PHYSICOCHEMICAL STUDIES OF THE CARCINOEMBRYONIC ANTIGENS OF THE HUMAN DIGESTIVE SYSTEM*

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Studies previously reported from this laboratory revealed that the human adenocarcinomas tested, arising from the entodermally derived digestive system epithelium, contained identical tumor-specific antigens (1, 2). Similar constituents were found in embryonic and fetal gut, pancreas, and liver during the first two trimesters of gestation. Because these antigenic components could not be detected in any other normal, diseased, or neoplastic human tissues, they were named carcinoembryonic antigens (CEA) of the human digestive system. It was suggested that the CEA may represent cellular constituents which are repressed during the course of differentiation of the normal digestive system epithelium and which reappear in the corresponding malignant cells by a process of derepressive dedifferentiation.

Preliminary experiments utilizing extraction in 0.6 M perchloric acid suggested that the CEA may be protein-polysaccharide complexes, and immunological data suggested that the tumor-specific antigenic determinants may contain carbohydrate components (3). More recent investigations demonstrated that the CEA are associated with the tumor cell surface (4) and are capable of inducing a tumor-specific antibody response in patients with primary digestive system cancers (5).

The objectives of the present investigation were (*a*) to attempt to purify and to characterize the chemical composition of the CEA found in a number of different digestive system cancers, (*b*) to compare the physical and chemical properties of the CEA obtained from different locations in different patients, and (*c*) to compare the CEA with identical fractions of comparable normal tissues.

Materials and Methods

Tumor Samples.—All specimens employed in the study were derived from adult tissues. Their sources are listed in Table I. Tissue specimens to be extracted were obtained either at

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operation or at autopsy within 12 hr of death. Tissue diagnoses were established by histologic examinations in all cases. Metastatic tumors were employed whenever possible in order to obtain large quantities of cancer tissue from single sources, thus eliminating the problems of allotypic variations within any single sample processed. In preparing specimen No. 6, the pool of primary large bowel tumors, and specimen No. 7, the pool of normal bowel mucosa, the pooling of samples was essential in order to obtain enough initial material for even a minimal number of analyses. Physicochemical studies are not feasible with embryos because of the small quantity of tissue available for investigation.

In processing specimens 1–5 and 8, a wet weight of 1000 g of tissue was employed in the initial extraction procedure for each specimen. For specimens 6 and 7, 56 g and 100 g of pooled tissue, respectively, were extracted.

Extraction Procedure.—The extraction procedure to be described is for 1000 g of tissue. The weights and volumes of reagents were decreased proportionately for specimens 6 and 7. Normal tissue remnants were removed as completely as possible from tumor specimens prior to extrac-

TABLE I

Samples Studied

Specimen No.	Source of tissue
1	Hepatic metastases from adenocarcinoma of the rectum
2	Pulmonary metastases from adenocarcinoma of the rectum
3	Hepatic metastases from adenocarcinoma of the colon
4	Omental metastases from adenocarcinoma of the cecum
5	Primary hepatoma
6	Pooled primary adenocarcinoma of the large bowel (7 donors)
7	Pooled normal colonic mucosa of the adult (2 donors)
8	Normal adult liver

tion. The presence or absence of CEA activity in the initial homogenate and at each stage of of the purification procedure was determined by precipitin-inhibition and direct Ouchterlony testing against specific anti-CEA antiserum (1, 2).

The tissue was cut into small pieces and put through a motor-driven meat grinder. The resultant mince was suspended in 2 liters of distilled water and homogenized in a water-cooled VirTis Chemixer at 15,000 rpm for 1 hr. An equal volume of 1.2 M perchloric acid was added to the homogenate and the mixture was stirred for 30 min at room temperature. The suspension was then centrifuged at 2000 rpm at 4°C for 15 min. The sediment was discarded. The supernatant was dialyzed in Visking tubing against cold running tap water for 24 hr and then concentrated under vacuum to a volume of 200 ml in a Rinco Rotovapor, model VE50GD, at room temperature. In order to clarify the concentrated dialyzed residue, it was centrifuged at 35,000 *g* at 4°C for 30 min in a Spinco model L preparative ultracentrifuge. The clear supernatant was decanted, dialyzed for 48 hr against four changes of distilled water, passed through a 0.22 μ Millipore filter (type GS), and finally lyophylized.

