Partial Purification of Carcinoembryonic-Reactive Antigen from Breast Neoplasms Using Lectin and Antibody Affinity Chromatography

Richard J. Santen, Julien Collette, and Paul Franchimont

ABSTRACT

The lability of carcinoembryonic antigen (CEA) under standard perchloric acid extraction techniques, its low concentration in breast neoplasms, and the small amounts of primary tumor tissue available have hindered the preparation of highly purified breast CEA. We report a new method which exploits the high resolving power of antibody and lectin affinity chromatography to obtain considerable enrichment of breast CEA. The antibody affinity step, using polyvalent antisera directed against crude breast tumor extracts, afforded a 25- to 30-fold purification with a single procedure and the wheat germ agglutinin lectin column, as an alternative to concanavalin A, afforded an additional 2- to 6-fold enrichment. Two further procedures (hydroxylapatite absorption and gel filtration) provided a total purification of 1400-fold (0.039 to >49.7 μg CEA per mg protein) under maximal conditions.

The degree of purity achieved by sequential biochemical procedures was monitored with the highly sensitive rocket and radirocket immunoelectrophoresis systems (using polyvalent antisera) and with radioimmunoassay. With the last preparative step, only one major immunocomplex, the CEA-reactive radirocket, was present in the leading portion of the gel filtration peak. The apparent molecular weight of the CEA-reactive material from breast which eluted on gel filtration chromatography in the range of 120,000 was considerably lower than that of colon CEA (M.W. 200,000), as further evidence that these materials were different, 7 to 20 times more colon CEA than breast CEA were necessary to produce rockets of similar height with the anti-breast CEA antibody. Serial dilutions of colon and breast CEA produced rockets with differing slopes. Finally, direct visualization of the immunocomplexes contained in the various gel filtration column fractions with radirocket immuno-electrophoresis allowed the demonstration that breast CEA possesses molecular weight and antigenic heterogeneity.

INTRODUCTION

The concept that CEA derived from different tissue sources can possess organ-specific, physicochemical, or antigenic determinants has received much recent support (10, 13, 17, 18, 28, 36). Several observations provided suggestive evidence that CEA derived from breast neoplasms has lectin binding and antigenic and molecular-weight characteristics which differ from those of CEA obtained from colon tumors (10, 13, 28). An assay specific for breast cancer-derived CEA would have potential usefulness for the early diagnosis and management of human breast carcinoma. However, highly purified extracts of breast CEA suitable for production of specific antisera or radiolabeling have not been prepared previously. With the ultimate goal of establishing a breast-specific CEA assay, we have partially purified CEA from human breast neoplasms.

The recently described problems of aggregation and perchloric acid lability of CEA (31) and the relatively low concentrations of CEA in breast compared to colon carcinomata (5-8, 10, 13-16, 23, 24, 28, 32, 33) led us to develop new purification techniques based upon antibody affinity and lectin chromatography (34). To follow sequential fractionations, rocket and radirocket IEP were used to exploit the ng range sensitivity of these techniques for demonstration of both CEA and contaminating proteins (2). Partial purification of at least 1400-fold was achieved, and the CEA-reactive material that was obtained differed by physicochemical and antigenic characteristics from that of colon CEA.

MATERIALS AND METHODS

Preparative Techniques

Polyvalent Antibody Affinity Column. Thirteen primary breast carcinomas weighing a total of 25 g were extracted by the perchloric acid method of Kruepy et al. (32, 33) and were injected with Freund's complete adjuvant into multiple intradermal sites in 2 rabbits. Polyvalent Antiseras 41 and 42 were obtained 2 months later after 3 booster injections at 2 weekly intervals. The γ-globulin fractions, from 70 ml antisera obtained by precipitation in 18% Na2SO4, were coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) by the method of Axen et al. (3, 4). Elution conditions of this affinity material were studied using 0.1- to 1.0-ml microcolumns and 125I-labeled, highly purified CEA obtained from a colon carcinoma (21, 22). Thirty to 55% of labeled colon CEA was bound to the column and could be eluted with either 0.1 M glycine-HCl, pH 2.0, or with 6 M guanidine-HCl, pH 2, at room temperature. A similar percent of labeled colon CEA bound to these antibodies under conditions of double antibody precipitation (antisera dilution, 1:640). Nonspecific binding determined with 125I-labeled α-fetoprotein was 0.5% in the glycine-HCl eluate and 2.9% in the 6 M guanidine fraction.

