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RADIOIMMUNOASSAY OF A GLYCOPROTEIN ASSOCIATED WITH MALIGNANCY

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ABSTRACT

A glycoprotein associated with malignancy was purified from the 0.6M perchloric acid-soluble fraction of serum obtained from cancer patients. The purified glycoprotein contained sialic acid, which was reponsible for binding to wheat-germ agglutinin–Sepharose. Gel electrophoresis showed one band with an apparent M_r of 50 000–55 000, and the isoelectric point was 4.4 ±0.1. The glycoprotein could be distinguished from carcinoembryonic antigen and α-fetoprotein. Iodination of this material with chloramine-T permitted development of a radioimmunoassay.

INTRODUCTION

Changes in the serum level of glycoproteins designated as acute-phase reactants have been reported for a variety of diseases including cancer[1]. Although the acute-phase glycoproteins in serum are primarily responsible for the observed increase in protein-bound carbohydrate in malignancy, the tumor cells may function as an additional glycoprotein source. Thus, direct products, such as α-fetoprotein[2,3] and carcinoembryonic antigen[4,5] have been described, as well as a variety of antigens characterized primarily by their interactions with monoclonal antibodies[6]. In addition, modification of normally circulating components by addition of glycosyl (sialyl) residues may prolong their serum half-lives and lead to net increases in concentration[7].

The isolation from human serum of a glycoprotein associated with the presence of a variety of malignancies has been reported[8]. Although the preliminary chemical and physical data indicated this material to be different from previously identified, circulating glycoproteins, detailed immunologic studies were not performed. In addition, convenient routine measurement of the levels of this glycoprotein in serum required a sensitive and relatively specific assay. This paper describes the development and application of a radio-immunoassay for the cancer-related glycoprotein.

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EXPERIMENTAL

Materials. — Wheat-germ agglutinin was prepared from crude wheat-germ, by us, using a modification of the method of Nagata and Burger[9]. The Agarose–IgG fraction of anti-rabbit IgG was purchased from Miles Laboratories Inc. (Elkhart, IN 46515). ^{125}Iodine was purchased from New England Nuclear (Boston, MA 02118) or Amersham Corp. (Arlington Heights, IL 60005). Sepharose 4 B, Sephadex G-25, and DEAE-Sephadex A-25 were from Pharmacia Inc. (Piscataway, NJ 08854); cyanogen bromide from Eastman Kodak Co. (Rochester, NY 14650); perchloric acid from J. T. Baker Chemical Co. (Phillipsburg, NJ 08865), and ampholytes from LKB (Bromma, Sweden). Acrylamide, *N,N*-methylbis-(acrylamide), 2-mercaptoethanol, and low-molecular-weight standards containing lysozyme, soybean trypsin-inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin (BSA), and phosphorylase were purchased from Bio-Rad laboratories (Richmond, CA 94804). *Vibrio cholerae* neuraminidase was purchased from Calbiochem-Behring Corp. (San Diego, CA 92112). All other chemicals were the best commercially available and were used without further purification.

Preparation of glycoprotein. — Plasma obtained by plasmapheresis of suitable donors was precipitated by a modification of the method of Krupey *et al.*[10]. Routinely, plasma (500 mL) was chilled in an ice bath and cold 0.6M perchloric acid (500 mL) added with stirring; additional conc. perchloric acid (23 mL) was added, and the mixture incubated in an ice bath for 20 min with continuous stirring. The perchloric acid-soluble fraction was centrifuged off and made neutral with M potassium hydroxide. The potassium perchlorate precipitate was centrifuged off, and the supernatant dialyzed against distilled water and lyophilized.