Purification of the Tumor-Specific Antigens.—Initial separation of the components of the lyophlized perchloric acid extract was performed by paper block electrophoresis on Shleicher and Schüll paper No. 2695 in borate buffer (pH 8.6, $\mu = 0.025$). A 130 ml volume of buffer was applied to the 30 cm × 20 cm paper block and allowed to distribute itself evenly. A 200 mg aliquot of a powdered perchloric acid extract was dissolved in 3 ml of distilled water and applied to the block in a line 13–15 cm long. The paper block was then placed in an electrophoresis apparatus similar to that described by Richter and Sehon (6). A drop of Gelman RBY

reference dye No. 51250 was placed below the sample to serve as a triple dye marker. Electrophoresis was performed at 400 v and a current varying from 18 to 20 ma at 4°C for 20 to 24 hr. After electrophoresis, the edges of the dye spots furthest from the line of sample application on the anodal side were 19 cm, 14 cm, and 1 cm for the Brilliant Blue, Amaranth, and Apalon Yellow markers, respectively. Separation of the components on the block was also followed visually by the use of shortwave ultraviolet light.

In the tumor specimens, the zones containing CEA activity were teased into small pieces and suspended in 50 ml of distilled water. The slurry was stirred for 10 min and filtered through a Gooch filter provided with a sintered glass disc. The residue was then washed with an additional 50 ml of distilled water. The total filtrate was dialyzed at 4°C against distilled water for 48 hr and finally lyophylized. In the case of normal tissue samples, identical areas of the paper block were eluted and processed.

The fractions obtained by the electrophoretic procedure were chromatographed on Sephadex G-200 at 4°C. The gel was previously washed by the usual method of decantation and then washed further with large volumes of distilled water under suction. In all samples except specimen 6, 50 mg of the sample dissolved in 3 ml of phosphate saline buffer (pH 4.5) was was applied to a 39 cm × 2.5 cm Pharmacia column (type K 25/45) filled with Sephadex G-200. The gel was previously equilibrated with the same buffer for 24 hr. The pressure was adjusted to give a flow rate of 20 ml/hr. The spectrophotometric absorption of the eluate at 280 mμ was constantly monitored and the eluate was collected in 4 ml fractions. Those fractions possessing CEA activity, and corresponding elution volumes of the normal tissue preparations, were dialyzed against distilled water at 4°C for 24 hr and finally lyophylized. The dried samples were stored in vacuo over calcium chloride. In the case of specimen 6, a 10 mg sample was processed in an identical manner.

Analytical Methods.—Carbohydrate analyses of the purified fractions were performed after hydrolysis of samples in 2 N HCl for 8 hr at 100°C in sealed Pyrex tubes. The acid was removed by a stream of nitrogen and the process of acid removal was repeated twice after the addition of water.

Qualitative descending paper chromatography was performed on Whatman No. 1 paper in solvent systems consisting of ethyl acetate:acetic acid:water (9:2:2) and ethyl acetate:pyridine:water (8:2:1). Each system was utilized for 24 hr sequentially at room temperature. The chromatograms were developed with the spray reagent *o*-aminodiphenyl (7).

