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Preparation and Properties of a Glycoprotein Associated with Malignancy†

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ABSTRACT: The protein and sialic acid contents of the perchloric acid soluble fraction of sera from cancer patients and normal volunteers indicate that patients with localized or metastatic malignancies have significantly elevated mean protein and sialic acid levels. Large volumes of plasma were obtained from cancer patients and normal volunteers by plasmapheresis, and the perchloric acid soluble material was collected. After subjection of this material to anion-exchange chromatography, isoelectric focusing over two different pH ranges, and affinity chromatography, ~100 μg of glycoprotein was isolated from 2500 mL of the cancer material. This material was radioactively labeled with [^{14}C]formaldehyde by reductive alkylation and with ^{125}I with chloramine-T. An apparent molecular weight of 50 000–55 000 was assigned on the basis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis using serum glycoproteins as molecular weight standards. A feature of the cancer glycoprotein was its affinity for wheat germ agglutinin (WGA) bound to Sepharose. Furthermore, it was found that the binding of the cancer glycoprotein was mediated by sialic acid since neuraminidase treatment of the bound material prevented its retention on the column. The WGA-binding fraction of the cancer glycoprotein was also retained on a *Ricinus communis* agglutinin I (RCA I) column, whose ligand specificity is terminal galactose. Neuraminidase treatment of the WGA-bound cancer glycoprotein resulted in quantitative retention on the RCA I column; the cancer glycoprotein was either not retained or poorly retained by a concanavalin A–Sepharose column. Reaction of the cancer glycoprotein with various antisera directed against normal serum glycoproteins established that it was not one of the major acute-phase reactant glycoproteins associated with malignancy and could be distinguished from carcinoembryonic antigen and α-fetoprotein by its molecular weight and chromatographic behavior.

Changes in normal serum glycoprotein levels have long been associated with malignancy; α_1-acid glycoprotein, α_1-antitrypsin, ceruloplasmin, and haptoglobin, members of the class of serum proteins known as the acute-phase reactants, have been correlated with neoplastic disease (Cooper & Stone, 1979). While changes in serum levels of these glycoproteins comprise the majority of the increase in protein-bound carbohydrate in serum in malignancy, tumors themselves have been shown to produce glycoproteins either not normally found in serum or found in very low amounts. In addition, elevations in sialic acid content cannot be accounted for by the rise in concentrations of these glycoproteins. The best studied of the tumor-associated glycoproteins are the fetal antigens carcinoembryonic antigen (Gold & Freedman, 1965a,b) and α-fetoprotein (von Kleist & Burtin, 1969; Abelev et al., 1963).

While some tumor antigens are secreted, as in the case of α-fetoprotein, others appear in the circulation by being shed from the cell surface of the tumor cells, a process which may be mediated by proteolysis. This cell-surface shedding of the tumor glycoproteins may serve a protective role for the cells, enabling them to escape the immune surveillance of the host. Thus, it has been suggested that shedding of epiglycanin, the major sialoglycoprotein of TA3-Ha mouse adenocarcinoma cells, blocks immune response to the tumor (Cooper et al., 1974).

A variety of cultured cells secrete or shed glycoproteins into their growth media. Chick embryo cells labeled with *N*-[^{3}H]acetylglucosamine have been shown to shed glycopeptides into the tissue culture media (Lapeller et al., 1973). Similarly, antigens associated with B16 mouse melanoma grown in culture and labeled with [^{3}H]leucine appear to be secreted into the medium (Bystryn, 1976; Bystryn et al., 1974; Bhavanandan et al., 1980). Glycopeptides produced by B16 mouse melanoma cells in culture have been isolated from the cells and the spent media and have been well characterized (Bhavanandan et al., 1977; Fareed et al., 1978; Bhavanandan & Davidson, 1976). The glycopeptides produced by the B16 melanoma, as well as similar glycopeptides produced by human melanoma and human mammary carcinoma cells, were apparently not expressed by comparable control cell populations (Chandrasekaran & Davidson, 1979). The characteristics of this type of sialoglycopeptide include a relatively high sialic acid content, an affinity for wheat germ agglutinin, linkage of the saccharide moiety via GalNAc[1] and a Pronase-resistant core structure

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[1] Abbreviations used: GalNAc, *N*-acetylgalactosamine; PCA, perchloric acid; DEAE, diethylaminoethyl; WGA, wheat germ agglutinin; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; α_1-AGP, α-acid glycoprotein; $NaDodSO_4$, sodium dodecyl sulfate.

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containing a cluster of saccharide units.

The finding that glycopeptides and glycoproteins are shed into the culture medium from tumor cells prompted the examination of serum from cancer patients and normal volunteers for the presence of sialoglycoproteins similar to those isolated from the medium of cultured malignant cells. We examined the levels of protein and sialic acid in the perchloric acid soluble fraction of sera from normal volunteers, cancer patients, and patients with nonmalignant diseases. Serum sialic acid and protein levels were compared among patients with widespread malignant disease, with localized and regionalized malignant disease, and on adjuvant chemotherapy following surgery. The data showed a clear correlation between sialic acid levels and tumor burden (Lipton et al., 1979; Harvey et al., 1981).