The lyophilized, perchloric acid-soluble material was dissolved, at a concentration of 10 mg/mL, in 10mM pyridinium acetate, pH 5.0, and applied to a column (3 × 47 cm) of DEAE–Sephadex A-25 equilibrated with 10mM pyridinium acetate. The column was eluted with a linear gradient of pyridinium acetate from 0.01 to 1M, and monitored for protein[11]. A major protein-peak, eluted at ~0.4M salt concentration, was pooled, dialyzed against distilled water, and lyophilized. Sequential, preparative isoelectric-focusing was performed with 440- and 110-mL columns in order. The fractions at pH 4.3–4.5 were combined, dialyzed exhaustively against distilled water, and freed from residual ampholyte by gel filtration in 50mM Tris, pH 8.0, in a column (1 × 60 cm) of Sephadex G-25. The void-volume peak fraction was applied to a wheat-germ agglutinin–Sepharose 4B affinity column which contained approximately 2.3 mg of wheat germ agglutinin/mL of settled gel. Bound glycoprotein was eluted with 50mM Tris, pH 8.0, containing 0.1M 2-acetamido-2-deoxy-D-glucose[8]. This product was designated CcW and was used for subsequent studies.

The material initially employed for radiolabeling as well as for antibody production was obtained from the plasma (1 L) of a patient with disseminated mammary carcinoma. Approximately 20 subsequent preparations have been carried out

utilizing plasma from patients with melanoma, or lung or mammary carcinoma and the protocol just described. The yield of CcW ranged from 100 to 300 μg/L.

Preparation of antiserum. — CcW (120 μg) was emulsified in complete Freund's adjuvant (1.0 mL) and the mixture injected subcutaneously into the foot pad of a male, New Zealand white rabbit. The antibody formation was boosted by intravenous injection, after 25 days, of addition of antigen (20 μg), and a second time after an additional 10 days. Blood was collected from the ear vein prior to immunization, and 10 days following the second booster. Subsequent bleedings have been performed at approximately 2-month intervals, 10 days after a booster injection. The blood was allowed to clot and the resulting serum adsorbed with normal human serum according to the following procedure.

The immunosorbent was prepared by adjusting the pH of dialyzed (against 0.15M sodium chloride) human serum (100 mL) to 7.0 with M phosphate buffer and treating with a 2.5% aqueous solution of glutaraldehyde (30 mL). Gel formation occurred after stirring for 20 min at 20°, and the gel was allowed to set for 3 h. The cross-linked serum was suspended in 0.2M phosphate buffer (1 L), pH 7.2, by homogenization in a loose-fitting Potter homogenizer. The suspended gel was recovered by centrifugation and the dispersal–recovery cycle repeated six times. Then, the washed gel was suspended in phosphate buffer (1 L), pH 7.2, containing 0.15M glycine and 0.02% sodium azide, and the mixture stirred for 18 h at 4°. The gel was recovered by centrifugation, washed thrice with the phosphate–glycine buffer, twice with distilled water, and freeze dried.

The lyophilized gel was added to the rabbit antiserum (10 mg/mL) and, after incubation for 2 h at room temperature, the suspension was held for 16 h at 4° with continuous mixing. The gel was removed by centrifugation and the serum clarified by filtration through a 0.22-μm pore filter. This process was repeated once. The resulting antiserum was stored at −20°; its antibody titer has been stable for at least two years. The adsorbed rabbit antiserum was employed for most subsequent experiments. The antibody titer against the glycoprotein was not significantly affected (less than a factor of 2) by the adsorption step.

^{125}I-Labeling of glycoprotein. — The glycoprotein (5 μg) was dissolved in 0.2M sodium phosphate buffer (100 μL), pH 7.5, and carrier free Na^{125}I (92.5 MBq) added, followed by chloramine-T (50 μg)[12]. After 60 s, a 0.24% solution of sodium hydrogensulfite (100 μL) and a 0.2% solution of cold NaI (50 μL) were added, and the free iodine was separated from the protein-bound iodine by gel filtration in a column (1 × 30 cm) of Sephadex G-25. The void-volume peak fraction was collected and dialyzed against 50mM Tris, pH 8.0. The specific activity of the iodinated protein was ~0.37 MBq/μg.