Galactose and mannose determinations were carried out by the quantitative chromatographic method of Spiro on washed Whatman No. 1 paper (8). The solvent system was *n*-butanol:ethanol:water (10:1:2). The sugars were located by comparison with developed paper strips containing standard sugars. The appropriate areas of the experimental paper strips were removed, the sugars eluted, and analyzed by a micromodification of the Nelson-Somogyi copper reduction method. Optical density measurements were carried out in a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer. Fucose was determined by the method of Dische and Shettles (9). Confirmatory evidence for the presence of methylpentose was obtained by the addition of appropriate volumes of water to each sample and noting the corresponding decrease in optical density (10).

For quantitative determinations of sialic acid, the samples were hydrolyzed in 0.1 N H_2SO_4 at 80°C for 90 min and analyzed by the thiobarbituric acid method of Warren (11).

Amino acid analyses were performed by a modification of the technique described by Lea and Sehon (12). Samples of 1.5 to 2.0 mg were dissolved in 0.001 N HCl and added to the sulfonic acid exchange resin Amberlite IR-120 (1 g, hydrogen form). Hydrolysis was carried out in a sealed Pyrex tube at 100°C for 8 hr. The supernatant was removed and adjusted to a 6 N HCl concentration with 10 N HCl. The peptides and amino acids still on the resin were released by the further addition of 6 N HCl.

The two resulting solutions were heated to 100°C for 24 hr in sealed tubes. Acid was removed

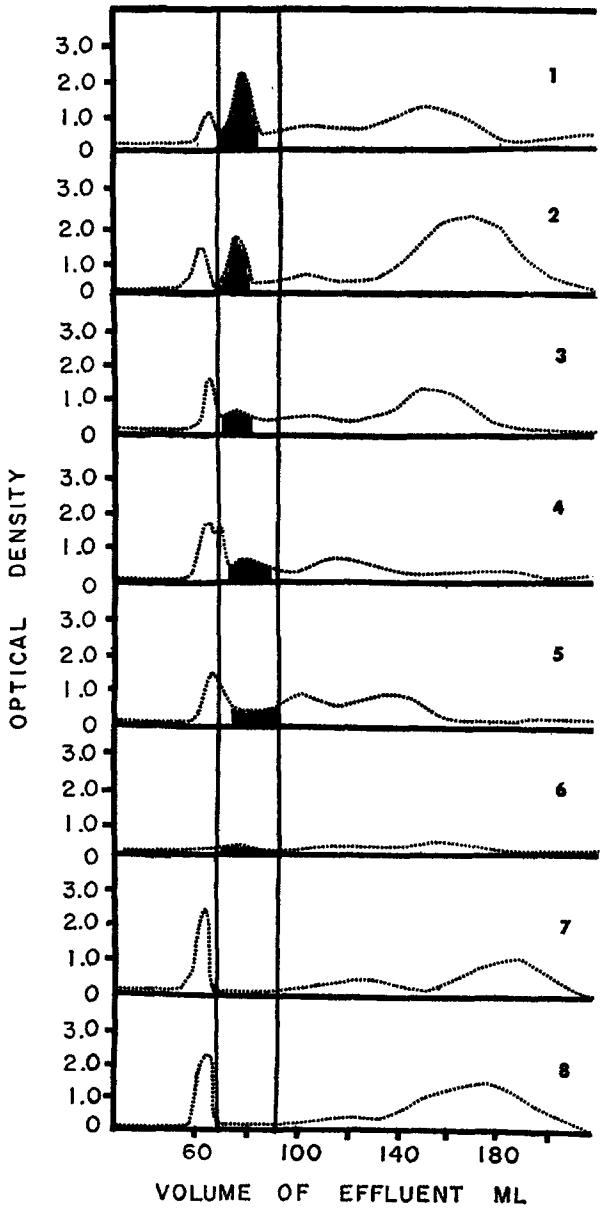

FIG. 1. Sephadex G-200 chromatography of the CEA preparation following extraction in 0.6 M perchloric acid and separation by paper block electrophoresis. The shaded areas indicate the zones containing antigenic activity.

and the samples taken to dryness as described above. The resultant fractions were then pooled and analyzed in a Technicon combination amino acid analyzer, model No. PNCL.