Large volumes of plasma were collected from cancer patients and normal volunteers by plasmapheresis and were treated with perchloric acid, and the soluble material was fractionated by ion-exchange chromatography and isoelectric focusing. Although an explicit biological property (e.g., enzyme activity) was not present, the purification strategy assumed properties analogous to those previously defined for the products of cultured mouse and human tumor cells (Bhavanandan et al., 1977; Chandrasekaran & Davidson, 1979). Parallel treatment of normal and cancer samples allowed direct assessment of differences.

Materials and Methods

Proteins used as standards in polyacrylamide gel electrophoresis, isoelectric focusing, and affinity chromatography came from the following sources: bovine serum albumin, transferrin, ceruloplasmin, fibrinogen, human serum albumin, and α_1-antitrypsin were from Sigma Chemical Co., St. Louis, MO; human α_1-acid glycoprotein was the generous gift of Professor Karl Schmid, Boston University, Boston, MA; [^{3}H]acetylglycophorin was from Dr. V. P. Bhavanandan, The Milton S. Hershey Medical Center, Hershey, PA. Chromatography media and reagents were obtained from commercial sources. Wheat germ agglutinin was purchased from Vector Laboratories, Burlingame, CA, or was prepared in this laboratory from crude wheat germ (Bhavanandan & Katlic, 1979) by a modification of the method of Nagata et al. (1974). *Ricinus communis* agglutinin I (M_r 60 000)– and agglutinin *II*–Sepharose 4B affinity columns and Phytohemagglutinin-E were also purchased from Vector. Rabbit anti-goat IgG and goat anti-rabbit IgG immunobeads were purchased from Miles Laboratories, Elkhart, IN, as were antisera to the normal human serum proteins and glycoproteins, serum albumin, transferrin, ceruloplasmin, fibrinogen, α_1-acid glycoprotein, haptoglobin, and α_1-antitrypsin. All radioactive chemicals were purchased from New England Nuclear, Boston, MA.

Large volumes of plasma were collected from normal donors and cancer patients by plasmapheresis. The latter were selected on the basis of serum sialic acid content (Lipton et al., 1979). Informed consent was obtained from all volunteers. Approximately 1 L of whole blood was collected in acid citrate–dextrose solution and the blood centrifuged to separate the cells and the plasma. The cells were resuspended and returned to the patients by transfusion. The yield of plasma from a single patient was routinely >500 mL. The plasma was stored at 4 °C and used within 36 h of collection. Additional normal plasma was obtained from the Hershey Medical Center blood bank.

Perchloric acid precipitation was performed by a modification of the method of Krupey et al. (1968). For analysis of small volumes of serum, 1 mL of serum was chilled on ice, and an equal volume of 1.2 M perchloric acid (PCA) added dropwise with stirring. The mixture was incubated in an ice bath for 20 min with occasional agitation and centrifuged at 10000*g* for 10 min. The pellet was dicarded and the supernatant neutralized by the addition of 1.2 M KOH with phenol red as an indicator. The potassium perchlorate was removed by centrifugation at 10000*g* for 2 min and the volume of the supernatant determined. The sialic acid content of the PCA-soluble material was determined by the periodate–resorcinol method of Jourdian et al. (1971) for total sialic acid. *N*-Acetylneuraminic acid was used as standard. Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard.

For the preparative isolation of PCA-soluble material, 400–600 mL of plasma from a single donor was processed as above. The supernatant was dialyzed against several changes of distilled water, lyophilized, and stored at −20 °C.

DEAE-Sephadex A-25 was equilibrated with 0.01 M pyridinium acetate, pH 5.2, and packed in a 3 × 47 cm column. The PCA-soluble fraction from 500 mL of serum was reconstituted in 10 mL of the 0.01 M pyridinium acetate and applied to the column. The column was then eluted with a 1-L linear gradient of pyridinium acetate from 0.01 to 1.0 M, and 8-mL fractions were collected and assayed for protein and sialic acid. The major peak fractions were combined, dialyzed against distilled water, and lyophilized. Sephadex G-25 and G-150 were equilibrated with 0.1 M pyridinium acetate pH 5.2, or with 0.15 M NaCl.

Analytical isoelectric focusing in polyacrylamide gel was performed in a Multiphor apparatus from LKB, Bromma, Sweden. For the pH range 2.5–6 a 3% polyacrylamide gel was poured containing ampholytes pH 2.5–4, 4–6, and 5–7 in a ratio of 2:2:1 to give a final concentration of 1%. The electrode solutions were 1 M H_3PO_4, anode, and 0.5% ampholyte, pH 5–7, cathode. Samples were applied in triplicate via vilter paper strips and focused for 2.5–3 h at 4 °C at 5 W of constant power (Karlsson et al., 1973). The gels were stained for protein with 0.3% Coomassie Blue G-250 in 45% methanol–10% acetic acid overnight and destained with 30% methanol–7% acetic acid.

Preparative isoelectric focusing was performed in either a 110- or 440-mL column from LKB by a modification of the method of Vesterberg & Svenson (1966), at 5 W (110-mL column) or 10 W (440-mL column) of constant power for 30 h at 4 °C. After focusing, 1- or 4-mL fractions, from the 110- and 440-mL columns, respectively, were collected and monitored for protein absorbance at 280 nm, and the pH was determined. Peak fractions were combined, dialyzed, and concentrated, and residual ampholyte was removed by gel filtration on Sephadex G-25 (1 × 15 cm column) equilibrated with 0.15 M NaCl.