Monospecific Antibody Affinity Column. Antiserum 41 was rendered monospecific for CEA by absorption according to the solid-phase method of Smith et al. (39) using purified antigens derived from normal liver and colon. The γ-globulin fraction from 4 ml of this observed antisera, demonstrated to be monospecific on radirocket IEP analysis, was coupled to...
Seprharose 4B for use in the monospecific antibody affinity column. Evaluation of the elution characteristics of this material on microcolumns revealed that the majority of 125I-labeled colon CEA was released by 3 m guanidine-HCl, pH 2.0.

Wheat Germ Agglutinin Lectin Column. Wheat germ agglutinin [10 mg lectin (Triticum vulgare); research grade; molecular weight, 18,000; obtained from Serva Feinbiochemica, Heidelberg, Germany] was incubated with 1 g of CNBr-activated Sepharose 4B under conditions identical to those used for antibody coupling. Monitoring of A280 revealed that 8.4 mg wheat germ agglutinin were bound to the Sepharose. Forty-six to 77% of 125I-labeled colon CEA bound to lectin microcolumns in 10 experiments performed at 4°C. The majority of counts eluted with 2% N-acetylglucosamine in Buffer A with Tween, pH 7.2. However, as observed by Chism et al. (10), an additional large percentage of cpm consistently remained on the column after carbohydrate elution.

Hydroxylapatite Absorption and Gel Filtration. Hydroxylapatite obtained from Bio-Rad Laboratories, Richmond, Calif. was equilibrated with 10 mM phosphate, pH 7.5, before use. Seventy % of 125I-labeled colon CEA used to evaluate column conditions did not bind to this material. With increase in phosphate concentrations, 7.9% 125I colon CEA cpm eluted with 25 mM phosphate, 3.3% with 50 mM phosphate, 4.8% with 100 mM phosphate, 3.2% with 250 mM phosphate, and 1% with 500 mM phosphate.

ACA 34 (3% acrylamide and 4% agarose gel beads; fractionation range, 20,000 to 380,000) and ACA 44 (range, 10,000 to 130,000) were obtained from LKB Instruments, Inc., Rockville, Md. and packed in 1.5- x 50-cm columns for gel filtration experiments. PBS at a pressure of 15 cm H2O was used for elution at a flow rate of 4 ml/hr. Standard proteins obtained from Boehringer Mannheim, Mannheim, Germany, were used for molecular weight standards and were measured by A280 elution patterns.

Analytical Techniques

Rocket and Radiorocket IEP. The methods for preparation, electrophoresis, Comassie blue staining, and agar plate development with Kodak XLI X-ray film, were used as described previously (2). Agarose L (electroendosmosis, <0.02) obtained from LKB Instruments was used to prepare the plates. Svendsen's buffer system (Tris-glycine:barbiturate buffer), pH 8.4, (0.02 M) was used as an electrophoresis buffer and as an agar diluent. Electrophoresis was carried out at 1 to 2 V/cm on an LKB multiphor system.