Two dimensional paper chromatography of the hydrolysate was carried out on Whatman No. 1 paper in solvent systems consisting of butanol:acetic acid:water (4:1:1) and pyridine:water (4:1).

TABLE II

Quantitative Amino Acid Composition of the Fractions Possessing CEA Activity

Amino acid	Micromoles of amino acid per mg CEA					
	Specimen No.					
	1	2	3	4	5	6
Asp	0.262	0.270	0.264	0.300	0.254	0.232
Thr*	0.180	0.200	0.194	0.190	0.234	0.176
Ser*	0.372	0.348	0.390	0.342	0.254	0.395
Glu	0.232	0.235	0.252	0.260	0.206	0.264
Pro	0.186	0.208	0.164	0.214	0.186	0.242
Gly	0.194	0.181	0.181	0.172	0.136	0.164
Ala	0.112	0.132	0.120	0.127	0.172	0.116
Leu	0.040	0.052	0.044	0.046	0.024	0.038
Leu	0.016	0.020	0.018	0.016	0.017	0.016
Tyr	0.024	0.053	0.057	0.037	0.022	0.027
Phe	0.024	0.032	0.030	0.040	0.022	0.050
Lys	0.036	0.056	0.034	0.042	0.024	0.060
His	0.072	0.049	0.070	0.057	0.022	0.070
Arg	0.066	0.041	0.062	0.062	0.024	0.067
½ Cys	—	—	—	—	0.050	—
Val‡	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

* The values were not corrected for losses during hydrolysis.

‡ Quantitative valine determinations were not possible due to the presence of a superimposed component, possibly galactosamine. (N.D., not determined.) Visual comparison with standards on paper chromatography suggest a valine content of not more than 0.1 μmoles.

Ultracentrifugal Studies.—Solutions of the samples possessing CEA activity in 0.1 M KCl were examined in a Spinco model E analytical ultracentrifuge at 25°C.

Immunoelectrophoresis.—Immunoelectrophoresis of the purified CEA preparation was performed against untreated rabbit anti-human colonic cancer antiserum as previously described (1).

RESULTS

Paper block electrophoresis of the perchloric acid extracts of both the cancerous and normal tissues resulted in resolution into a number of components detected by short-wave ultraviolet light. Although similarities were noted between specimens, each specimen had its own distinctive pattern. Regardless of the pattern, however, the antigenic activity of all tumor samples was contained in a 3 cm length of the paper block extend-

ing from 9 cm to 12 cm anodal to the line of application. No comparable antigenic properties were detected in identical zones of paper blocks on which the normal tissue specimens underwent electrophoresis.

Each of the fractions eluted from the 3 cm length of paper block, described above, manifested chromatographic heterogeneity on Sephadex G-200 (Fig. 1). In all of the tumor specimens, except for the primary hepatoma, antigenic activity was found in a portion of the eluate giving a definite peak at the spectrophotometric absorption wave length of 280 mμ. This peak began to appear after 68 to 72 ml of eluate had passed through the column. Depending on the tumor, the CEA-containing eluate was collected in a volume ranging from 12 to 16 ml. In the case of the primary hepatoma (specimen 5), no comparably distinct peak was found. However, a significant increase in optical density above the base line was observed in the CEA-containing region. The chromatographed fractions of normal bowel and liver showed no significant optical density at corresponding elution volumes.

TABLE III

Quantitative Carbohydrate Composition of the Fractions Possessing CEA Activity

Carbohydrate	μMoles of carbohydrate per mg CEA					
	Specimen No.					
	1	2	3	4	5	6
Fucose	0.834	0.965	0.902	1.060	0.623	-*
Mannose	0.282	0.208	0.342	0.367	0.417	-
Galactose	1.000	0.924	0.867	1.104	0.562	-
Sialic acid	0.156	0.278	0.109	0.305	1.046	-

* Not performed.