Wheat germ agglutinin–Sepharose 4B or 6B was prepared by using cyanogen bromide activation of the gel (Cuatrecasas & Anfinsen, 1971); columns contained 2–3 mg of bound WGA/mL of gel. Glycoprotein behavior was substantially the same on the two column types. The gel was collected by filtration and washed several times with 0.1 M sodium acetate–1 M NaCl, pH 4.0, and 0.1 M Tris–1 M NaCl, pH 8.0. The lectin column was equilibrated with 0.5 M Tris buffer, pH 8.0, and bound protein eluted with 0.05 M Tris buffer, pH 8.0, containing 0.1 M *N*-acetylglucosamine.

The *R. communis* agglutinin I (RCA I)–Sepharose 4B was equilibrated with 0.1 M phosphate buffer, pH 7.4, containing 0.15 M NaCl; bound protein was eluted with this buffer containing 0.1 M lactose. The concanavalin A (Con A)–Sepharose 4B was equilibrated with 0.1 M Tris buffer, pH 7.2,

containing 0.15 M NaCl, and bound protein was eluted from the gel with this buffer containing 0.1 M α-methyl mannoside.

Sialic acid was removed from glycoproteins either by mild acid hydrolysis, 0.2 N H_2SO_4 for 1 h at 80 °C, or by *Vibrio cholerae* neuraminidase (VCN) treatment. Enzymatic release of sialic acid was performed in 0.1 M sodium acetate buffer, pH 5.6, with 0.001 M $CaCl_2$, at 37 °C for 18–24 h. Release of sialic acid was followed by using α_1-AGP as substrate; the reaction was complete after 18 h. Free sialic acid was separated from the glycoprotein by gel filtration on Sephadex G-25. Asialo-α_1-AGP used in many of the experiments as a standard was prepared by treatment of 52 mg of α_1-AGP with 75 units of *V. cholerae* neuraminidase in a 3-mL reaction volume as described above.

$NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to the method of Weber & Osborn (1969) using 1 × 10 cm gels with 6% cross-linking. The following proteins and glycoproteins were used as molecular weight standards: cytochrome *c* (12 800), α_1-AGP (40 000), asialo-α_1-AGP (~37 000), α_1-antitrypsin (54 000), bovine serum albumin (66 000), transferrin (76 500), and ceruloplasmin (151 000). The proteins were denatured before electrophoresis by incubation in phosphate-buffered saline, pH 7.0, containing 1% $NaDodSO_4$ for 2 h at 40 °C or for 30 min at 80 °C. Electrophoresis was carried out at 70 V of constant voltage for 3–4 h, until the tracking dye, bromphenol blue, was within 2 cm of the bottom of the gels. Protein bands were stained with Coomassie Brilliant Blue G-250.

Radioactive labeling of glycoproteins was performed as follows. (a) On sialic acid, sialic acid residues of sialoglycoproteins were modified to the 7 and 8 carbon analogues and labeled with sodium borotritide as described by Van Lenten & Ashwell (1972). (b) On protein, reductive methylation with [^{14}C]formaldehyde was performed. Lysine residues in proteins were converted to dimethyllysine derivatives and labeled with [^{14}C]formaldehyde and sodium borohydride by a modification of the method of Winklehake (1977). Sample proteins were dissolved in 0.2 M borate buffer, pH 8.0, and the solution was chilled on ice. A 3-fold molar excess of [^{14}C]formaldehyde (10 mCi/mmol) compared to lysine was added and the solution stirred on ice under a stream of nitrogen gas for 5 min. A 1.5-fold molar excess of sodium borohydride compared to formaldehyde was then added and the solution stirred under nitrogen gas for 5 min on ice, followed by 5 min at room temperature. The reaction was terminated by the addition of 1.0 M Tris buffer, pH 6.0. The solution was dialyzed against the following solutions: 0.1 M Tris, pH 6.0, then 0.05 M Tris, pH 8.0, and finally distilled water. By use of α_1-AGP as a model, >95% of the starting material was recovered with a specific activity of 6.6 μCi/mg. (c) On protein, iodination was performed. Three methods of iodination were examined: (1) Bolton–Hunter (Bolton & Hunter, 1973), (2) lactoperoxidase, and (3) chloramine-T (Hunter & Greenwood, 1962). The latter procedure was found most suitable.

For iodination of protein using chloramine-T, the protein was dissolved in 100 μL of 0.2 M phosphate buffer, pH 7.5. Chloramine-T (50 μg) was added, followed by the addition of 1.25 mCi of Na^{125}I. The reaction was immediately terminated by addition of sodium metabisulfite and cold NaI. Protein-bound iodine and free iodine were separated by gel filtration on G-25 Sephadex. A total of 87% of the α_1-AGP labeled was recovered with a specific activity of 41 μCi/μg.

Antibodies to α_1-AGP and to the glycoprotein obtained after isoelectric focusing were raised in female New Zealand White rabbits. The rabbits were bled to collect preimmune serum prior to multisite multiroute injection of the proteins suspended in 2 mL of Freund's complete adjuvant (Garvey et al., 1977). The amounts of injected protein were as follows: 1.2 mg of α_1-AGP and 74.1 μg of Cc. The rabbits were bled by ear vein puncture 25 days later, with collection of ~30 mL of blood from each rabbit. Blood was allowed to clot for 2–3 h at 4 °C, centrifuged at 850*g* for 20 min, divided into 5-mL aliquots, and stored at −70 °C.