For each IEP plate, the y-globulin equivalents of 180 pL of polyclonal Antibody 41 and 42 (see below) were added to 9 ml 1% agarose:2% polyethylene glycol 6000 at 52°C. The agarose was then immediately poured onto 63- x 98-mm sections of 88- x 98-mm glass plates and allowed to cool. Three ml of agarose:polyethylene glycol were then poured onto the remaining 25- x 98-mm sections, into which 4 mm holes were punched for sample application. Finally, as trace for radio-rocket experiments, 1 x 10^6 cpm of 125I-labeled colon CEA (specific activity, 50 to 150 pCi/µg) were incorporated into 350 µl agar at 52°C and were immediately pipetted into 2- x 80-mm troughs cut on the agar plates just above the area of sample application.

Proteins and CEA. Protein concentrations were measured either by the method of Lowry, by determination of absorbance at 280 or 230 nm, or by a fluorescence method using the fluorescamine reagent (29). All results are expressed in terms of a bovine serum albumin standard. The radioimmunoassay system previously described and characterized was utilized for CEA measurements (21, 22). Because this assay used a colon CEA trace and standard and anti-colon CEA antibody, the values obtained for CEA in breast extracts were considered relative estimates to judge the efficacy of various purification steps.

Extraction and Purification of Breast Extracts

Breast Extract 1. Forty-five g of tissue obtained from 90 histologically confirmed primary and soft-tissue metastatic human breast carcinomas were homogenized in 4 volumes 0.15 m NaCl and then sonicated for 5 min at high-power Setting 5 on an Analis sonicator at 4°C. The homogenate was centrifuged at 16,000 x g, and the lipid material was removed with a spatula and filtration through glass wool.

The extract was slowly recycled 4 times through the polyvalent affinity column (Sepharose bed, 70 ml) at 4°C over a 24-hr period. Column elution consisted of 250 ml PBS, 0.5% Tween, and 1700 ml PBS (to remove nonspecifically absorbed protein) (4, 8), followed by 250 ml 0.1 m glycine-HCl, pH 2.0, and 250 ml 6 m guanidine-HCl, pH 2.0. The 2 latter fractions were collected in 10-ml tubes containing 2 ml 0.5 m phosphate, pH 7.5, to produce immediate neutralization and then were dialyzed exhaustively against distilled H2O and lyophilized. Recycling of the nonbound homogenate fraction onto the affinity column and repeat elution produced only 10% additional CEA in the glycine-HCl fraction. Thus, column capacity for CEA was not exceeded.

Lyophilized material from the antibody affinity column was then dissolved in Buffer A and incubated with constant turning for 24 hr at 4°C with 1 ml wheat germ agglutinin coupled to Sepharose 4B. After transfer to a siliconized glass column, the Sepharose was eluted sequentially with 20 ml Buffer A with 0.1% Tween, 20 ml Buffer A alone, and 5 ml 0.1 M N-acetylglucosamine:0.1% Tween. The N-acetylglucosamine fraction was then frozen for later purification on hydroxylapatite. Recycling of nonbound material on the column again produced less than 10% of additional CEA in the carbohydrate fraction. Aliquots of the N-acetylglucosamine eluate were dialyzed against 10 mM phosphate buffer and mixed with an equal volume of hydroxylapatite. After incubation for 30 min at room temperature and centrifugation, the supernatant was collected, and the precipitate was washed once. Thereafter, sequential elutions with identical volumes of 50 and 250 mM phosphate were carried out. One analytical run also utilized additional phosphate concentrations of 25 and 100 mM.

The 10 mM fraction of the hydroxylapatite-absorbed extracts was concentrated to 1 ml in a dialysis bag against Sephadex G-100 and then applied to an ACA 34 column for further purification.