The quantity of purified CEA recovered differed with each of the tumor specimens. While 10–15 mg of purified material was obtained from each kilogram of metastatic tumor tissue (0.001–0.0015%), only 5 mg was recovered from the kilogram of primary hepatoma (0.005%). A 2 mg quantity was recovered from 56 g of pooled primary adenocarcinomata of the large bowel (about 0.004%).

Analyses of the Purified Fractions.—Hydrolysates of the purified samples obtained from normal bowel and liver (specimens 7 and 8) failed to stain with ninhydrin, indicating absence of significant amounts of amino acids in these fractions. Table II lists the quantitative amino acid composition of the tumor samples. The principal amino acid constituents present in all samples were aspartic acid, threonine, serine, glutamic acid, proline, glycine, and alanine. Leucine, isoleucine, valine, tyrosine, phenylalanine, lysine, histidine, and arginine were present in lesser amounts. Only the CEA fraction from the primary hepatoma contained a demonstrable amount of cysteine. Confirmation of the presence of these amino acids was obtained by qualitative paper chromatography.

Comparison of the hydrolysates from normal and tumor tissues by descending paper

chromatography revealed relatively minute quantities of monosaccharides in the normal tissue samples. Qualitatively, both the purified normal and tumor specimens contained galactose, mannose, glucose, glucosamine, galactosamine, and sialic acid. Fucose was detected in the tumor samples but not in those from normal tissues. The quantitative carbohydrate analyses are shown in Table III. In the metastatic tumor samples, the quantities of fucose and galactose were approximately equal and showed

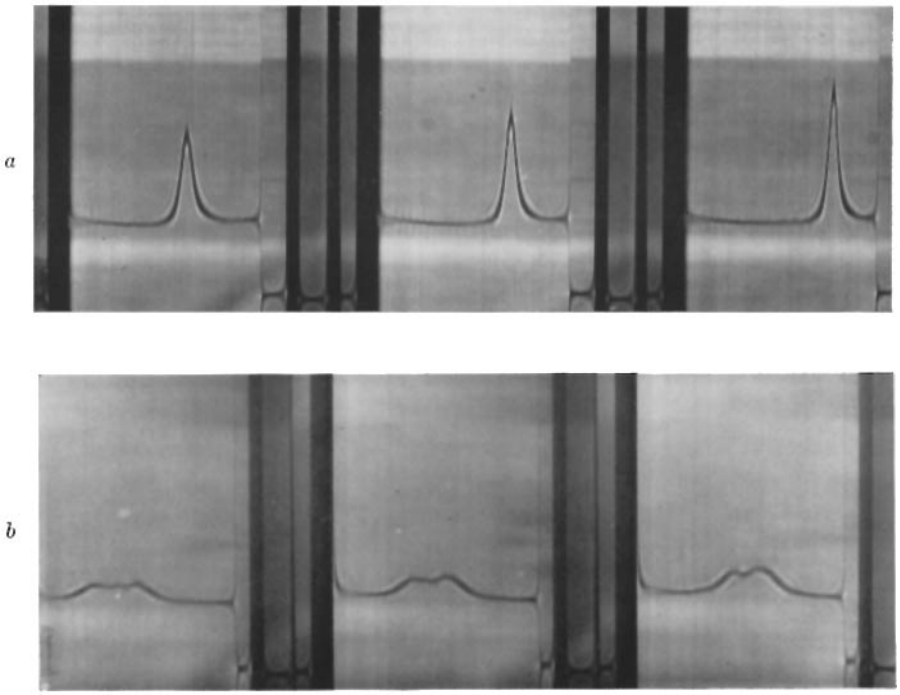

FIG. 2. Ultracentrifugal analysis of the purified CEA preparations. (*a*) Specimen 1 (hepatic metastases from adenocarcinoma of the rectum). Specimens 2–4 were identical to specimen 1. (*b*) Specimen 5 (primary hepatoma).

only minor variation from sample to sample. Mannose and sialic acid were present in relatively smaller quantities. The concentration of sialic acid was found to vary considerably in the different samples. The monosaccharide content of the specimen of primary hepatoma was distinctly different from that seen in the metastatic tumor samples, particularly with regard to the higher sialic acid content.