Table I: Protein and Sialic Acid Content of Plasma Collected by Plasmapheresis from Cancer Patients and Normal Volunteers

source	sample	mean (mg/mL)
normal (75 samples)	crude plasma protein	72.8 ± 9.2
	crude plasma sialic acid	0.85 ± 0.09
	perchloric acid soluble protein	0.53 ± 0.11
	perchloric acid soluble sialic acid	0.064 ± 0.01
cancer (150 samples)	crude plasma protein	73.6 ± 8.7
	crude plasma sialic acid	1.22 ± 0.25
	perchloric acid soluble protein	1.34 ± 0.49
	perchloric acid soluble sialic acid	0.13 ± 0.04

Radioimmunoprecipitation was performed by incubating either ^{14}C- or ^{125}I-labeled antigen with antibody and separating the antigen–antibody complex from free antigen, either by precipitation with 50% saturated ammonium sulfate (Minden & Farr, 1978) or by reaction with immobilized second antibody, goat anti-rabbit IgG bound to agarose beads. When ^{14}C was counted, the pellets were dissolved in phosphate-buffered saline and transferred to scintillation vials for counting; ^{125}I-labeled samples were counted directly.

Amino acid analyses were performed on 24- or 72-h 6 N HCl hydrolysates by utilizing an automated chromatographic system. Results from different preparations were within experimental error.

Results

Cancer patients with PCA-soluble protein and sialic acid levels >1.50 and 0.150 mg/mL, respectively, were chosen for plasmapheresis. Protein levels in the unfractionated plasma of the cancer patient and normal donors were similar, whereas the sialic acid levels in the cancer patients were higher than those of the normal volunteers as shown in Table I. Following PCA precipitation, the protein levels in the normal PCA-soluble supernatant dropped to 0.6–0.8% of the unfractionated levels and the sialic acid to 6–9% of the untreated levels; the PCA-soluble fraction from cancer patients contained 1.1–2.2% of the protein of untreated plasma and 6.7–25.5% of the sialic acid of the unfractionated plasma. The sialic acid content was related to the stage of malignancy (Lipton et al., 1979).

The results shown in Figure 1 are typtical of chromatography of the PCA-soluble material from cancer and normal donors. The recovery of protein and sialic acid from the column was routinely 85–90%, and there were no qualitative differences between the cancer and normal elution patterns. The material which came through the column at the start of the gradient was high in protein and low in sialic acid and was not retained on rechromatography. The second peak, B, contained little sialic acid compared to protein. Consequently, further investigation concentrated on the major fraction, containing 68% of the protein and 95% of the sialic acid applied to the column. Although this fraction could be arbitrarily divided into three parts, isoelectric focusing experiments showed that these contained the same material, and in most experiments this peak was collected as a whole. The major

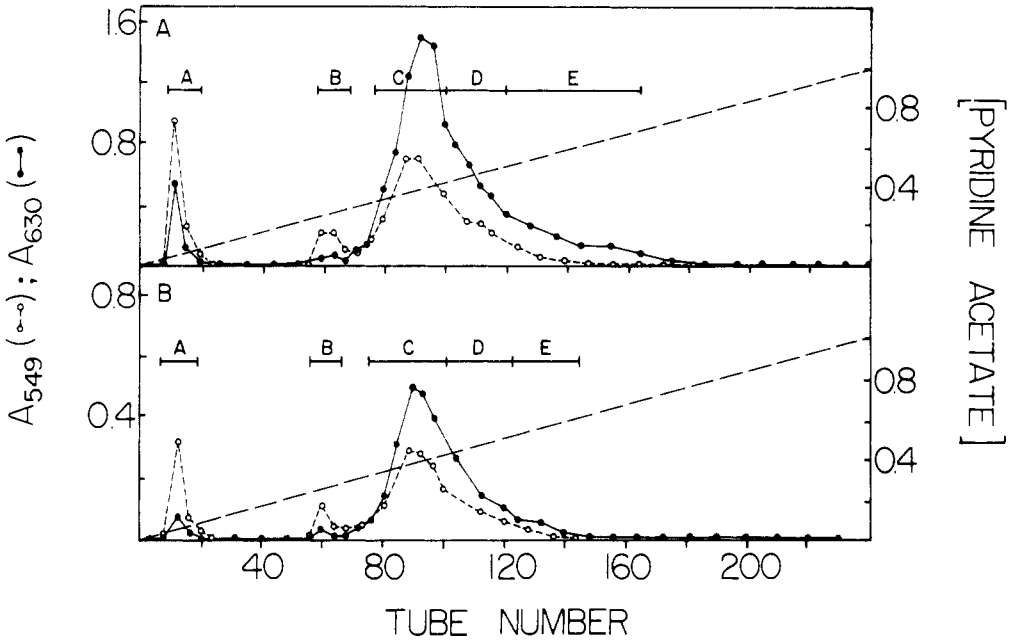

FIGURE 1: DEAE-Sephadex chromatography of perchloric acid soluble fraction of (A) cancer plasma and (B) normal plasma. A solution containing 100–300 mg of protein in 0.01 M pyridinium acetate, pH 5.2, was applied to a DEAE-Sephadex A-25 column (3 × 47 cm) equilibrated in the same buffer. After the column was washed with 50 mL of starting buffer, material was eluted with 1000 mL of a linear gradient of 0.01–1.0 M pyridinium acetate. Fractions (8 mL) were collected and aliquots assayed for protien and sialic acid. Symbols: (○) A_{549}, sialic acid; (●) A_{630}, protein.

