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Isolation of Tumor Cell Surface Binding Sites for Concanavalin A and Wheat Germ Agglutinin [1]

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SUMMARY

Novikoff ascites tumor cells were found to be agglutinable by both concanavalin A and wheat germ agglutinin (WGA). A sialoglycopeptide-containing fraction, released by papain digestion of Novikoff tumor cell suspensions, inhibits the agglutination reactions, indicating that receptor sites for these plant lectins are components of this glycopeptide fraction. The purpose of this research was to develop methods for separation of the glycopeptide fraction into its component molecules and to characterize these components regarding their ability to inhibit agglutination of Novikoff tumor cells by concanavalin A and WGA.

The sialoglycopeptide-containing fraction was digested with Pronase to minimize variation in the peptide chain length of the component glycopeptides. The Pronase-digested glycopeptide fraction was submitted to gel filtration on Sephadex G-50 and subsequently to ion-exchange chromatography on DEAE-cellulose. Gel filtration resolved the glycopeptides into two molecular weight classes, a low-molecular-weight fraction (M.W. 2000 to 3300) and a higher-molecular-weight fraction (M.W. >3300). This latter fraction consists of molecules of widely different molecular weights, some of the material being excluded from Sephadex G-100. Ion-exchange chromatography of the low-molecular-weight material resolved four sialoglycopeptide fractions which inhibited agglutination of Novikoff tumor cells by concanavalin A but which were inactive in inhibiting agglutination by WGA. Ion-exchange chromatography of the higher-molecular-weight material resulted in the isolation of a sialoglycopeptide fraction which possessed high specificity as an inhibitor of agglutination by WGA.

INTRODUCTION

Two of the fundamental manifestations of the cancer cell are its invasiveness and ability to metastasize. It is these characteristics of cancer cells which have provoked investigators to consider cancer as a disorder of the social interactions of cells that evolves from chemical alterations at the cell periphery (16, 19, 24). Recently, a new tool has been introduced to demonstrate chemical alteration at the cell periphery; *i.e.*, the agglutinability of cells by certain plant lectins, specifically Con A[3] and WGA. Binding sites for these plant lectins are expressed during transformation of cells by virus, chemical carcinogens, or X-irradiation, indicating that the expression of these binding sites involves a membrane alteration common to cancer cells of etiologically different origin (1, 3, 5, 13, 14). Loss of contact inhibition of mobility and cell division occurs concomitantly with appearance of the binding sites (7, 14).

Normal cells become agglutinable by either of the agglutinins after treatment with trypsin, a fact which has been interpreted to indicate that the binding sites for the agglutinins are present in the membranes of normal cells in a nonavailable or cryptic form (5, 14). It has been proposed that upon transformation the binding sites for these agglutinins become exposed in some as yet undetermined manner (5, 14).

Recent evidence has demonstrated the involvement of Con A receptor sites in the cell-to-cell interactions of normal and transformed cells. Con A receptor sites are exposed on embryonic cells undergoing morphogenesis (19). Trypsinization of these embryonic cells results in reduced agglutinability by Con A. The reappearance of these binding sites during neoplastic transformation may be related to the ability of tumor cells tc metastasize (19). Trypsin-inactivated, monovalent Con A will restore contact inhibition of cell division in transformed cells by covering the agglutinin-binding sites (8). Conversely, trypsin-treated normal cells are released from contact inhibition until the trypsin-labile surface material is replaced (8). The importance of the "new" surface structure to the social behavior of a cell makes investigation of the chemical nature of these binding sites imperative.

A glycopeptide fraction (23) isolated from the surface of Novikoff tumor cells has been found to be a potent inhibitor of the agglutination of Novikoff tumor cells by these plant lectins. This report describes a procedure for the resolution of this glycopeptide fraction into its components and the characterization of each of these components with respect to

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[3] The abbreviations used are: Con A, concanavalin A; WGA, wheat germ agglutinin; C-SGP, crude sialoglycopeptide; TCA, trichloroacetic acid; P-SGP, Pronase-digested, sialoglycopeptide-containing fraction; DEAE-, diethylaminoethyl.

their ability to inhibit agglutination of Novikoff tumor cells by Con A and WGA.

MATERIALS AND METHODS

The Preparation of C-SGP. The C-SGP was prepared from Novikoff cells by the method of Walborg *et al.* (23), modified as follows: (*a*) a 10-fold decrease in papain concentration was utilized for enzymatic digestion of the cell suspension; (*b*) 10% TCA was utilized to terminate proteolytic digestion; and (*c*) the procedure was scaled up to process up to 160 ml of packed cells. The above modification did not alter the recovery of glycopeptide from that previously reported (23).

Sephadex G-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) was utilized for gel filtration of concentrated, nondialyzable, sialoglycopeptide-containing material obtained from papain digestion of 150 to 220 ml of packed cells (Chart 1).

Separation of the C-SGP on Sephadex G-75 and G-100. C-SGP was submitted to gel filtration on Sephadex G-75 or G-100 (Chart 2).

Pronase Digestion of C-SGP. For maximal removal of peptide material from the glycopeptide fraction, the C-SGP was digested with Pronase (Grade B, Calbiochem, Los Angeles, Calif.). For determination of the extent of digestion, one reaction was monitored with a Radiometer pH titrator (Radiometer, Copenhagen, Denmark). The C-SGP, dissolved in 0.02 M $CaCl_2$, pH 7.5, was digested for 12 hr at 37°. The concentration of C-SGP was 10 mg/ml and the enzyme to glycopeptide ratio was 1:50 (w/w). At 12 hr, a 2nd addition of the same quantity of enzyme was made. The reaction was terminated at 24 hr. The reaction, performed under N_2 atmosphere, was maintained at pH 7.5 by addition of 0.02 N NaOH. Toluene was added to retard bacterial growth.