Breast Extract 2. Fifteen g of tumor obtained from an additional 23 histologically confirmed primary and metastatic human breast neoplasms were homogenized as with Extract 1. This preparation, phenylmethyl sulfonyl fluoride (250 µl), e-aminocaproic acid (1 ml of 0.4 m solution), EDTA (400 µl of a

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3 mM solution), sodium azide (1 ml of a 0.4% solution), and trypsin (250 µg), was added to inhibit proteolysis. In an attempt to improve purification achievable with the antibody affinity chromatography, Sepharose 4B coupled to monospecific antiserum was used. Elution of this column was identical to that for Breast Extract 1 except that 3 M guanidine-HCl, pH 2.0, was used rather than 0.1 M glycine-HCl. After neutralization with 0.5 M phosphate (pH 7.5), dialysis against PBS, and concentration to 1 ml on an Amicon PM30 membrane, this extract was run on an ACA 44 column without use of the wheat germ antigen or hydroxylapatite columns.

Breast Extract 3. Techniques were identical to those used for Extract 1 with 2 exceptions: (a) protease inhibitors similar to those in Breast Extract 2 were used; and (b) all procedures were carried out without lyophilization or freezing between steps.

RESULTS

Breast Extract 1

Antibody Affinity Column. After homogenization in 0.15 M NaCl and equilibration on the antibody affinity column, 28% of the CEA present eluted with 0.1 M glycine-HCl (Chart 1A). The total protein of 20 mg in this fraction represented less than 1.0% in the original homogenate (Table 1). With this single step, the specific activity of CEA increased 30-fold from 0.049 µg/mg protein to 1.49 µg/mg protein. Although the guanidine-HCl eluate also contained enriched CEA, this fraction was not used further. Rocket IEP analysis of the glycine-HCl eluate revealed the presence of one heavily stained rocket antigen: antibody complex and several more weakly stained ones where height progressively decreased with serial dilution (Chart 1B). CEA could be detected only with the more sensitive radiolabeled rocket IEP system. After radioautography of the agar plate, CEA was visualized as a diffusely stained rocket which did not correspond to any of the Coomassie blue-stained complexes (Chart 1C). Consequently, the combination of the rocket and radiolabeled rocket IEP techniques revealed several proteins in addition to CEA. It is of note that several additional rocket complexes (Chart 1C) were also outlined by thin linear patterns of radiolabeling.

To further verify that the substances being measured in these extracts were CEA, serial dilutions were introduced into the colon CEA radioimmunoassay system. As shown in Chart 2, the 0.15 M NaCl PBS:Tween, glycine-HCl, and guanidine-HCl extracts all produced displacement parallel to that of the purified colon CEA standard.

Wheat Germ Agglutinin Affinity Column. Prior to application on this column, the glycine-HCl extract had been dialyzed, lyophilized, and redissolved in Buffer A. These procedures produced a 2-fold decrease in the amount of CEA measured by radioimmunoassay. When applied to the wheat germ agglutinin column (Chart 3A), 47% of CEA bound and was eluted with 0.1 M N-acetylglucosamine. This fraction contained 3.0 µg CEA per mg protein (Table 1), which represented a 2-fold enrichment in specific activity compared to the original glycine-HCl extract before lyophilization or a 6-fold purification of the redissolved material applied to the column as shown in Chart 3A. Rocket IEP of Fractions 42 to 44 (eluted with N-acetylglucosamine) revealed the disappearance of the major Coomassie

Hydroxylapatite Absorption. After dialysis against 10 mM phosphate buffer, the pool of wheat germ agglutinin Fractions 42 to 46 was sequentially eluted from hydroxylapatite. Fifty % of the CEA in the original wheat germ agglutinin fraction did not bind to hydroxylapatite at a phosphate concentration of 10 mM. An additional 2% was recovered in the two 5-m1 washes with 10 mM phosphate, but negligible amounts were eluted with 40 or 250 mM phosphate (Chart 4A). Twenty-eight % of protein appeared in the nonbound fraction, and the remainder eluted with high buffer concentrations. Specific activity of nonbound CEA increased to 9.88 µg/mg protein.