Ultracentrifugal Analysis.—Ultracentrifugal analysis of the fractions possessing CEA activity revealed the presence of a single peak for all metastatic tumors (Fig. 2). The sedimentation coefficients listed in Table IV range from 6.9S to 8.0S. Three peaks were observed for the CEA fraction from primary hepatoma with sedimentation value of 7.6, 6.0, 5.47S.

Immunoelectrophoresis.—As shown in Fig. 3, the CEA fractions from the metastatic tumors and primary adenocarcinomas gave a single band when reacted against non-absorbed rabbit antiserum. Two bands were observed with the CEA fraction from primary hepatoma (Fig. 3).

TABLE IV

Sedimentation Coefficients of the Fractions Possessing CEA Activity

Specimen No.	$S_{20,w}$
1	8.02
2	7.79
3	8.04
4	6.90
5	7.64, 6.00, 5.47

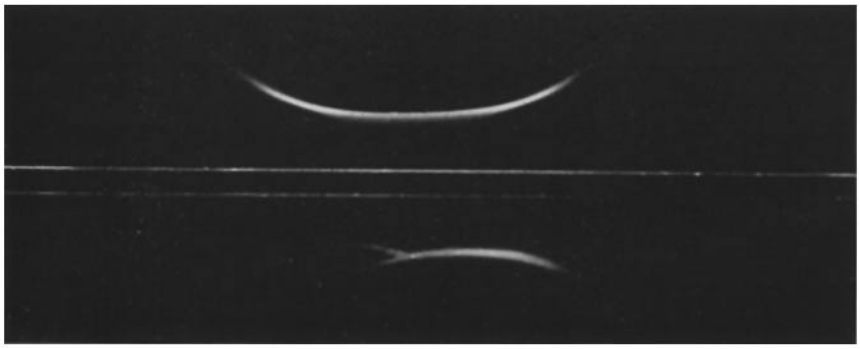

FIG. 3. Immunoelectrophoretic patterns of purified CEA preparations against untreated rabbit anti-human colonic cancer antiserum. The upper well contains purified CEA from specimen 1 (hepatic metastases from adenocarcinoma of the rectum) and the lower well contains purified CEA from specimen 5 (primary hepatoma).

DISCUSSION

In the present series of experiments, the use of perchloric acid extraction followed by paper block electrophoresis and column chromatography on Sephadex G-200 yielded highly purified final preparations (purified CEA) in which the carcinoembryonic antigenic components were concentrated approximately 100,000-fold. The degree of purity of the final samples obtained from the metastatic tumors is indicated by two findings. Each purified CEA preparation gave rise to a single peak in the ultracentrifuge and a single band against untreated rabbit anti-tumor antiserum on immunoelectrophoresis. However, the fact that the purified CEA contained components other than carcinoembryonic antigens is apparent from a number of observations.

It was found that there were relatively wide variations in the concentration of sialic acid amongst the various tumor specimens. Of particular significance was the difference in sialic acid content between the hepatic and pulmonary metastases from an adenocarcinoma of the rectum (specimens 1 and 2, respectively) obtained from the same patient at autopsy. This finding would suggest that the quantity of sialic acid in the purified CEA samples may be dependent on the tissue of growth of the tumor rather than on the tissue of origin, and that it may reflect the presence of constituents other than CEA.