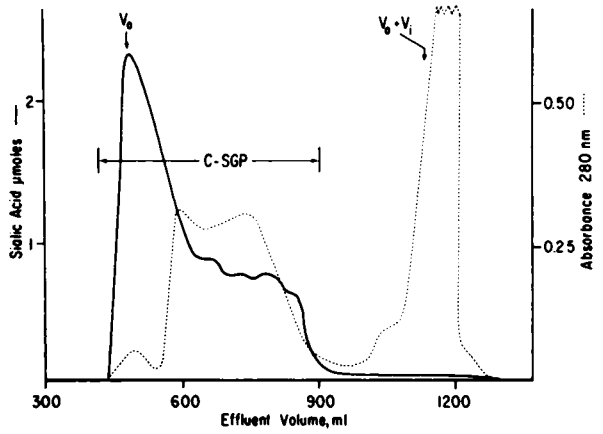

Chart 1. Gel filtration of TCA-soluble, nondialyzable, sialoglycopeptide-containing material released by papain digestion of Novikoff tumor cell suspensions. The column of Sephadex G-50, 5.5 x 55 cm, was eluted with deionized water at a flow rate of 60 ml/hr at 23°. The sample volume was 30 ml. The effluent was assayed for sialic acid by the method of Warren (25). Sialic acid concentration is expressed as μmoles/15-ml fraction. The material eluted at an effluent volume of 435 to 915 ml was pooled to yield C-SGP. V_o was determined by measuring the effluent volume of Blue Dextran 2000 (Pharmacia). The sum of the V_o and the V_i was determined by measuring the effluent volume of NaCl.

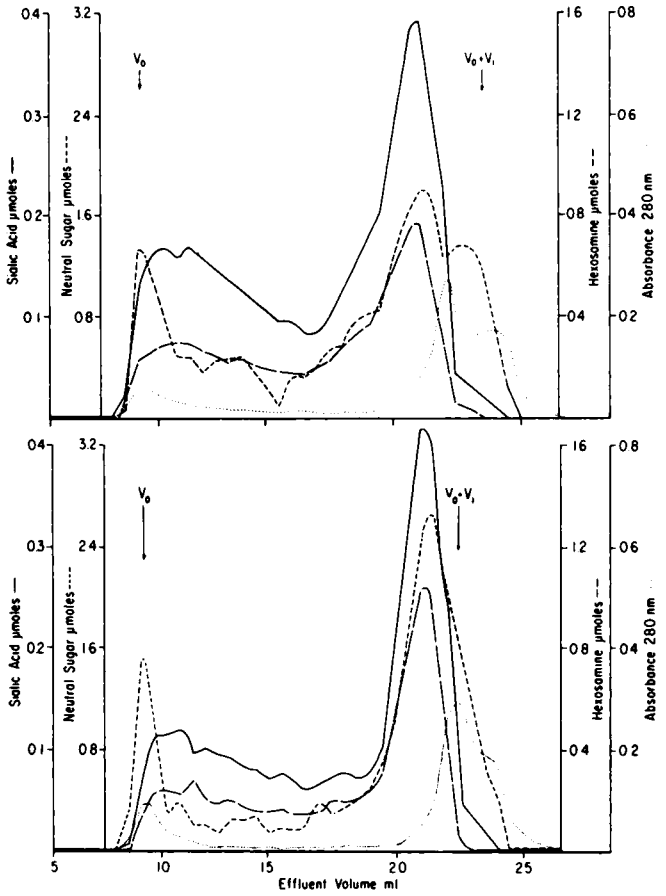

Chart 2. Gel filtration of C-SGP on Sephadex G-75 and Sephadex G-100. Columns of Sephadex G-75 (*upper*) and Sephadex G-100 (*lower*), 0.6 x 90 cm, were eluted with deionized water at a flow rate of 1 ml/hr at 23°. The sample volume was 0.5 ml, containing 10 mg of C-SGP. Sialic acid was determined by the method of Warren (25), neutral sugar was determined by the method of Dubois *et al.* (9), and hexosamine was determined by the method of Gatt and Berman (10). The saccharide concentrations are expressed as μmoles/0.5-ml fraction. V_o was determined by measuring the effluent volume of Blue Dextran 2000 (Pharmacia). The sum of the V_o and the V_i was determined by measuring the effluent volume of NaCl.

Subsequent Pronase digestions of C-SGP preparations were buffered at pH 7.5 with 0.05 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (11) obtained from Calbiochem. Otherwise, the digestion conditions were the same as described above. At termination, the digest was brought to a final concentration of 3% TCA. After standing 30 min at 4°, the solution was centrifuged. Occasionally, a precipitate formed during the digestion. This precipitate was removed by centrifugation prior to acidification with TCA. After removal of the TCA precipitate, the solution was neutralized to pH 7 with dilute NaOH and submitted to gel filtration on Sephadex G-50 (Chart 3). The column effluent was collected and pooled via 2 schemes. By the 1st scheme, all of the sialic acid-containing fractions were pooled to provide material representing a fraction, P-SGP, comparable to C-SGP. By the