To characterize the pattern of protein recovery in more detail, 50 µl wheat germ agglutinin Fraction 43 were added to 50 µl of hydroxylapatite and was eluted with 10, 25, 50, 100, and 250 mM phosphate. Rocket IEP (Chart 4B) revealed 2 Coomassie blue-stainable complexes in the 10 mM phosphate fractions.
Purification of CEA from breast carcinoma extracts

<table>
<thead>
<tr>
<th>Extract</th>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>CEA (µg)</th>
<th>Specific activity (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.15 M NaCl extract Antibody affinity column wheat germ agglutinin lectin column Hydroxylapatite absorption ACA 34 gel filtration</td>
<td>3867</td>
<td>190</td>
<td>0.049</td>
</tr>
<tr>
<td>2</td>
<td>0.15 M NaCl extract Antibody affinity column ACA 44 gel filtration</td>
<td>755</td>
<td>276</td>
<td>0.37</td>
</tr>
<tr>
<td>3</td>
<td>0.15 M NaCl extract Antibody affinity column wheat germ agglutinin lectin column Hydroxylapatite absorption ACA 44 gel filtration</td>
<td>4165</td>
<td>163</td>
<td>0.039</td>
</tr>
</tbody>
</table>

IEP analysis. To evaluate this difference further, a different anti-CEA antibody raised against colon CEA and used in radioimmunoassay was also used in the radiorocket system. With this antibody, heavily labeled rockets were observed in the region of higher molecular weight (Fractions 26 to 30), and faintly labeled ones were observed in the latter fractions (Chart 5D). These observations, taken together, suggested that different antigenic determinants were present in the species of CEA eluted at higher and lower molecular weights.

Breast Extract 2

For purification of this material, the monospecific antibody affinity column was utilized, protease inhibitors were added to 0.15 M NaCl extract, and the hydroxylapatite and wheat germ agglutinin lectin steps were omitted. Although yields from the antibody affinity column were low [276 µg CEA applied, 7.92 µg recovered (Table 1)] the final specific activity of 14.0 µg/mg protein was similar to that obtained in Extract 1.

Breast Extract 3

After recognizing from Breast Extract 1 that CEA activity was lost during lyophilization and freezing, an additional purification run was carried out without intervening lyophilization or freezing. The specific activity obtained after gel filtration >49.7 µg CEA per mg protein (Table 1) represented a 1400-fold purification from the starting material (0.039 µg/mg).

Chart 2. Parallel displacement of purified colon CEA standard and breast tissue extracts in radioimmunoassay using anti-colon CEA antibody and 125I-labeled colon CEA tracer. ●, colon CEA standard; ●, 0.15 M NaCl extract; ■, glycine-HCl; □, guanidine-HCl; ◊, PBS-Tween; ▼, undiluted. BIBo is the ratio of bound labeled antibody (B) to total bound antibody (Bo) in the absence of unlabeled antigen.

and additional rockets which appeared during elution with higher salt concentrations. On radiorocket IEP analysis, CEA was found predominantly in the 10 mM phosphate eluates (Chart 4C).

Gel Filtration. When applied to an ACA 34 column, the CEA from the 10 mM hydroxylapatite fraction eluted asymmetrically with its peak in the region preceding human albumin. The majority of CEA appeared before the major contaminating protein, and the specific activity of CEA in Fractions 30 to 32 was 16.8 µg/mg protein (Chart 5A). Unexpectedly, on radiorocket IEP, the pattern of CEA elution differed from that observed by radioimmunoassay. The shoulder preceding the main CEA peak on radioimmunoassay (Fractions 25 to 29) corresponded in the radiorocket system to poorly developed and faintly radiolabeled complexes (Chart 5C). The main radioimmunoassay peak in Fractions 30 to 35, however, was reflected by clearly defined, heavily labeled complexes on radiorocket
Affinity Isolation of Breast CEA

An aliquot of colon CEA was bound and eluted from the antibody affinity column with 3 M guanidine-HCl, pH 2.0, and then applied to the ACA column. Material subjected to these conditions coeluted with the original colon CEA aliquot (Chart 6).