Table II: Isoelectric Points of Cancer and Normal Proteins Separated by Isoelectric Focusing, pH Range 4–6

source	constituent	p*I*	source	constituent	p*I*
cancer	Ca	4.0	normal	Na	4.0
	Cb	4.1		Nb	4.1
	Cc	4.4		Nc	4.55
	Cd	4.8		Nd	4.8
	Ce	5.0		Ne	5.0
	Cf	5.2		Nf	5.2
	Cg	5.5			

sialic acid containing material (Figure 1, peak CDE) chromatographed as a single peak on G-150 Sephadex; no component resolution was observed.

It was expected that a large proportion of the material retained on the DEAE-Sephadex column was α_1-AGP, since this protein elutes from the column at ~0.38 M pyridinium acetate. α_1-AGP has an isoelectric point which varies somewhat with sialic acid content, but under typical conditions, the p*I* is between 2.7 and 2.9 (Kawai, 1973). An analytical isoelectric focusing gel of the DEAE-Sephadex fraction CDE over the range 2.5–6 showed two bands. One focused at pH 2.8 and comigrated with α_1-AGP; the second was more diffuse, stained more heavily in the cancer-derived material, and focused over the pH range 3.7–5.5. There was no material detected at pH >6.

Accordingly, the sialic acid containing peak from the DEAE-Sephadex column was subjected to preparative isoelectric focusing. The results of a typical experiment with cancer and normal material are shown in Figure 2. The peak at p*I* 2.8 in both experiments was shown to be α_1-AGP by analytical isoelectric focusing and by polyacrylamide gel electrophoresis. In general, the pattern from the cancer-derived material showed an increased percentage of material in the pH range 4–6.

The material that focused in the pH 4–6 range was combined and subjected to preparative isoelectric focusing over the pH range 4–6. The results are summarized in Table II and Figure 3. The cancer plasma contained material at pH

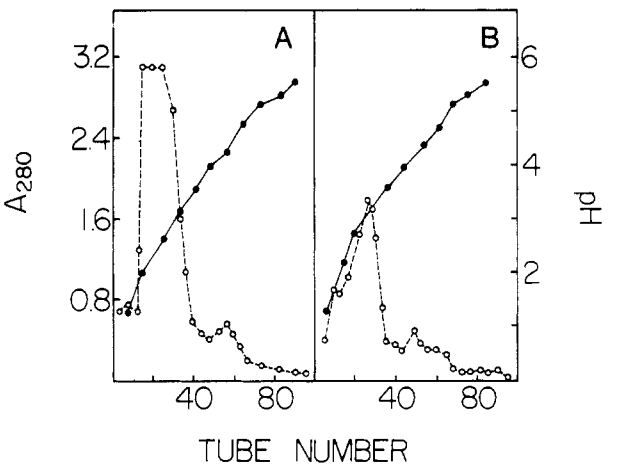

FIGURE 2: Isoelectric focusing, pH range 2.5–6, of the major sialic acid containing peak from DEAE-Sephadex chromatography of (A) cancer and (B) normal material. Symbols: (○) A_{280}; (●) pH. Protein (50–100 mg) was dissolved in the heavy density gradient solution and introduced into the LKB 110-mL column during the formation of the sucrose density gradient. The anode and cathode solutions were 1 M H_3PO_4 and 0.5% ampholyte, pH 5–7, respectively, with the anode at the bottom. Focusing was carried out at 4 °C at 5 W of constant power for 30 h. Fractions (1 mL) were collected and monitored for pH and for absorbance at 280 nm (protein).

4.4 apparently not present in normal plasma. This result was reproducible in other isoelectric focusing runs in that the single consistent difference between the cancer and normal material was the protein focusing at pH 4.4, designated Cc; the peak noted at pH 5.5 did not appear routinely and was not further studied.

The individual fractions from the focusing runs were dialyzed exhaustively against distilled water, and residual ampholytes were removed by gel filtration. The yield of Cc glycoprotein was ~100 μg from 2.5 L of plasma. The initial isolation utilized material pooled from two patients with breast cancer and one patient with colon cancer. Fifteen subsequent preparations from single sources indicated concentration ranges

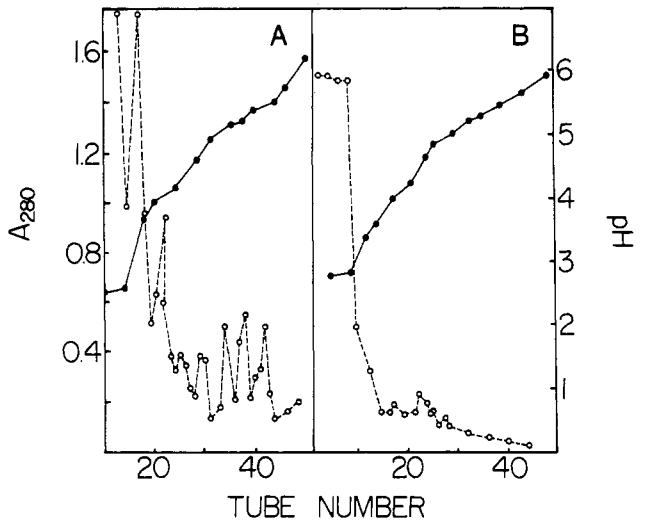

FIGURE 3: Isoelectric focusing, pH range 4–6, of (A) cancer and (B) normal material focusing above pH 3.0 in the previous isoelectric focusing experiment, pH range 2.5–6. Symbols: (○) A_{280}; (●) pH. Protein (10 mg) was dissolved in the heavy density gradient solution, introduced into the LKB 440-mL column, and focused for 30 h at 4 °C at 10 W of constant power. The anode and cathode solutions were 0.5% ampholyte, pH 2.5–4 and 5–7, respectively, with the anode at the bottom. Fractions (4 mL) were collected and monitored for protein and pH. Peak fractions were combined, dialyzed, and concentrated. There was a peak at pH 4.4 which is present in the cancer fraction but not in the normal.