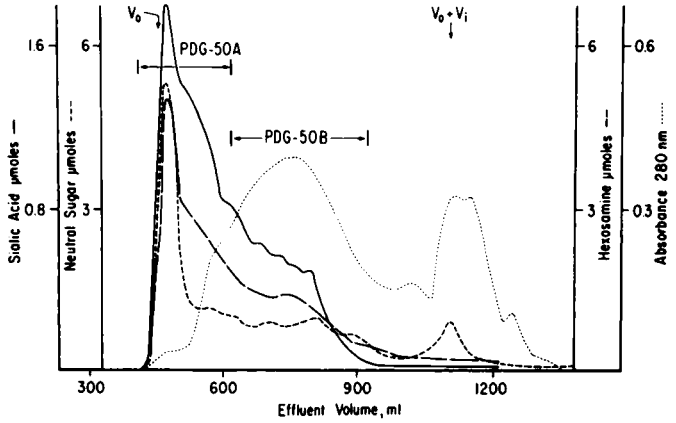

Chart 3. Purification of Pronase-digested C-SGP by gel filtration on Sephadex G-50. Columns of Sephadex G-50, 5.5 x 55 cm, were eluted with deionized water at a flow rate of 60 ml/hr at 23°. The sample volume was 20 ml. The effluent was assayed for sialic acid by the method of Warren (25), neutral sugar was assayed by the method of Dubois *et al.* (9), and hexosamine was assayed by the method of Gatt and Berman (10). Results are expressed as μmoles/15-ml fraction. Material eluted at an effluent volume of 435 to 615 ml was pooled to yield PDG-50A, and material eluted at an effluent volume of 615 to 915 ml was pooled to yield PDG-50B. V_o was determined by measuring the effluent volume of Blue Dextran 2000 (Pharmacia). The sum of the V_o and the V_i was determined by measuring the effluent volume of NaCl.

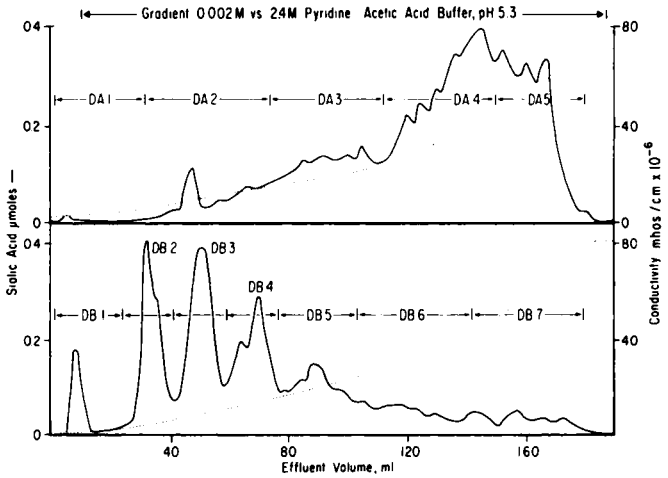

Chart 4. Ion-exchange chromatography of PDG-50A and PDG-50B on DEAE-cellulose. The sample (20 to 50 mg) was dissolved in 2 ml of 0.002 M pyridine-acetic acid buffer, pH 5.3. Elution of the material from the column (1 x 10 cm) was accomplished by sequential application of the following buffers: (*a*) 10 ml of 0.002 M pyridine-acetic acid, pH 5.3; (*b*) a concave gradient of 116 ml of 0.002 M pyridine-acetic acid buffer, pH 5.3, *versus* 27 ml of 2.4 M pyridine-acetic acid, pH 5.3; and (*c*) 10 ml of 1.2 M pyridine-acetic acid, pH 5.3. The concentration of each buffer is expressed as final concentration of pyridine. All buffers were adjusted to pH 5.3 with acetic acid prior to bringing the solutions to volume. The mixing chamber for the 2-chambered gradient apparatus was a cylinder with dimensions of 5.5 x 14 cm, while the reservoir was a cylinder with dimensions of 2.4 x 11 cm (Glenco Scientific, Inc., Houston, Texas, Catalog Nos. 3130-A-300 and 3130-A-50). The flow rate was 10 ml/hr. The fraction volume was 2 ml. Sialic acid was assayed by the method of Warren (25) and expressed as μmoles/2-ml fraction. The conductivity of the column effluent was measured on 5-μl aliquots of each fraction diluted to 3 ml with deionized water.

2nd scheme, the column effluent was divided into 2 pools, PDG-50A and PDG-50B (Chart 3).

Resolution of PDG-50A and PDG-50B by Chromatography on DEAE-cellulose. Ion-exchange chromatography was performed with Whatman Microgranular DE 32 (DEAE-cellulose, 1.0 meq/g dry exchange agent), obtained from H. Reeve Angel and Co., Ltd., London, England (Chart 4). Preparation and equilibration of the DEAE-cellulose was performed according to the procedure outlined by Thompson (21).

Assay of Column Effluents. Sialic acid was determined according to the method of Warren (25). *N*-Acetylneuraminic acid, obtained from Pierce Chemical Co., Rockford, Ill., was used as the standard. Samples for analysis were hydrolyzed in 0.1 N H_2SO_4 for 1 hr at 80° prior to assay. Samples of 0.5 ml were utilized instead of the 0.2 ml described by Warren. A molecular extinction coefficient of 53,400 ± 240 was obtained with this modified procedure.

Neutral sugar was determined by the method of Dubois *et al.* (9). D-Galactose (Mann Research Laboratories, Inc., New York, N. Y.) was utilized as the standard. For assay of material of limited quantity, the sample and reagent volumes were reduced by one-half.

Hexosamines were assayed by the procedure of Gatt and Berman (10). D-Glucosamine-HCl (Mann) was utilized as the standard. Prior to colorimetric analysis, the samples were hydrolyzed in 2.0 N HCl for 8 hr at 100°.