The presence of components other than CEA in the purified fraction derived from the primary hepatoma (specimen No. 5) is suggested by the finding of three peaks in the ultracentrifugal analysis and two bands in the immunoelectrophoretic analysis of this specimen. The 7.6S peak in the ultracentrifuge corresponded with the CEA-containing peaks noted with the metastatic tumors. However, the other two peaks, of lower sedimentation coefficients may well represent non-CEA constituents. The material contained in all three peaks was originally eluted from a Sephadex G-200 column in the same, relatively small elution volume. Furthermore, the purified CEA derived from the hepatoma was obtained at the same effluent volume as all of the other purified CEA samples. Presumably then, all of the constituents of the CEA eluate from the hepatoma were initially of similar sedimentation coefficients of the order of 7–8S. The finding of cysteine exclusively in the purified CEA of primary hepatoma suggests the possibility of a breakdown of a 7–8S component, at a relatively labile disulfide linkage, to give rise to two unequal fragments of lower sedimentation coefficients.

Despite the probable presence of non-CEA components, the finding of a single band on immunoelectrophoresis would suggest that the purified CEA preparations derived from metastatic tumor specimens (specmens 1–4) are of relative antigenic purity. The contaminants indicated by the varying sialic acid concentrations are either of very low antigenicity or are present in very small quantities. Except for the sialic acid concentrations, all of the purified CEA samples derived from metastatic tumors had similar amino acid and monosaccharide contents. Thus, the present investigation has demonstrated the presence of a physicochemically distinct component(s) found only in human digestive system cancers, and absent from comparable fractions of normal tissues. The data previously obtained solely by immunological methods (1, 2) have therefore been confirmed by physicochemical techniques.

Recently, Karitzky and Burtin (13) described the partial characterization of a protein-polysaccharide complex isolated from human gastric cancer tissue. This substance was designated as the autoantigen responsible for the formation of autoantibodies in patients suffering from gastric cancer. The demonstration of such an antibody response had previously been reported by Burtin and his associates (14, 15). However, there are a number of differences between the

gastric autoantigen described by Burtin et al. and the purified CEA described in the present investigation.

First, the gastric autoantigen was found to be present in normal gastric mucosa, although in a much lower concentration than in gastric tumor tissue. Hence, the corresponding autoantibody activity found by Burtin et al. in the sera of gastric cancer patients was neutralzed by absorption with normal gastric mucosal extracts. The CEA, however, have not been detected in normal digestive system tissues by either the immunological techniques reported previously (1, 2), or by the physicochemical procedures described in this report. Furthermore, the anti-CEA antibody activity detected in the sera of patients with primary digestive system cancers could not be removed by absorption with highly concentrated normal digestive system tissue extracts (5).

Another striking difference between the gastric autoantigen and the CEA is that of molecular size as determined by analytical ultracentrifugal data. The sedimentation coefficient of the gastric autoantigen was found to be 1.83S while that of the CEA is 7–8S. Finally, Karitzky and Burtin found that the gastric autoantigen has a hexose content of 6%, while the carbohydrate analysis of the CEA would indicate that the hexose content is about four-fold greater.

The relative contributions of the protein and polysaccharide moieties toward the tumor-specific antigenicity of the CEA remain to be determined. Makari (16, 17) has postulated a central role for "Tumor Polysaccharide Antigenic Substances" (TPS) in all human tumor growth, but this concept, as initially proposed, has lacked substantial support (18, 19). Although previous immunologic data, from our laboratory (1, 3) suggested that the polysaccharide component of the CEA is involved in the tumor-specific antigenic determinant group, further studies are presently under way to evaluate this possibility more fully.

SUMMARY

A procedure has been described for the purification of the carcinoembryonic antigens (CEA) of the human digestive system. Tumor tissue extraction in 0.6 M perchloric acid followed by paper block electrophoresis and column chromatography on Sephadex G-200 resulted in highly purified CEA preparations as determined by both immunological and physicochemical criteria.