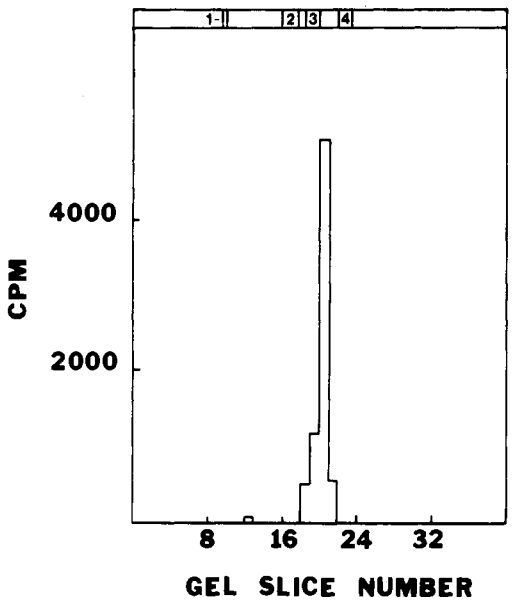

FIGURE 4: Polyacrylamide gel electrophoresis of [^{125}I]CcW using glycoprotein molecular weight markers. A 200-μL aliquot of [^{125}I]CcW was incubated with 5 μL of 5% $NaDodSO_4$ and 0.04% bromophenol blue for 2 h at 40 °C. A 10-μL sample was then applied to the 6% gels and electrophoresis carried out at 70 V until tracking dye ran to within 2 cm of the bottom of the gel. Glycoprotein molecular markers are (1) ceruloplasmin (151 000), (2) transferrin (76 500), (3) α_1-antitrypsin (55 000), and (4) α_1-AGP (40 000).

of 30–200 ng of Cc/mL of plasma.

Because the amount of isolated normal and cancer proteins from the pH 4–6 isoelectric focusing experiments was small, it was considered essential to radioactively label the components for subsequent analytical studies. Between 4 and 45 μg of protein components obtained from the pH 4–6 focusing experiments was labeled. The products had specific activities between 0.156 and 0.539 μCi/μg. Although iodination using the Bolton–Hunter reagent was successfully used to label the Gp52 glycoprotein produced by mammary carcinoma cells (Ritzi et al., 1976), the Cc protein labeled in this way did not react with anti-Cc antiserum. The chloramine-T procedure gave quantitative protein recovery with specific activities in the range of 22 μCi/μg.

Polyacrylamide gel electrophoresis of [^{14}C]Cc gave one sharp band whose apparent molecular weight was between 50 000 and 70 000, whereas the Nc protein (nearest analogous product) had an apparent molecular weight of 25 000. The Cd component had a molecular weight of ~13 500, the same as that of the Nd protein. The other fractions from the focusing experiments were examined and found to have apparent molecular weights different from that of the Cc protein.

Previous studies have shown that the sialoglycopeptides prepared from glycoproteins produced by B16 mouse melanoma cells in culture have an affinity for wheat germ agglutinin (Bhavanandan et al., 1977; Bhavanandan & Davidson, 1976) which is dependent on the number and spatial array of the sialic acid residues. Accordingly, we investigated the WGA affinity of several of the isolated components. A total of 81% of α_1-AGP applied was bound to a WGA–Sepharose column and eluted with 0.1 M *N*-acetylglucosamine. Contrastingly, when 88% of the sialic acid was removed from the α_1-AGP by neuraminidase treatment, only 6% of the applied asialo-α_1-AGP was retained on the column.

The Cc fraction was the only one in which significant material was bound to the WGA–Sepharose column; the bound fraction was designated CcW. Reapplication of the unbound material did not result in retention of any additional radioactivity. The CcW was dialyzed, concentrated, and reapplied to the WGA column; this material was completely retained and could be quantitatively eluted with 0.1 M *N*-acetylglucosamine.

[^{125}I]Cc, α_1-[^{14}C]AGP, and [^{3}H]acetylglycophorin were tested for WGA affinity by chromatography on a WGA–Sepharose 6MB column. A total of 81.2% of the α_1-AGP and 78.4% of the glycophorin were bound by the WGA column and eluted with *N*-acetylglucosamine. When [^{125}I]Cc was applied, 49.4% of the radioactivity was retained in good agreement with the 47% of the [^{14}C]Cc bound to the WGA column in the previous experiment.