Preparation of Plant Lectins. WGA was prepared essentially according to the method described by Burger and Goldberg (7). Wheat germ lipase was obtained from Pentex Biochemicals Inc., Kankakee, Ill. Further purification of the material, obtained from gel filtration on Sephadex G-75, was accomplished by gel filtration of 25 mg of the WGA on a column (0.6 x 90 cm) of Sephadex G-25, eluted with deionized water at a flow rate of 5 ml/hr at 23°. The fractions (0.5 ml) were assayed for their ability to agglutinate Novikoff tumor cells and for UV absorbance at 230 and 280 nm. Those fractions (effluent volume, 11.5 to 14.0 ml) exhibiting the highest agglutinin activity were pooled and lyophilized.

Con A (3 times crystallized, Lot No. 10) was obtained as 1 g of protein suspended in 32 ml of 30% saturated ammonium sulfate (Miles Laboratories, Inc., Elkhart, Ind.).

Agglutination of Novikoff Tumor Cells by WGA and Con A. WGA and Con A were made up in stock solutions of 1 mg/ml of deionized water. In the case of Con A, the solution was titrated to pH 7.5 with 5% $NaHCO_3$ before being brought to volume. Serial dilutions of each agglutinin were prepared with deionized water, giving a series of solutions of 1.0, 0.5, 0.33, . . . 0.01 mg/ml. In preparation for an agglutination assay, samples of each dilution of Con A (10 μl) or WGA (25 μl) were placed in separate wells of a Plasmacrit flocculation slide (Scientific Products, Inc., Evanston, Ill., Catalog No. M6233) and allowed to dry.

The Novikoff ascites tumor was maintained in 7- to

9-week-old female Sprague-Dawley rats[4] (A. R. Schmidt, Inc., Madison, Wis.). At 5 to 8 days after inoculation, the ascites fluid was collected as previously described (23), diluted with 3 volumes of Buffer 1 (23), and centrifuged at 25 X *g* for 12 min. After removal of the supernatant fluid by aspiration, the cells were resuspended in Buffer 1 and the centrifugation was repeated. After the 2nd wash, the cells were resuspended in Buffer 2, containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.5. After centrifugation at 25 X *g* for 15 min, the supernatant fluid was removed, and 1.1 ml of the loosely packed cells were diluted to 100 ml with Buffer 2, yielding a suspension which contained 1×10^6 cells/ml. An aliquot of this suspension (0.05 ml) was added to each well on a flocculation slide. The contents of each well were stirred with a toothpick immediately after addition of the cells. All agglutinations were scored 30 min after addition of the cells. The degree of agglutination was determined by phase-contrast microscopy (16X objective and 10X oculars). All assays were scored (blind) by the same observer. The degree of agglutination in each well was rated as 0, +1, +2, +3, or +4. The degree of agglutination was based on the presence of agglutinated cells, the size of the cell clumps, and the number of nonagglutinated cells present, according to the following criteria: +1, at least 2 clumps of 5 to 15 cells in the majority of fields viewed; +2, at least 2 clumps of 15 to 30 cells in the majority of fields viewed; +3, clumps of >30 cells, but with smaller clumps or single cells still present; +4, clumps of >100 cells with only few smaller clumps or single cells visible.

Assay of Inhibition of Agglutination by C-SGP, P-SGP, DEAE Fractions from PDG-50A and PDG-50B, and Monosaccharides. C-SGP, P-SGP, and fractions obtained from DEAE-cellulose chromatography were prepared as 1-mg/ml stock solutions. The monosaccharide inhibitors methyl-α-D-mannoside (chemically pure grade) and 2-acetamido-2-deoxy-D-glucose (M.A. grade) were obtained from Mann. The same quantity of inhibitor was placed in each well of a flocculation plate and allowed to dry. Con A or WGA was added to the flocculation wells in quantities described previously. For ensurance of uniform mixing of the inhibitor and the agglutinin, the solution in each well was stirred and spread evenly over the area of the well. After the inhibitor-agglutinin mixture dried, the agglutination assay was performed and scored as described previously.

RESULTS

Agglutination of Novikoff Tumor Cells by Con A and WGA. Novikoff tumor cells were agglutinated by both Con A and WGA. With the assay system described, an average +2 agglutination was obtained with 0.5 μg of Con A and with 0.8 μg of WGA. The results from all assays for each agglutinin have been combined and are shown in Charts 5 and 6. The *shaded area* represents the variability of the assays. Omission of Ca^{++} and Mg^{++} from Buffer 2 did not significantly alter the degree of agglutinability of Novikoff tumor cells.

Preparation of C-SGP. The nondialyzable, sialoglycopeptide-containing fraction was analyzed for sialic acid prior to gel filtration on Sephadex G-50. These analyses indicated that papain released 0.14 ± 0.03 μmole of sialic acid per ml of packed cells. Chart 1 illustrates the purification of nondialyzable, sialoglycopeptide-containing material isolated from 210 ml of packed cells. The elution profile of sialic acid was similar for each batch of C-SGP isolated; however, the UV absorbance varied considerably from preparation to preparation. The recovery of sialic acid from gel filtration was 90 ± 12%. The yield of C-SGP was 390 ± 70 μg/ml, packed cell volume. The sialic acid content of lyophilized C-SGP was 0.30 ± 0.04 μmole/mg.

Gel filtration of the nondialyzable, sialoglycopeptide-containing fraction utilizing 0.1 N acetic acid as eluant, yielded better resolution of the high- and low-molecular-weight classes of glycopeptides; however, the maximum elution volume of C-SGP was unchanged, indicating that there was no significant adsorption of low-molecular-weight sialoglycopeptides to the gel during elution with deionized water.