The properties and composition of five different purified CEA preparations derived from digestive system cancer metastases were examined. The findings demonstrated a high degree of uniformity amongst these samples. Sedimentation coefficients ranged from 6.9 to 8S. Each sample showed the presence of 14 different amino acid residues and six different carbohydrate constituents (four of which could be quantitated with the amount of material available for analyis). Studies of a purified CEA preparation from a primary hepatoma yielded results which, in some respects, differed from those obtained with the CEA samples of

metastatic tumor origin. The implications of these variations were discussed with regard to the probable presence of non-CEA components in the hepatoma preparation.

Of primary importance was the observation that the few normal adult digestive system tissues tested failed to show the presence of constituents similar to the CEA. This finding would seem to indicate that, in the adult, the carcinoembryonic antigens of the human digestive system are qualitatively tumor-specific and are not dectectable in comparable normal tissues.

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BIBLIOGRAPHY

1. Gold, P., and S. O. Freedman. 1965. Demonstration of tumor-specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. *J. Exptl. Med.* **121**:439.
2. Gold, P., and S. O. Freedman. 1965. Specific carcinoembryonic antigens of the human digestive system. *J. Exptl. Med.* **122**:467.
3. Gold, P., J. Jonson, and S. O. Freedman. 1966. The effect of the sequence of erythrocyte sensitization on the bis-diazotized benzidine hemagglutination reaction. *J. Allergy.* **37**:311.
4. Gold, P., M. Gold, and S. O. Freedman. 1968. Cellular location of carcinoembryonic antigens of the human digestive system. *Cancer Res.* In press.
5. Gold, P. 1967. Circulating antibodies against carcinoembryonic antigens of the human digestive system. *Cancer.* **20**:1663.
6. Sehon, A. H., M. Richter, J. G. Harter, and B. Rose. 1956. Studies on ragweed pollen. I. Preliminary fractionation of ragweed pollen. *J. Allergy.* **27**:236.
7. Timell, T. E., C. P. J. Glaudemans, and A. L. Currie. 1956. Spectrophotometric method for determination of sugars. *Anal. Chem.* **28**:1916.
8. Spiro, R. G. 1960. Studies on fetuin, a glycoprotein of fetal serum. I. Isolation, chemical composition and physicochemical properties. *J. Biol. Chem.* **235**:2860.
9. Dische, Z., and L. Shettles. 1948. A specific color reaction of methyl pentoses and a spectrophotometric micromethod for their determination. *J. Biol. Chem.* **175**:595.
10. Dische, Z., and L. Shettles. 1951. A new spectrophotometric test for the detection of methyl pentoses. *J. Biol. Chem.* **192**:579.
11. Warren, L. 1959. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* **234**:1971.
12. Lea, D. J., and A. H. Sehon. 1962. The chemical characterization of non-dialyzable constituents of ragweed pollen. *Int. Arch. Allergy Appl. Immunol.* **20**:203.
13. Karitzky, D., and P. Burtin. 1967. Isolement de l'autoantigene responsable de la formation d'autoanticorps chez les malades atteints de cancer gastrique. *European J. Biochem.* **1**:411.

14. Burtin, P., S. v., Kleist, W. Rapp, F. Loisillier, A. Bonatti, and P. Grabar. 1965. Auto-anticorps chez les cancereux. *Presse Med.* **73**:2599.
15. Kleist, S. v., and P. Burtin. 1966. On the specificity of autoantibodies present in colon cancer patients. *Immunology.* **10**:507.
16. Makari, J. G. 1958. The polysaccharide behaviour of cancer antigens. *Brit. Med. J.* **2**:355.
17. Makari, J. G. 1963. An immunologic unitarian concept of cancer. *J. Am. Geriat. Soc.* **11**:167.
18. Gluck, E. 1963. Immunohistochemical investigations of human neoplasia. *Acta, Unio Intern. Contra Cancrum.* **19**:154.
19. Prehn, R. T. 1963. Tumor-specific antigens and the homograft reaction. *Am. J. Surg.* **105**:184.