Antigenic Characteristics. Two antibodies raised against breast CEA extracts (Antibodies 41 and 42) were used in the radirocket IEP system to compare the antigenic properties of colon and breast CEA preparations. With both antibodies, serial

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Physicochemical and Antigenic Properties of Breast and Colon CEA

Gel Filtration. The elution pattern of a highly purified colon CEA preparation was compared to that of Breast Extracts 1 and 2 on ACA 34 and ACA 44 gel filtration columns. On ACA 34, both 125I-labeled and unlabeled colon CEA eluted identically but in earlier fractions than did the CEA in Breast Extract 1 (not shown). To obtain better separation, the more favorable fractionation range of the ACA 44 column (M.W. 10,000 to 130,000) was exploited. Breast Extracts 1 and 2 eluted between human albumin (M.W. 68,000) and aldolase (M.W. 160,000) and clearly later than labeled colon CEA. To ensure that the acid conditions used during the antibody affinity column elution did not artifactually produce this difference, an

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Chart 4. Hydroxyapatite absorption. A, elution pattern of hydroxyapatite absorption experiment. Symbols identical to those used in Chart 1. B, rocket IEP analysis of repeat hydroxyapatite run (see "Results") using elutions with 10, 25, 50, 100, and 250 mM phosphate buffer. C, radirocket IEP of plate with identical magnification; ng CEA/fraction.

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Chart 5. A, elution pattern of ACA 34 gel filtration column. CEA measured by radioimmunoassay and proteins by Coomassie blue-stained rocket height. All fractions had an A280 of <0.10, and those illustrated as containing no protein had A280 of <0.010. B, rocket IEP analysis of ACA 34 column Fractions 25 to 40 stained with Coomassie blue and run with the polyvalent anti-breast extract antibody; C, radirocket IEP of A developed on Kodak XL1 film at an identical magnification; D, radirocket IEP analysis of repeat experiment using anti-colon CEA antibody to assay the same fractions. Each rocket corresponds to the same fraction tube from the ACA 34 column as in B and C.
Classical methods for purification of CEA utilize initial extraction with perchloric acid followed by preparative ion-exchange and gel filtration chromatography (1, 5, 6, 14-16, 19, 20, 24, 25, 31-33, 35). Recently, Kimball and Brattain (31) demonstrated that perchloric acid produces a 90% degradation of CEA during standard purification procedures requiring 1 hr of incubation. Exposure to such strongly acidic and oxidizing conditions also modifies isoelectric focusing properties of CEA. Since primary and hepatic-metastatic colon carcinomas contain high concentrations of CEA, procedural losses resulting from the perchloric acid extraction of these tissues have been acceptable. However, CEA concentrations are lower in breast than in colon carcinomas (10, 28), available tumors are smaller, and hepatic metastases may contain structurally different CEA than contained in primary breast tumors (28). For these reasons, we sought to develop an alternate method to purify CEA from the breast and chose to exploit the high resolving power afforded by affinity chromatography.

The fixation of antibodies to an inert material for affinity chromatography provides the potential for a high degree of purification with a single step (26). While monospecific antisera are usually used with this technique, our data illustrate the possibility of achieving an initial 30-fold purification of CEA with the use of polyvalent antibodies raised against crude breast extracts. Consequently, this technique may be applied before pure material obtained by other methods is available to produce monospecific antibodies. This preparative step, while not capable of one-step purification, compares favorably to standard biochemical methods in its resolving power and provides a simple, initial step for purification.

Attempts to improve the antibody affinity step by using absorbed monospecific antisera for Breast Extract 2 did not increase the degree of purification (Table 1) but lowered considerably the yield of recovered material. As shown by Gospodarowicz (26), antibody titer must be high to obtain pure proteins with a single antibody affinity column step. During absorption, the titers of our antibody were reduced, and the binding capacity of the monospecific antibody column (determined by competitive displacement experiments) was only 22 μg CEA. Consequently, higher titer antisera must be generated before full exploitation of this technique is possible.