Gel Filtration of C-SGP on Sephadex G-75 and G-100. As shown in Chart 2, the C-SGP is heterogeneous with respect to the molecular weight of its components. The high-molecular-weight fraction, eluting near the volume outside the gel (V_o) of Sephadex G-50, represents components of widely different molecular weight, some components being excluded from Sephadex G-100. The recovery of sialic acid, neutral sugar, and hexosamine was quantitative.

Pronase Digestion of C-SGP. Further digestion of the peptide moiety of C-SGP by Pronase was demonstrated with a Radiometer pH-stat to monitor the extent of digestion. Observable proteolytic activity ceased after 6 hr. The acid groups released by proteolytic cleavage of peptide bonds were equivalent to 0.1 μmole/mg C-SGP. Controls measuring CO_2 absorption and Pronase autodigestion showed no significant changes in pH when N_2 atmosphere was present in the reaction vessel.

Subsequent batches of C-SGP were digested with Pronase, with the use of a buffered reaction. Precipitates that formed during the digestion were removed by centrifugation. Assay of these precipitates for sialic acid indicated that the precipitate contained less than 2% of the total sialic acid. The neutralized supernatants obtained after TCA precipitation of the digests were rechromatographed on the same Sephadex G-50 column used for initial purification of C-SGP (Chart 3). Recovery of sialic acid from these columns was 94 ± 9%, while weight recovery of P-SGP was 74 ± 6%. The effluent volume for material representing P-SGP was 435 to 915 ml. PDG-50A (effluent volume, 435 to 615 ml) contained 62% of the total sialic acid and 58% of the total weight of sialoglycopeptide recovered from the column. PDG-50B (effluent volume, 615 to 915 ml) contained 38% of the total sialic acid and 42% of the total weight of sialoglycopeptide recovered from the column.

Commercially available Pronase preparations are known to be contaminated with saccharide material (15). The Pronase

[4] The research described in this report utilized animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

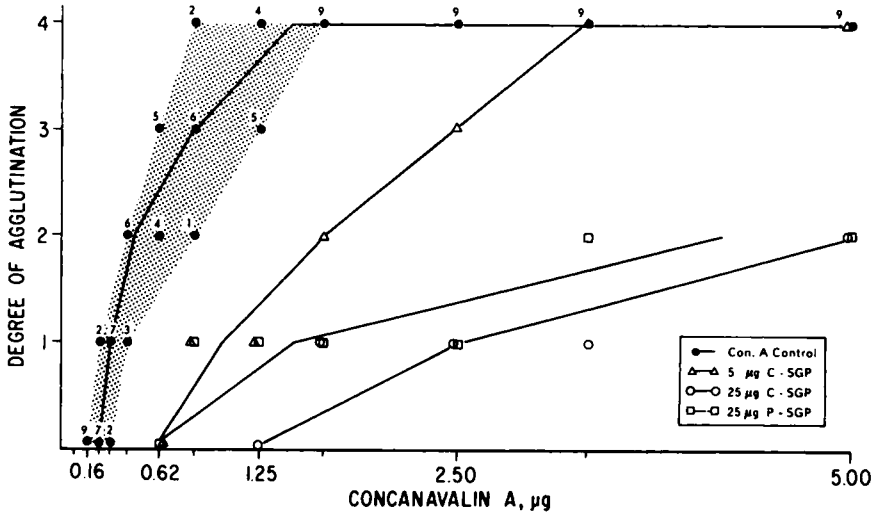

Chart 5. Agglutination of Novikoff tumor cells by Con A in the presence and absence of C-SGP and P-SGP. Agglutination assays were performed in Plasmacrit flocculation plates. Each well contained 50,000 washed Novikoff tumor cells in a total volume of 0.05 ml. The degree of agglutination was scored after 30 min. The concentrations of Con A, C-SGP, and P-SGP are expressed as μg/well. The degree of agglutination, determined microscopically, was based on the presence of agglutinated cells, the size of the cell clumps, and the number of nonagglutinated cells present. Numbers associated with the various control points represent the number of times that degree of agglutination was scored for each concentration of agglutinin. *Shaded area,* degree of variability of the assay.