Final purification of the radiolabeled product was achieved by affinity chromatography[13] on a column (1.5 × 6 cm) of wheat-germ agglutinin–Sepharose 6MB. The density of the ligand was 2 mg/mL of packed gel. After dialysis, the iodinated components in the retentate were transferred to the column, and the unbound material was eluted with Tris buffer, pH 8.0 (10 mL). Bound glycoprotein was eluted

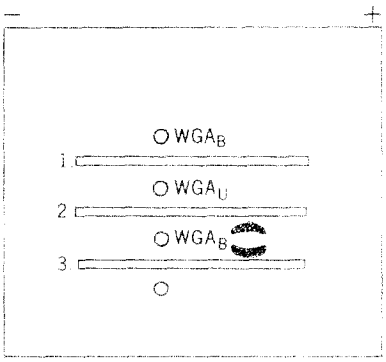

Fig. 1. Immunoelectrophoresis of CcW against rabbit anti-CcW. The wells were filled with ^{125}I-labeled, wheat germ agglutinin-bound (WGA_B) or ^{125}I-labeled, wheat-germ agglutinin-unbound (WGA_U) CcW. The electrophoresis was carried out at 6 V/cm for 45 min. The troughs were filled with either rabbit anti-normal human serum (1) or rabbit anti-CcW (2 and 3). The slide was incubated for 24 h in a moist chamber at room temperature, and then washed with saline solution extensively for 48 h. The autoradiograph was developed after exposure for 24 h.

with buffer containing 0.1M 2-acetamido-2-deoxy-D-glucose; ~50% of the non-dialyzable radioactivity was specifically displaced by the ligand.

Immunoelectrophoresis was carried out by use of rabbit anti-CcW or anti-normal human serum vs. the WGA-bound and -unbound fractions for 45 min at 6 V/cm. At the conclusion of the experiment, the slides were incubated for 24 h at room temperature in a moist chamber and washed with saline extensively prior to development of a radioautograph. The results are shown in Figs. 1 and 2.

Radioimmunoassay. — The antibody titers were established by treating a fixed amount of labeled antigen (5×10^3 c.p.m.) with measured dilutions of the adsorbed rabbit-antibody. Incubations were carried out at pH 7.4 (10mM phosphate buffer) in a total volume of 200 μL with mechanical shaking for 18 h at room temperature. Antigen and antibody were prepared in a 1:1000 dilution of pre-immune rabbit serum in 10mM phosphate buffer, pH 7.4. Goat anti-rabbit immunobeads were obtained from Miles Laboratories and washed several times with the phosphate buffer prior to use. The gel-bead pellet was resuspended in an equal volume of phosphate buffer and aliquots (300 μL) were added to the reaction tubes containing antigen and rabbit antibody; the suspension was incubated for 18 h at room temperature with continuous shaking. The gel was recovered by centrifugation for 10 min at 9000*g*, the pellet washed twice by centrifugation with buffer, and the washed-pellet samples were counted directly in a gamma counter. Blanks with pre-immune rabbit serum showed <10% of binding. Standard competition-curves were developed by inclusion of various amounts of unlabeled antigen in the assay system as described. In a similar procedure, competition data were obtained for the following serum proteins: α_1-acid glycoprotein, α_1-antitrypsin, α_1-antichymotrypsin, transferrin, and ceruloplasmin, as well as the tumor-marker carcinoembryonic antigen.