$NaDodSO_4$–polyacrylamide gel electrophoresis was performed to determine an apparent molecular weight of the WGA-bound (CcW) and unbound material. The results indicate that both fractions appear as single molecular weight species. Comparison with several serum glycoproteins used as molecular weight standards is shown in Figure 4. The CcW migrated slower than α_1-AGP (40 000) and slightly faster than α_1-antitrypsin (55 000), indicating an apparent molecular weight of ~50 000 for [^{125}I]CcW; CcW was also shown to migrate differently from transferrin (76 500) and ceruloplasmin (151 000).

In order to determine whether the WGA affinity of the [^{125}I]CcW was mediated by sialic acid, we diluted a solution of [^{125}I]CcW in a volume of 300 μL 1:1 with 0.2 M sodium acetate, pH 5.6, containing 2 mM $CaCl_2$. *V. cholerae* neuraminidase (12.5 units) was added and the mixture incubated at 37 °C for 18 h; a second aliquot of enzyme was added and the incubation continued for an additional 24 h. α_1-AGP was treated in a parallel experiment as was an aliquot of the cancer-derived material labeled in the sialyl residues by periodate oxidation followed by reduction with NaB^3H_4 (Van Lenten & Ashwell, 1972). The bulk (86%) of the neuraminidase-treated CcW was no longer bound to WGA–Sepharose in concordance with the properties of the asialo-α_1-AGP. The CcW at this stage was considered to be substantially sialic acid free. The data from the WGA affinity

Table III: Summary of WGA-Sepharose 6MB Affinity Chromatography Experiments

sample	%[a] unbound	%[a] bound	%[b] recovery
α_1-[^{14}C]AGP	18.8	81.2	98.2
asialo-α_1-[^{14}C]AGP	93.4	6.6	100.2
[^{3}H]acetylglycophorin	21.6	78.6	84.0
[^{125}I]Cc	50.6	49.4	85.5
[^{125}I]CcW, dialyzed and reapplied	9.1	90.8	93.3
[^{125}I]CcW, treated with VCN once	50.9	49.1	99.0
[^{125}I]CcW, treated with VCN twice	86.4	13.5	89.5

[a] Percent of recovered counts. [b] (Recovered counts/total counts applied) × 100.

Table IV: Summary of RCA I-Sepharose 4B Affinity Chromatography Experiments

sample	%[a] unbound	%[a] bound	%[b] recovery
α_1-[^{14}C]AGP	50.7	49.3	83.7
asialo-α_1-[^{14}C]AGP	0	100	85.0
[^{3}H]acetylglycophorin	21.8	78.2	89.2
[^{125}I]CcW	12.8	87.2	90.1
[^{125}I]CcW, treated with VCN	0	100	85.0
WGA-unbound [^{125}I]Cc	92.4	7.6	86.1
WGA-unbound [^{125}I]Cc, treated with VCN	87.5	12.5	85.1

[a] Percent recovered counts. [b] (Recovered counts/total counts applied) × 100.

chromatographic experiments are summarized in Table III.

An RCA I–Sepharose 4B column, whose ligand specificity is for terminal β-D-galactosyl residues, was tested for reactivity with asialo-α_1-[^{14}C]AGP and [^{3}H]asialoglycophorin (Codington et al., 1975; Sharon, 1975). A total of 100% of the asialo-α_1-AGP was retained on this column and could be eluted with 0.1 M lactose; 78% of the asialoglycophorin was similarly retained on the column. α_1-[^{14}C]AGP which had not been neuraminidase treated was not well retained on the RCA-I column; 49.3% of the material was bound.

The percentage of [^{125}I]CcW bound to RCA-I was increased to 100% by treatment with neuraminidase. From the results of the WGA affinity chromatography, it was thought that the [^{125}I]WGA-unbound material might be a partially desialylated counterpart of the WGA-binding material. However, when the WGA-unbound [^{125}I]Cc was applied to the RCA-I column, only 7.6% of the applied radioactivity was retained. Further, neuraminidase treatment increased the percentage of retained counts to only 12.5%. A summary of the RCA-I affinity chromatography experiments is shown in Table IV.

Con A–Sepharose 4B was tested with α_1-[^{14}C]AGP and [^{14}C]transferrin, because both contain α-mannosyl residues in their oligosaccharide chains. A total of 43% of the applied α_1-AGP was retained and could be eluted with 0.1 M α-methyl mannoside, while 52.5% of the transferrin was retained on the Con-A column; desialylation did not significantly increase the α_1-AGP binding. When the column was tested with [^{3}H]-acetylglycophorin, which contains 15 O-linked oligosaccharide chains and only 1 mannose-containing chain per molecule, <4% of the applied radioactivity was retained on the column. Although it seems difficult to identify a glycoprotein as containing asparagine-linked saccharides on the results of the Con A column, because α_1-AGP and transferrin were not completely bound, it may be possible to recognize O-glycosidically linked glycoproteins by their inability to bind to Con A–Sepharose. Less than 10% of the [^{125}I]CcW was retained on the Con A–Sepharose column; no desialylated CcW was retained.

Table V: Amino Acid Analysis of CcW

	CcW		CcW
$CySO_3H$	9	Met	
Asp	62	Ile	19
Thr	41	Leu	30
Ser	168	Tyr	12
Glu	162	Phe	17
Gly	158	His	24
Ala	81	Lys	76
Pro	40	Arg	11
Val	32		

Because of their molecular weight, isoelectric point, or WGA-binding behavior, three of the acute-phase reactants generally associated with malignancy were examined for their immunological similarity to CcW. Antisera to α_1-AGP, α_1-antitrypsin, and ceruloplasmin were utilized. When the antisera to the three normal serum glycoproteins were reacted with their respective antigens, there was a specific antibody dilution-dependent binding of radioactivity. However, when the antisera were reacted with the labeled CcW, there was no significant binding of [^{125}I]CcW counts. In other studies, labeled CcW did not react with anti-haptoglobin or anti-whole human serum. Current preparations of antiserum are active in radioimmunoassay at dilutions of 1:2500.