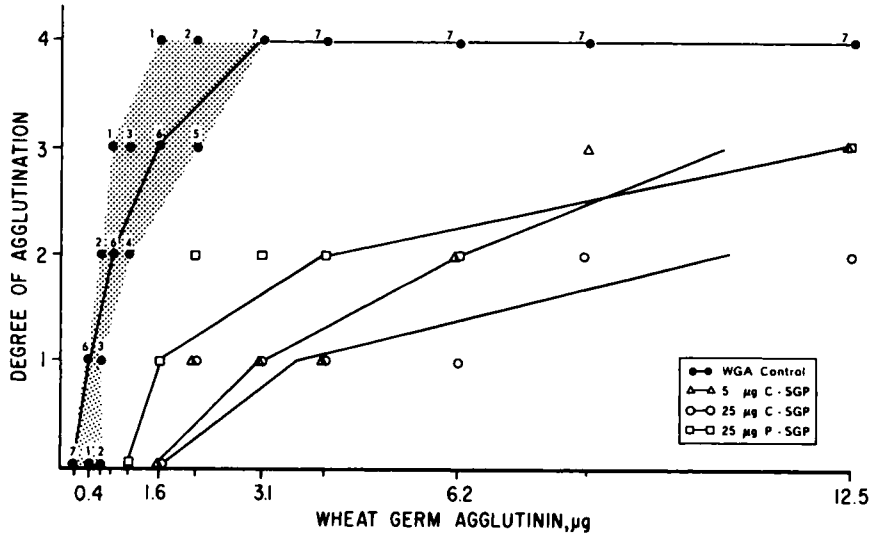

Chart 6. Agglutination of Novikoff tumor cells by WGA in the presence and absence of C-SGP and P-SGP. Agglutination assays were performed in Plasmacrit flocculation plates. Each well contained 50,000 washed Novikoff tumor cells in a total volume of 0.05 ml. The degree of agglutination was scored after 30 min. The concentrations of WGA, C-SGP, and P-SGP are expressed as μg/well. The degree of agglutination, determined microscopically, was based on the presence of agglutinated cells, the size of the cell clumps, and the number of nonagglutinated cells present. Numbers associated with the various control points represent the number of times that degree of agglutination was scored for each concentration of agglutinin. *Shaded area,* degree of variability of the assay.

utilized for digestion of C-SGP was assayed and found to contain 0.077 μmole of neutral sugar per mg, < 0.010 μmole of hexosamine per mg, and <0.002 μmole of sialic acid per mg. An autodigest of 100 mg of Pronase was performed. The fraction, soluble after precipitation with TCA, was submitted to gel filtration under conditions similar to that utilized for the preparation of P-SGP. Neutral sugar was present in all fractions of the column between the V_o and the $V_o + V_i$ (sum of the volume inside and outside the gel); 24% appeared as high-molecular-weight material, and 50% eluted at the same effluent volume as P-SGP. The extent of contamination of the P-SGP by carbohydrate material in the Pronase is minimal due to the small amounts of Pronase used for each digest. The maximum neutral sugar contamination from Pronase was calculated to be 0.07%.

Chromatography of PDG-50A and PDG-50B on DEAE-cellulose. PDG-50A and PDG-50B were each submitted to chromatography on DEAE-cellulose columns with a pyridine-acetic acid buffer elution system (Chart 4). The material resolved on DEAE-cellulose was pooled on the basis of the sialic acid assay and/or the conductivity of the collected fractions. The fractions obtained from several columns were pooled, lyophilized, and weighed. The fraction designations are shown on Chart 4. The recovery of sialic acid from these columns was 90 ± 12%, while the weight recovery was 89 ± 9%.

Fractions DA1 and DB1 were not readily soluble in deionized water. The insoluble material, a white, flocculant precipitate, was not characterized further. Pepper and Jamieson have also reported insoluble fractions which were the result of lyophilization (20). DB6 and DB7 were light brown and only partially soluble in deionized water. The sialic acid content and weight recoveries of the fractions resolved on DEAE-cellulose are summarized in Table 1.

Inhibition of Con A and WGA Agglutination of Novikoff Tumor Cells by C-SGP, Fractions Resolved on DEAE-cellulose, and Monosaccharides. As shown in Charts 5 and 6, C-SGP and P-SGP effectively inhibit agglutination of Novikoff cells by Con A and WGA. The fractions resolved on DEAE-cellulose were also assayed for their ability to inhibit the agglutination of Novikoff tumor cells by Con A and WGA. The results of this investigation are summarized in Table 2. Fraction DB4 was contaminated with material of higher molecular weight, known to inhibit agglutination by WGA; consequently, data

Table 1
Sialic acid content and weight recoveries of fractions resolved on DEAE-cellulose

Fraction	Sialic acid[a] (μmole/mg)	Weight recovery (% of P-SGP)
PDG-50A		
DA1	0.01	17.0
DA2	0.4	6.0
DA3	0.6	8.4
DA4	0.6	17.0
DA5	0.6	11.0
PDG-50B		
DB1	0.1	4.7
DB2	0.4	6.1
DB3	0.5	6.6
DB4	0.6	4.9
DB5	0.6	3.8
DB6	0.2	5.6
DB7	0	10.6

[a] Expressed as *N*-acetylneuraminic acid.

on its inhibitory properties must await purification of more material.

The presence of 0.025 μmole of methyl-α-D-mannoside per agglutination well resulted in a 3-fold increase in the Con A concentration necessary to yield half-maximal agglutination (an average +2 degree of agglutination), whereas the presence of 0.5 μmole of 2-acetamido-2-deoxy-D-glucose per agglutination well resulted in a 3-fold increase in the WGA concentration necessary to yield half-maximal agglutination.

DISCUSSION

Novikoff tumor cells were agglutinated by the plant lectins, Con A and WGA. A sialoglycopeptide-containing fraction (C-SGP), released from Novikoff tumor cells by digestion with papain (23), inhibited agglutination by Con A or WGA, thus indicating that binding sites for these plant lectins resided in this fraction. Since the C-SGP represents a mixture of components, a procedure was devised for resolution of C-SGP into its components. The resolved components were assayed for their ability to inhibit agglutination by Con A and WGA.