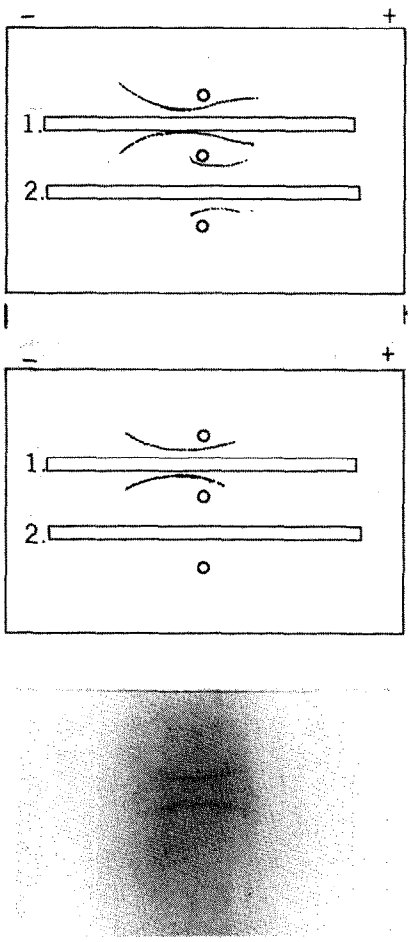

Fig. 2. Wells contained rabbit anti-CcW antiserum. After the electrophoretic run (6 V/cm, 45 min), the troughs were filled with goat anti-rabbit IgG (1) or IgM (2). Precipitin lines developed after incubation overnight at room temperature (upper diagram). The slide was washed extensively with saline solution for 48 h, and then ^{125}I-labeled CcW was layered over the slide which was incubated for 24 h. The slide was washed extensively with saline solution for 48 h and radioactivity detected by autoradiography (lower diagram and photograph).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. — $NaDodSO_4$–polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn[14] on 6% gels (0.5 × 10 cm) in the presence of low-molecular-weight standards, and, α_1-acid glycoprotein, unlabeled and radiolabeled by reduction alkylation with [^{14}C]formaldehyde[15], as markers. The proteins were denatured before electrophoresis by incubation for 3 min at 100° in 2-mercaptoethanol and sodium phosphate buffer, pH 7.0, containing 1.0% $NaDodSO_4$; Bromophenol Blue was used as tracking dye. Protein bands were stained with Coomassie Brilliant

Blue according to the method of Fairbanks *et al.*[16]. Gels containing radiolabeled proteins were cut into 2-mm slices, and the slices dissolved in 30% hydrogen peroxide (1 mL) and incubated overnight at 37°. Scintillation fluid was then added and samples counted. In the case of ^{125}I-labeled protein, the 2-mm slices were counted directly in a gamma counter.

Preparation of asialoglycoprotein. — Glycoprotein was treated[17] with *Vibrio cholerae* neuraminidase in 0.1M sodium acetate buffer, pH 5.6, with mM calcium chloride for 24 h at 37°. Free sialic acid was separated from the glycoprotein by gel filtration on a column (0.6 ×60 cm) of Biol-Gel P-2.

RESULTS

The isolated CcW glycoprotein appeared homogeneous by $NaDodSO_4$–polyacrylamide gel-electrophoresis and isoelectric focusing. Comparison with several proteins used as molecular-weight standards is shown in Fig. 3. The CcW glycoprotein migrated slightly slower than α_1-acid glycoprotein and slightly faster than bovine serum albumin, indicating an apparent M_r of 50 000–55 000 for both labeled and unlabeled compound.

Immunoelectrophoresis of CcW and reaction with anti-CcW showed a single band with a mobility similar to that of albumin (Fig. 1). The rabbit antibodies were primarily of the IgG class as indicated by immunoelectrophoresis, followed by treatment with goat anti-rabbit IgG or IgM antiserum, and then with ^{125}I-CcW (Fig. 2).

The purity of the radioiodinated preparation was examined by gel electrophoresis. ^{125}I-Labeled CcW migrated as a single molecular weight species (Fig. 4). Although the amount of CcW employed as an immunogen was relatively small, the rabbit mounted a suitable antibody response. A typical antibody dilution curve is shown in Fig. 5. In the double-antibody radioimmunoassay, maximum binding ranged from 70 to 80% at antibody dilutions of 1:1000, and 40 to 50% at a dilution of 1:10 000; this value decreased gradually with further dilutions of antiserum. The wheat germ-agglutinin-unbound fraction was not reactive.