The amino acid analysis of CcW is presented in Table V.

Discussion

An examination of the protein and sialic acid levels in the PCA-soluble fraction of serum revealed significant differences between normal donors and those with widespread malignant disease (Lipton et al., 1979; Harvey et al., 1980). Similar findings have been reported when whole serum was analyzed by a carbocyanine dye-binding assay (Salo et al., 1976) or by the direct Ehrlich assay (Nixon, 1973).

Many components normally present in blood are increased in various diseases other than cancer (e.g., α_1-AGP in rheumatoid arthritis or serum ceruloplasmin in acute leukemia) (Poulik & Weiss, 1975). However, serum ceruloplasmin concentrations may be elevated to "leukemic" levels by administration of estrogens and by several other nonmalignant conditions as well (Kawai, 1973).

Besides the acute-phase reactants which are increased in the PCA-soluble fraction of cancer serum, sialoglycoproteins produced by the tumor cells themselves may be present. The shedding of glycoproteins and glycopeptides from cancer cells in culture (Bhavanandan et al., 1977; Fareed et al., 1978) and production by solid tumor in vivo (Anglin et al., 1977) have indicated that the PCA-soluble fraction of serum may contain sialoglycoprotein markers arising from malignant cells. The primary constituent of the PCA-soluble fraction was α_1-AGP, a reasonable finding since this glycoprotein along with α_1-antitrypsin comprises the majority of the sialic acid in normal serum and is known to rise dramatically in malignancy and in other diseases (Poulik & Weiss, 1975). Advantage was taken of α_1-AGP's characteristically low isoelectric point, pH 2.7, to separate it by isoelectric focusing. A glycoprotein with p*I* 4.40, not present in normal material, was isolated from cancer sera. Ceruloplasmin has a p*I* of 4.4 but differs from Cc in molecular weight, solubility, and lectin affinity properties.

Characterization using lectin affinity chromatography revealed that CcW was the only PCA-soluble protein with p*I*

between 4 and 6 either from cancer or from normal plasma which was retained on a WGA–Sepharose column. This finding was particularly interesting since mucin-type glycopeptides produced by B16 mouse melanoma and two lines of human mammary carcinoma cells in culture have a characteristic affinity for WGA mediated by clustered sialic acid residues. The binding of the CcW glycoprotein to WGA–Sepharose was similarly shown to be dependent on sialic acid.

The CcW was partially retained on an RCA-I column, and neuraminidase treatment increased the proportion of bound material to 100%, implying that CcW contains terminal sugar sequences of sialic acid→galactose. This structure is analogous to that found in the mucin-type glycopeptide from mouse melanoma cells (Bhavanandan et al., 1977), the small oligosaccharide unit of epiglycanin (Codington et al., 1975), and glycopeptides produced by cultured human mammary carcinoma cells (Chandrasekaran & Davidson, 1979). The WGA-unbound component has not been further characterized other than to establish that it is not a desialylated derivative of CcW.

The N-linked glycoproteins α_1-AGP and transferrin were partially retained on a Con A–Sepharose column, while glycophorin which contains mainly O-linked saccharides was not. CcW was similarly unretained by the Con A column. However, on the basis of lectin chromatography alone, it is not possible to define the exact nature of the oligosaccharides of CcW, but an external sequence of sialic acid→Gal and several oligosaccharides per molecule seem likely features. The amino acid composition is distinguished by high levels of glycine, serine, and glutamic acid.

It is possible to distinguish CcW from a number of serum glycoproteins which are known to be elevated in malignancy. 3.8S histidine-rich α_2-glycoprotein, α_1-AGP, α_1-antichymotrypsin, hemopexin, C-reactive protein, and α_2-glycoprotein I differ from CcW in molecular weight and/or isoelectric point. Some of the common acute-phase reactants also have both molecular weight and isoelectric points different from those of CcW: α_1-AGP, α_1-antitrypsin, and haptoglobin. Still others may be distinguished from CcW by their solubility in 0.6 M PCA, e.g., ceruloplasmin, α_1-antitrypsin, α_1-β-glycoprotein, transferrin, transcortin, and the C_3 component of complement. The glycoproteins, α-fetoprotein, associated with hepatocellular carcinoma, CEA, associated with colon cancer, and β_2-microglobulin, associated with lymphoproliferative disease, are commonly recognized markers. CcW can be distinguished from these by its molecular weight which is much smaller than CEA, slightly smaller than α-fetoprotein, and much larger than β_2-microglobulin.

The glycoprotein CcW does not appear to be a normal serum component, nor is it one of the cancer-associated glycoproteins currently being used as a diagnostic marker. It appears homogeneous on the basis of gel electrophoresis, isoelectric focusing, and lectin affinity chromatography. However, a definitive statement that CcW is a cancer-specific glycoprotein awaits the establishment of a functional immunoassay and the examination of sera of normal volunteers and patients with both malignant and nonmalignant diseases.

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