Gel filtration of C-SGP on Sephadex G-50, Sephadex G-75, and Sephadex G-100 demonstrated that the C-SGP was composed of molecules differing in molecular weight and composition. The ratio of sialic acid to hexosamine to neutral sugar varied with effluent volume, indicating a multiplicity of components. With gel filtration on Sephadex G-50, it was shown that C-SGP contained molecules with a molecular weight of 2000 and greater. This estimation of molecular weight was based on the calibration of Bhatti and Clamp (2). Although Bhatti and Clamp (2) utilized gels calibrated with oligosaccharides and glycopeptides, they did not investigate highly charged sialoglycopeptides; consequently, the molecular weight estimates based on their calibration must be considered an approximation until other methods of molecular weight determination are applied to the components of C-SGP. Gel filtration of C-SGP on Sephadex G-75 demonstrated the presence of 2 general molecular size classes of sialic acid-containing material: a class of molecules with a molecular weight of >5000 and another with a molecular weight of <5000. Such an assessment was made by comparison to a similar fractionation of sialoglycopeptide material released by papain digestion of suspensions of human platelets (20). The higher-molecular-weight material is extremely heterogeneous with respect to molecular weight, since some sialoglycopeptide material was eluted at the V_o on a column of Sephadex G-100. Such heterogeneity is not unique, since similar results have been reported for the sialoglycopeptides released from human

Table 2
Inhibition of plant lectin agglutination of Novikoff tumor cells by cell-surface glycopeptides

Fraction	Amount (μg)	Average +2 ratio[a] ConA	WGA
C-SGP	5	4	7
	10	12	8
	25	12	12
P-SGP	5	3	2
	10	6	3
	25	9	5
PDG-50A			
DA1[b]	10	4	1
	20	10	1
DA2	5	4	
	10	6	3
	20		3
DA3	5	2	
	10	6	4
	20		6
DA4	5		4
	10	3	6
	20	4	8
DA5	5		4
	10	2	5
	20	2	6
PDG-50B			
DB1[b]	5	5	
	10	8	1
	20		2
DB2	5	7	
	10	>10	1
	20		1
DB3	5	4	
	10	>10	1
	20		1
DB5	5	2	
	10	4	1
	20	8	1

[a] The degree of inhibition has been expressed as the ratio:
(the average amount of agglutinin which produces a +2 degree of agglutination when inhibitor is present)/
(the average amount of agglutinin which produces a +2 degree of agglutination when no inhibitor is present)
[b] Amounts of Fractions DA1 and DB1 represent the total weight of the fraction prior to partial solubilization.

erythrocytes by trypsin digestion (26) and from platelets by Pronase and papain digestion (20).

Pronase digestion of C-SGP resulted in a 25% weight loss without loss of sialic acid. This weight loss was due to further hydrolysis of the peptide moieties, as evidenced by diminished UV absorbance of the P-SGP on Sephadex G-50 and by release of titratable acid groups in a nonbuffered enzyme incubation. Although Pronase digestion of C-SGP resulted in a reduction in its inhibitory activity (Table 2), P-SGP remained a potent inhibitor of agglutination by Con A and WGA. Similar losses of hapten activity have been observed following proteolytic digestion of MN blood substance (17) and the phytohemagglutinin receptor from human erythrocytes (18). Such a decrease in activity was attributed to changes in molecular arrangement accompanying proteolysis (22).

P-SGP was submitted to gel filtration on Sephadex G-50 and separated into 2 fractions, PDG-50A and PDG-50B, each fraction representing molecules of a more restricted molecular weight range. On the basis of the calibration of Bhatti and Clamp (2), PDG-50B contained molecules in the molecular weight range of 2000 to 3300, while PDG-50A contained molecules with a molecular weight of >3300. Submission of PDG-50A and PDG-50B to ion-exchange chromatography on DEAE-cellulose resulted in further resolution of the sialoglycopeptide-containing material. The best resolution was obtained with PDG-50B, probably because this fraction contains a family of sialoglycopeptides of very restricted molecular weight range. The sialic acid content of the resolved components (Table 1) indicated that the resolution was based primarily on the number of charged groups contributed by sialic acid residues. The glycopeptide components derived from PDG-50B inhibited only cell agglutination by Con A. DB6 and DB7 were not assayed since they were only partially soluble in water. In the cases of DB2, DB3, and DB5, the inhibitory activity, expressed on a weight basis, was inversely related to the sialic acid content. On a weight basis, DB2 was the most potent inhibitor of agglutination of Novikoff tumor cells by Con A.

Submission to PDG-50A to ion-exchange chromatography resulted in the resolution of DA1, a fraction which is rich in neutral sugar but contains only trace quantities (0.01 μmole/mg) of sialic acid. DA1 inhibited only agglutination by Con A. Sialoglycopeptides eluted with the concave buffer gradient were not resolved into any distinct fractions; consequently, the eluate was pooled on the basis of the conductivity of the eluate. These components derived from PDG-50A possessed both inhibitory activities, each component being characterized by a different ratio between the 2 activities. The inhibitory activity against agglutination by Con A decreased with the order of elution, while inhibitory activity against agglutination by WGA increased with the order of elution. Thus, DA5 represents the sialoglycopeptide fraction most highly enriched in WGA-inhibitory activity. DA4 and DA5, which are both rich in sialic acid, represent 28% of the weight of P-SGP. Although glycopeptides with specific and distinct inhibitory activities have been isolated, the use of papain to isolate the cell-surface-binding sites precludes a decision as to whether or not these determinants reside on the same macromolecule.

A factor released by hypotonic shock of L1210 cells and of polyoma-transformed BHK-21 cells has been reported to inhibit agglutination of these cells by WGA (4). Hakomori *et al.* (12) have reported that a fucose-containing glycolipid obtained from a human adenocarcinoma inhibited agglutination by WGA. The activity of these substances as inhibitors of agglutination by Con A has not been reported. The work described herein indicates that sialoglycopeptides, released from Novikoff tumor cells by papain digestion, can serve as binding sites for Con A or WGA. The isolation of several glycopeptides, exhibiting only inhibitory activity against agglutination by Con A, and of a sialoglycopeptide fraction, enriched in inhibitory activity against agglutination by WGA, emphasizes the complexity of the carbohydrate-binding sites present at the cell surface. The methods developed for the isolation of cell-surface sialoglycopeptide fractions showing specificity for inhibition of cell agglutination by Con A and WGA will allow preparation of material for investigation of the structural requirements of the Con A- and WGA-binding sites.