^{125}I-CcW was treated with neuraminidase and the asialo product isolated by exclusion chromatography. Analysis indicated that >90% of the sialic acid had been removed. The asialo-CcW was further purified by affinity chromatography on a column of ricin–Sepharose (RCAI). Bound material (90%) was eluted with 0.1M lactose in 50mM Tris, pH 8.0. The binding curve of the asialo derivative is shown in Fig. 5.

A standard curve was generated by adding various concentrations of unlabeled CcW to a constant amount of ^{125}I-CcW at a 1:1000 dilution of anti-CcW serum (Fig. 6). The concentration of unlabeled antigen was varied between 1 and 100 ng/tube. The results are expressed as percent of radioactivity bound in the absence of unlabeled antigen *vs.* concentration of unlabeled antigen added. The percentage of bound radioactivity decreased slowly at low concentrations of added un-

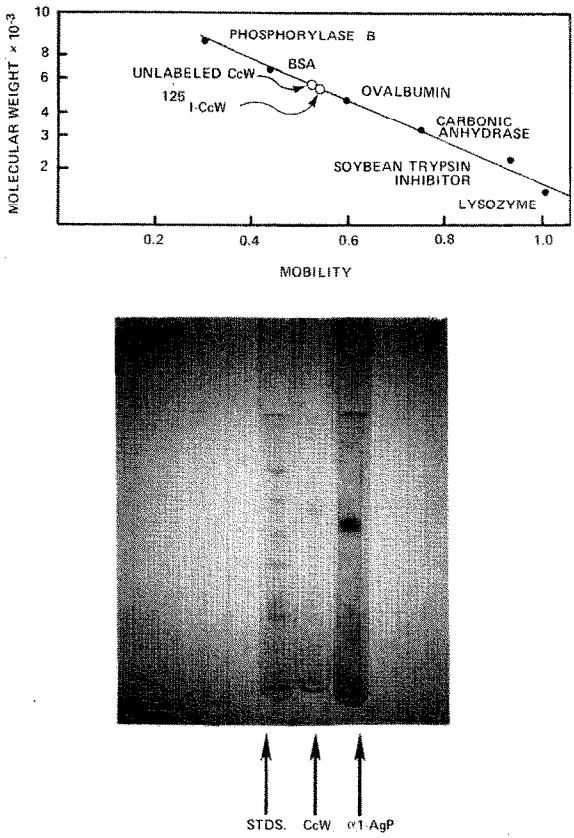

Fig. 3. Polyacrylamide-gel electrophoresis of CcW (~10 μg) and protein standards. Details of the procedure are described in the Experimental section. Abbreviations: Stds., protein standards; α1-AgP, α_1-acid glycoprotein.

labeled antigen, and in a semi-logarithmic fashion at antigen concentrations of 4–100 ng/tube. The amount of labeled CcW used in each assay was ~300 pg.

In competition binding (see Table I), the cross-reactivity of α_1-acid glycoprotein was marked and suggested a close structural relationship to CcW. In view of the failure of the absorption step to remove the cross reactivity with α_1-acid glycoprotein, the antiserum was tested against a number of other serum proteins, especially those identified as acute-phase reactants. The data (summarized in Table I) show that none of the other serum components was recognized by the antiserum. In a separate experiment, an antibody-affinity column was prepared utilizing the absorbed rabbit-antibody coupled to Sepharose 4B. The density of the antibody on the matrix was estimated, on the basis of amino acid analyses of a gel aliquot, as 2 mg/mL of packed gel. The material from serum selectively bound by this column was eluted with 0.4M acetic acid and examined by polyacrylamide–gel electrophoresis. The gel pattern revealed essentially one broad protein band with an

