). However, in the Farr assay system, both G-21 and G-23 bind CCA-III and CEA-H, although, as noted above, less efficiently for CCA-III than G-80. G-81 (another goat anti-colon cancer CEA antiserum) gives similar findings (Chart 3). These results are compatible with those of other laboratories (9, 23, 39, 41) indicating that the associated antigen CCA-III has cross-reactive determinants with colon cancer CEA.

Possible antigenic identity was further studied by competitive inhibition in radioimmunoassay. In the reference radioimmunoassay system (G-21/125I-labeled CEA-H), only colon CEA gave complete inhibition, and it did so at relatively low concentrations of inhibitor (Chart 4a). The inhibition of G-21 125I-labeled colon CEA by CCA-III could be explained by a less than 5% contamination with colon cancer CEA-H molecules. Alternatively, as both CCA-III and breast CEA gave only partial inhibition at even higher inhibitor concentrations, it may be that these latter 2 molecular types share 1 antigen in common with colon cancer CEA but do not carry a 2nd colon cancer-unique antigen. The relative inhibition curves given by these preparations would thus also depend upon the relative concentrations of the 2 respective antibody populations in the G-21 antiserum, upon the dilution used, and upon the possible number of such antigenic determinants per molecule.

In the G-80/125I-labeled CCA-III assay, the degree of inhibition with colon cancer CEA-H is most probably due to an

multiple antibody activities. Thus, the relationship between the titration curves for G-21 and G-80 (Chart 3) used with antigens CCA-III and CEA-H suggests that each antiserum may contain an antibody to a common determinant, but that G-80 also contains an antibody to a unique CCA-III determinant and G-21 contains an antibody to a unique CEA-H determinant. G-80 also precipitates ovarian CEA, and yet ovarian CEA cannot be identical with CCA-III, as it is not precipitated by G-21. Furthermore, the R-153/G-80 relationship with colon CEA is quite different from that with CCA-III. The colon CEA-L material, although of a size similar to CCA-III and ovarian CEA, is clearly different, lacking virtually all of these antigenic determinants.

The breast CEA material appears to be identical with ovarian CEA and is not identical with CCA-III (see below). A disparity is noted between the inhibitory activity of breast and ovarian CEA in the G-21, CEA-H, radioimmunoassay, and the precipitability of these antigens by G-21. This fact may relate to the relatively greater concentration of the material used as inhibitor, compared to the low concentration of breast and ovarian CEA used as a diluted 125I-labeled antigen.
approximately 1% contamination with CCA-III molecules (Chart 4d). Breast cancer CEA, however, shows complete inhibition, consistent with its precipitation by G-80 (Chart 3d), and ovarian CEA gives results identical with those of breast CEA. When G-80 (anti-CCA-III) is used with $^{125}$I-labeled colon CEA and G-21 (anti-colon CEA) is used with $^{125}$I-labeled CCA-III, the inhibitory activity of the 2 preparations is similar (Chart 4, c and d). CEA from breast cancer also shows clear evidence of possessing a shared antigen.

CEA from breast, ovarian, and colon cancer was tested for its inhibitory activity in the G-21/$^{125}$I-labeled colon CEA and R-153/$^{125}$I-labeled breast CEA assays. Chart 5a presents evidence that colon relative to a breast and ovarian cancer CEA shares an antigen but is nonidentical. The use of identical preparations in the R-153/$^{125}$I-labeled breast CEA assay reveals that colon CEA is markedly less effective in its inhibitory activity (Chart 5b). These findings, along with those of Chart 4, corroborate the antiserum-binding data and support the concept of shared and nonshared determinants existing on the different CEA preparations.

Con A and WGA Lectin-binding Sites of CEA-related Materials. Since CEA is a glycoprotein, specific sugars in the carbohydrate moiety should interact with lectins (1, 6, 16, 42). We have previously described the use of lectins to analyze the carbohydrate moiety in CEA-related materials in a simple Farr-type precipitation assay (5).

Precipitation of $^{125}$I-labeled antigen by lectin occurs when the assay is performed in either serum diluent or DBS. Although precipitation is facilitated at lower lectin concentrations in DBS, the maximum percentage of precipitation is the same in serum diluent or DBS.

The comparative precipitability of colon CEA-H, breast CEA, CCA-III, and ovarian CEA with the use of the lectins Con A and WGA diluted in DBS is shown in Chart 6. Colon CEA-H is precipitated by both lectins, but to a greater extent by WGA (Chart 6a). Colon CEA-L is not precipitated by either lectin (5). CCA-III is similar to Con A- and WGA-binding sites (Chart 6c) and resembles colon CEA-H. Breast and ovarian CEA, however, resemble each other in having WGA sites but lacking Con A sites. These latter materials differ from CCA-III and colon CEA-H, again indicating that they are different molecular species.