The second affinity column purification step utilized the lectin wheat germ agglutinin as binding reagent (30). Molecules containing N-acetylglucosamine, the sugar comprising 30% of the carbohydrate of CEA (19), were bound to wheat germ agglutinin as binding reagent (30). Molecules containing N-acetylglucosamine, the sugar comprising 30% of the carbohydrate of CEA (19), were bound to wheat germ agglutinin specifically and with high affinity. Chism et al. (10) first demonstrated that CEA derived from breast or colon tumors binds to this lectin and can be eluted by competition with N-acetylglucosamine. While methods of CEA isolation using concanavalin A as lectin have been described previously for colon CEA (7, 9-12, 27, 28, 37, 38), that from breast does not bind well to this material. In our experiments, the wheat germ agglutinin lectin column produced a 2- to 6-fold purification of CEA. Yields, however, were lower than expected because of persistent binding of a fraction of CEA not eluted with 2% N-acetylglucosamine. This phenomenon was observed by Chism et al. (10). Experiments carried out after these studies were completed revealed that from 12 to 32% of additional radiolabeled CEA could be released from these columns after addition.

**DISCUSSION**

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of 10% N-acetylglucosamine. This step should diminish procedural losses and increase the degree of purification in subsequent experiments.

Another factor which enhanced overall procedural losses was the apparent instability of CEA. A 3-fold decrease in the amount of CEA measured by radioimmunoassay was observed following lyophilization of the glycine-HCl extract. This effect could be due to aggregation, to continued proteolysis, or to other factors. Similar phenomena have been observed by others during extraction of colon carcinoma under neutral conditions (8). This effect was avoided in the preparation of Breast Extract 3 and, consequently, a 1400-fold purification was attained (Table 1).

It is of note that the specific activity of Breast Extract 2 was 10-fold higher than that of the others. This suggests that the expression of CEA activity varied in different tumors. Preliminary experiments have confirmed this possibility, and we are now selecting only high-specific-activity tumors for CEA purification.

Our results demonstrated that CEA-reactive material isolated from breast tumors differed both physicochemically and antigenically from colon CEA. On gel filtration, breast CEA consistently eluted in a region of lower molecular weight than did colon CEA. An antibody directed against breast CEA produced radiorockets which differed in height and in slope when breast or colon CEA preparations were compared (Chart 7). An additional finding was that the radirocket IEP technique, which allows direct visualization of antigen:antibody complexes, clearly demonstrated antigenic differences between subspecies of breast CEA. Anti-colon CEA antibodies better recognized larger molecular variants of breast CEA (Chart 5D), and those against breast recognized smaller subspecies (Chart 5C). These results support those of Chism et al. (10), who also demonstrated that CEA-reactive material from breast contains a relatively unique antigenic determinant, while also sharing a common site with colon CEA. Our data, in addition, suggest that the unique breast CEA antigen resides in a lower-molecular-weight subspecies.

The material isolated from breast tumors does not appear to be colon carcinoma antigen-III, a glycoprotein extractable from normal tissues and human plasma (10). Colon carcinoma antigen-III elutes in the region of M.W. 60,000 and does not cross-react in the colon CEA-anticolon CEA radioimmunoassay which was used to monitor purification (21, 22).

In summary, a new method for preparation of breast CEA has been described which takes advantage of specific affinity interactions with anti-breast CEA antibodies and with wheat germ agglutinin. The major advantage of this procedural approach is the potential for a high degree of purification under relatively mild conditions. Slight modifications of the procedures described such as avoidance of lyophilization, use of 10% acetylglucosamine for wheat germ agglutinin elution, and greater amounts of anti-breast CEA antibody should allow greater yields. The usefulness of the rocket and radirocket IEP methods to follow protein purification was demonstrated.

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