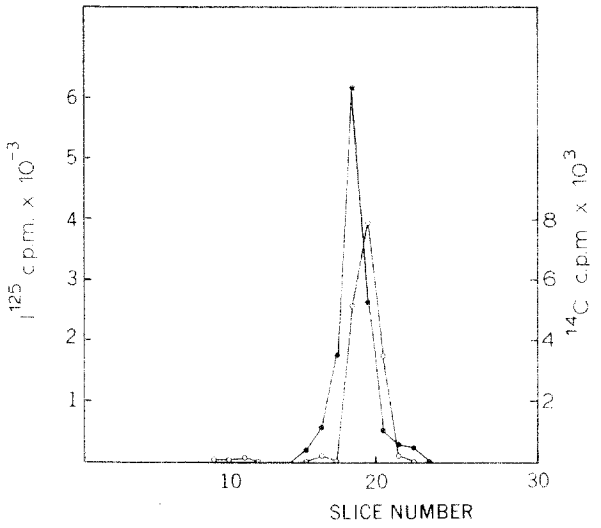

Fig. 4. Gel electrophoresis of ^{125}I-CcW and α_1-acid [^{14}C]glycoprotein. Similar amounts of protein were utilized; at the conclusion of the electrophoretic run, the gels were sectioned into 2-mm segments and the radioactivity was counted: (●——●) CcW; (○——○) α_1-acid glycoprotein.

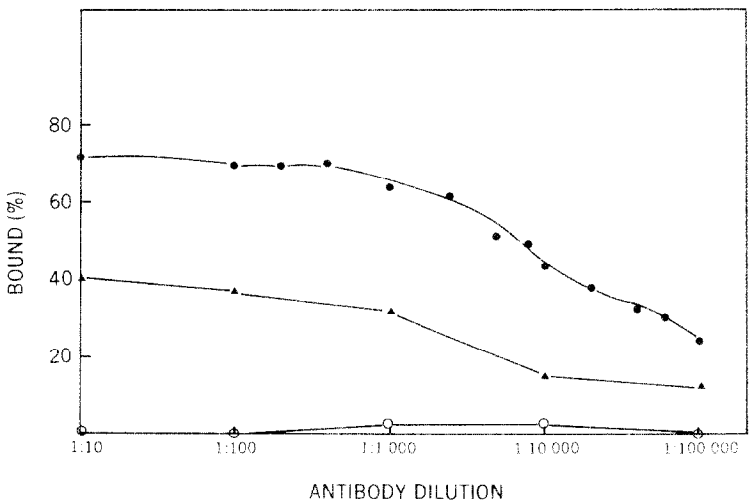

Fig. 5. Precipitation of CcW (●——●), asialo CcW (▲——▲), and the WGA-unbound fraction (○——○) by increasing dilutions of adsorbed rabbit-antibody; ~3×10^3 c.p.m. (300 pg)/reaction tube were utilized.

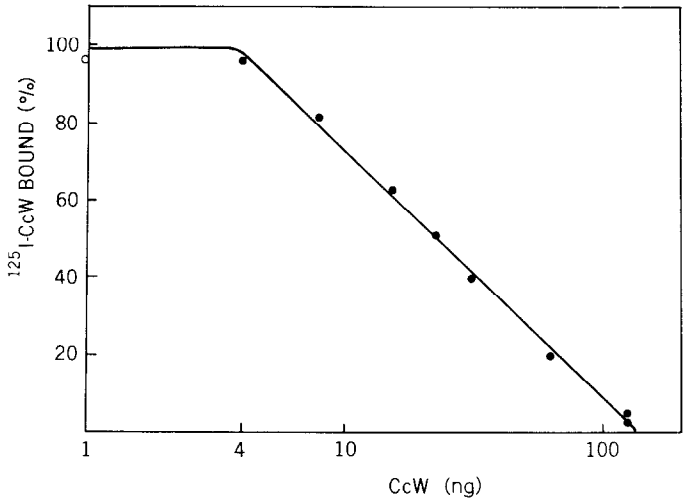

Fig. 6. Semi-logarithmic plot of CcW antigen-competition data at 1:1000 dilution of antiserum. Details of assay are given in the Experimental section.