More rigorous evidence that the Farr-type lectin assay measures a specific carbohydrate lectin interaction comes from monosaccharide competitive inhibition experiments. Con A has a broad specificity for sugars, which it binds with different affinities. The strongest affinity is with mannopyranosides, followed by glucopyranosides and then N-acetylglucosamides (35). In accordance with this pattern, MMP effectively inhibits the precipitation of colon CEA-H by Con A, while the unrelated sugar β-GalNAc does not (Chart 7). Con A binding to colon CEA-H can also be competitively inhibited by β-mannose and β-GlcNAc, but with relatively less avidity than by MMP. Similarly, colon CEA-H binding to WGA is inhibited by β-GlcNAc but not by MMP, confirming the lectin carbohydrate interaction specificity (Chart 7). In contrast, Con A binding to CCA-III, although completely inhibited with MMP and β-mannose, shows only partial inhibition with β-GlcNAc, indicating possible heterogeneity in the lectin-binding sites on these 2 preparations (Chart 8). The ovarian CEA/WGA interaction is depicted in Chart 8 and is similar to the colon CEA/WGA interaction.

Heterogeneity Assessed by Association of Antiserum- and Lectin-binding Sites. By combination of the above 2 approaches of antiserum or lectin precipitability of CEA, the percent of molecules with either lectin- or antiserum-binding sites, or both, may be calculated. In Chart 9, 75% of colon CEA-H molecules carried Con A-binding sites, and 81% of the molecules were precipitated by G-21 antiserum. When G-21 and Con A were mixed as coprecipitins, a total of 89% of the molecules was precipitated. The difference in precipitation suggests that 6 to 8% of the molecules with a G-21-recognized antigenic site do not have the Con A carbohydrate-binding site. In contrast, the CCA-III preparation appears to be very homogeneous, in that all molecules bound by lectin were also precipitated by G-21 antiserum.
Heterogeneity of CEA from Different Tumor Sources

With this approach the association of G-21-, Con A-, and WGA-binding sites has been determined for several CEA preparations (Table 1). The majority of CEA-H and CCA-III molecules possess both lectin- and antiserum-binding sites, whereas most colon CEA-L molecules lack all 3 sites. One CEA-H preparation (CEA-G) also contains a Blood Group A site, as approximately 42% of the labeled molecules were precipitated with an anti-blood group antiserum. All other preparations failed to react with the anti-A serum, indicating the presence of an A-like site that is not an essential compo-

tent of the CEA molecule (24). The results in Table 1 also show that, in the 2 colon CEA-H preparations, small but significant populations of molecules exist that share the antiserum- and WGA-binding sites but lack Con A sites.

**Lectin Immunoadsorbent Binding of CEA-related Molecules.** Confirmation of the Farr-type radioimmunoassay findings was made by using lectins covalently bound to Sepharose 4B. Iodinated CEA preparations containing a known amount of radioactivity were passed through lectin-Sepharose columns, and elution was performed with the relevant monosaccharide solution. The number of counts/min bound, eluted, and retained was determined. Two examples are shown in Chart 10. Of the CEA-H (CEA-R) counts applied to the Con A-Sepharose column, 26% were not bound. This material was then applied to a 2nd Con A-Sepharose column, and again none of this material bound, indicating that the binding capacity of the 1st lectin column had not been exceeded. Following application of 2% MMP to the bound material in the Con A column (Chart 10), approximately 47% of the original applied radioactivity was eluted. However, the remaining radioactive material was not

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**Table 1**

| Lectin/antiserum binding characteristics | % of molecules with indicated binding patterns
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>CEA-H (R)</td>
</tr>
<tr>
<td>Ag+ W+ C+</td>
<td>68</td>
</tr>
<tr>
<td>Ag+ W+ C-</td>
<td>13</td>
</tr>
<tr>
<td>Ag+ W- C+</td>
<td>8</td>
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<tr>
<td>Ag+ W- C-</td>
<td>0</td>
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<td>Ag- W+ C-</td>
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<tr>
<td>Ag- W+ C+</td>
<td>0</td>
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<tr>
<td>Ag- W- C+</td>
<td>10</td>
</tr>
</tbody>
</table>

* This column lists the 8 possible combinations of reaction patterns. Ag+, positive reactive with the G-21 anti-colon CEA antiserum; W+, precipitation by WGA; C+, precipitation by Con A.