TABLE I

COMPETITION RADIOIMMUNOASSAY DATA FOR VARIOUS GLYCOPROTEINS[a]

Protein	*50% Inhibition level (ng)*
α_1-Anti-trypsin	>500
α_1-Acid glycoprotein	35
Ceruloplasmin	>500
Transferrin	>500
Fibrinogen	>500
Human serum albumin	>500
Haptoglobin	>500
Carcinoembryonic antigen	>500

[a]Assay conditions as described in Experimental section.

apparent M_r of 42–50 000. Minor components were completely removed by a second absorption-elution step from the affinity column.

DISCUSSION

Strongly acidic reagents, such as perchloric acid and trichloroacetic acid, are known to cleave off sialyl residues[18]. However, if the acid step is performed at low temperature, the glycosyl bond resists a short exposure. Prior studies from our laboratory and others have demonstrated significant differences between the protein and sialic acid levels in the perchloric acid-soluble fraction of serum from normal individuals and patients with malignant disease[19–23].

The proportions of several of the acute-phase reactants of serum are in-

creased in the perchloric acid-soluble fraction as is the cancer-associated carcinoembryonic antigen. The isolated CcW glycoprotein could be differentiated from previously described tumor markers (carcinoembryonic antigen, α-fetoprotein, and acid phosphatase) on the basis of molecular weight, solubility, and immunologic and chromatographic properties. In addition, CcW differs from α_1-antitrypsin, α_1-antichymotrypsin, transferrin, and ceruloplasmin in either molecular weight, isoelectric point, or lectin affinity, in addition to immunologic properties. Cross reactivity was exhibited with α_1-acid glycoprotein, which could compete effectively in the radioimmunoassay (50% inhibition at 35 ng). However, differences from α_1-acid glycoprotein included apparent M_r ($\Delta \sim 2000$), isoelectric point (4.4 *vs.* 2.9), and lectin-affinity properties. Although asialo-α_1-acid glycoprotein has a pI of 4.4, CcW is fully sialylated and shifts to a higher pI when the sialyl residues are removed. The generalized elevation of the level of CcW observed in a variety of malignancies, together with physical, chemical, and immunologic data, suggest that CcW is closely related to α_1-acid glycoprotein, possibly an incompletely processed precursor or a non-liver-tissue isoform[24–27]. The synthesis of acute-phase-reactant proteins by liver has been reviewed in detail[28]. The failure of the adsorption step to significantly alter the titer of the antibody is not clearly understood but may be related to the method of preparation of the adsorbent since an adequate amount of α_1-acid glycoprotein is present in serum to interact effectively with the antibody. An explicit definition of the chemical differences between CcW and α_1-acid glycoprotein requires detailed structural work, currently underway.

CcW had an affinity for wheat-germ agglutinin that was mediated by sialyl groups; the presence of subterminal D-galactosyl residues is also likely in view of the affinity of the asialo-CcW for ricin, and the usual attachment loci of sialyl groups. The interaction of WGA with sialic acid has been noted by several laboratories including our own. Although sialic acid itself has very low affinity for the lectin, clustered sialyl groups, such as those present in multi-branched (antennary), complex glycoproteins are effective ligands[29–31]. The radioimmunoassay was sensitive to ~10 ng of the glycoprotein with material iodinated *via* the chloramine-T procedure. Alternative iodination procedures were also examined. Lactoperoxidase-catalyzed peroxide generation[32] yielded a product having significantly lower specific activity, whereas use of the Bolton–Hunter reagent[33] gave an immunologically inactive product.

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