* The 4 columns list the percentage of molecules for the 4 iodinated CEA preparations, showing the relevant binding characteristics. CEA-H (R) and CEA-H (G) are 2 different colonic carcinoma CEA preparations weighing approximately 200,000 daltons; CEA-L (G) is the 60,000-dalton material derived from iodinated colonic carcinoma CEA; CCA-III also weighs approximately 60,000 daltons.

* Of the total population, 42% was precipitated by anti-Blood Group A.
eluted, even by 3.5 M sodium thiocyanate. The results of such binding experiments with the use of different labeled CEA preparations are given in Table 2. It essentially confirms the data of the Farr-type assay. Most colon cancer CEA-H molecules bind to Con A, whereas fewer colon CEA-L or breast and ovarian CEA molecules do. Similarly, there was strong binding of CEA-H to WGA-Sepharose (Chart 10). In this latter instance, 17% of the counts were not bound, 52% were bound and eluted, and 32% were irreversibly bound.

The relationship of G-21 antiserum-binding site to the Con A-binding sites was also studied (Table 3). The CEA-H Con A-bound and MMP-eluted material contained a high proportion of molecules with G-21-binding sites. In contrast, Con A-bound and MMP-eluted breast and ovarian CEA-L molecules lacked the G-21 site. Colon CEA molecules that did not bind to Con A-Sepharose had little reactivity with G-21, although the observation that some precipitation occurred is consistent with the data from Table 1 indicating the presence of some molecules with G-21 binding sites but no Con A-binding sites.

**Nonidentity of Antiserum- and Lectin-binding Sites.** As most of the molecules in the colon CEA preparation carry both G-21 antisera and both lectin-binding sites, it is relevant to question whether the immunodominant site is directly associated with the carbohydrate component. Using the same concentration of sugars that causes inhibition of lectin precipitation of colon CEA-H, we were unable to demonstrate any inhibiting activity by sugars on G-21 precipitation, confirming similar previous observations (6, 29). This result is consistent, however, with several possibilities: (a) nonidentity of G-21- and lectin-binding sites; (b) the possibility that the G-21-binding site is relatively large, and although it includes the lectin-binding site simple monosaccharides are unable to inhibit completely; and (c) the possibility that the site for antiserum and lectin binding is identical, but that the affinity of antiserum binding is considerably greater than that of monosaccharides.

**DISCUSSION**

Despite the considerable knowledge that has accumulated in the past decade concerning the immunochemical nature of colonic carcinoma CEA, relatively little information is available on the nature of the CEA molecular species from noncolonic tumor types. The fact that CEA is a large molecule (200,000 daltons) (3, 20, 37), is heterogeneous (4, 7, 11, 12, 34), and possesses several different carbohydrates (3, 37) raises the possibility that multiple antigenic determinants may be present on the molecule (43). Furthermore, recent studies have established that CEA, as currently defined, represents a set of related or isomeric glycoproteins (10, 31).

In this study we have demonstrated that CEA from breast and ovarian cancer shares a determinant with "classical" 200,000-dalton colon CEA but differs in size, lectin-binding sites, and G-21 antisera-binding sites. Although the related antigen CCA-III shares several characteristics with colon CEA (G-21 and lectin precipitability), it is not identical (molecular weight; lectin inhibition by monosaccharide).
Similarly, breast and ovarian CEA share characteristics with colon CEA (G-21 inhibitory activity in radioimmunoassay; WGA precipitability) but differ in other ways (G-21 and Con A precipitability; molecular weight). A comparison of several characteristics is summarized in Table 4.

Although these data clearly require further assessment by absorption studies, we feel that our preliminary evidence, consistent with several other publications (10, 19, 28, 31), is compatible with the existence of at least 4 different antigenic determinants (Table 4), defined as follows: (a) colon cancer CEA-specific antigen not present on CCA-III, breast CEA, or ovarian CEA molecules; (b) an antigenic determinant shared by all of these molecules; (c) a determinant present on CCA-III but not found in colon cancer CEA (the data suggest that this determinant is also present on breast and ovarian CEA, all being molecules with a molecular weight of approximately 60,000); (d) an antigenic determinant detected on breast and ovarian cancer CEA but not present on colon CEA or CCA-III.

Based on published data and our findings, it appears that molecules with CEA activity (or at least 1 cross-reactive determinant) may be found in many tumor extracts, but unique or tissue-specific epitopes probably also exist. With a combination of techniques, it may be possible to elucidate the carbohydrate and antisemur sites and further characterize glycoproteins associated with cancer.

**REFERENCES**

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