Digestion of Novikoff tumor cells by papain does not result in the reduction of their agglutinability by either Con A or WGA. Such an observation is consistent with other investigations concerning the agglutinability of transformed cells following digestion with proteases (5, 14). In the case of Novikoff tumor cells, papain releases papain-sensitive binding sites while uncovering other papain-resistant binding sites. This research raises several questions. Are the protease-sensitive sites (or a portion thereof) responsible for the agglutination of Novikoff tumor cells by Con A and WGA? Are the binding sites exposed after protease digestion similar to those of normal cells which become exposed only after protease digestion? Do the binding sites exposed after protease digestion represent glycolipid or protease-resistant glycopeptide? Further experimentation is in progress to answer these questions.

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REFERENCES

1. Aub, J. C., Sanford, B. H., and Cote, M. N. Studies on Reactivity of Tumor and Normal Cells to a Wheat Germ Agglutinin. Proc. Natl. Acad. Sci. U. S., *54:* 396–399, 1965.
2. Bhatti, T., and Clamp, J. R. Determination of Molecular Weight of Glycopeptides by Exclusion Chromatography. Biochim. Biophys. Acta, *170:* 206–208, 1968.
3. Biddle, F., Cronin, A. P., and Sanders, F. K. The Interaction between Wheat Germ Agglutinin and Receptors on Normal and Transformed Cells and on Erythrocytes. Cytobios, *5:* 9–17, 1970.
4. Burger, M. M. Isolation of a Receptor Complex for a Tumor Specific Agglutinin from the Neoplastic Cell Surface. Nature, *219:* 499–500, 1968.
5. Burger, M. M. A Difference in the Architecture of the Surface Membrane of Normal and Virally Transformed Cells. Proc. Natl. Acad. Sci. U. S., *62:* 994–1001, 1969.

6. Burger, M. M. Proteolytic Enzymes Initiating Cell Division and Escape from Contact Inhibition of Growth. Nature, *227:* 170–171, 1970.
7. Burger, M. M., and Goldberg, A. R. Identification of a Tumor-specific Determinant on Neoplastic Cell Surfaces. Proc. Natl. Acad. Sci. U. S., *57:* 359–366, 1966.
8. Burger, M. M., and Noonan, K. D. Restoration of Normal Growth by Covering of Agglutinin Sites on Tumor Cell Surface. Nature, *228:* 512–515, 1970.
9. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. Colorimetric Methods for Determination of Sugars and Related Substances. Anal. Chem., *28:* 350–356, 1956.
10. Gatt, R., and Berman, E. R. A Rapid Procedure for the Estimation of Amino Sugars on a Micro Scale. Anal. Biochem., *15:* 167–171, 1966.
11. Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S., and Singh, R. M. M. Hydrogen Ion Buffers for Biological Research. Biochemistry, *5:* 467–477, 1966.
12. Hakomori, S., Koscielak, J., Bloch, K. J., and Jeanloz, R. W. Immunologic Relationship between Blood Group Substances and a Fucose-containing Glycolipid of Human Adenocarcinoma. J. Immunol., *98:* 31–38, 1967.
13. Inbar, M., and Sachs, L. Structural Difference in Sites on the Surface Membrane of Normal and Transformed Cells. Nature, *223:* 710–712, 1969.
14. Inbar, M., and Sachs, L. Interaction of the Carbohydrate-binding Protein Concanavalin A with Normal and Transformed Cells. Proc. Natl. Acad. Sci. U. S., *63:* 1418–1425, 1969.
15. Jamieson, G. A. Glycoproteins. I. The Carbohydrate Portion of Ceruloplasmin. J. Biol. Chem., *240:* 2019–2027, 1965.
16. Kalckar, H. M. Galactose Metabolism and Cell "Sociology." Science, *150:* 305–313, 1965.
17. Klenk, E., and Uhlenbruck, G. Uber neuraminsaurehaltige Mucoid aus Menschenerythrocytenstroma ein Beitrag zur Chemie der Agglutinogene. Z. Physiol. Chem., *319:* 151–160, 1960.
18. Kornfeld, R., and Kornfeld, S. The Structure of a Phytohemagglutinin Receptor Site from Human Erythrocytes. J. Biol. Chem., *245:* 2536–2545, 1970.
19. Moscona, A. A. Embryonic and Neoplastic Cell Surfaces: Availability of Receptors for Concanavalin A and Wheat Germ Agglutinin. Science, *171:* 905–907, 1971.
20. Pepper, D. S., and Jamieson, G. A. Isolation of a Macroglycopeptide from Human Platelets. Biochemistry, *9:* 3706–3713, 1970.
21. Thompson, C. M. (ed.), Whatman Advanced Ion-Exchange Celluloses Laboratory Manual. Maidstone, Kent, England: W. and R. Balston, Ltd. Technical Bulletin IE3.
22. Uhlenbruck, G. Erythrocyte Mucoids. Vox Sanguinis, *9:* 377–384, 1964.
23. Walborg, E. F., Jr., Lantz, R. S., and Wray, V. P. Isolation and Chemical Characterization of a Cell-Surface Sialoglycopeptide Fraction from Novikoff Ascites Cells. Cancer Res., *29:* 2034–2038, 1969.
24. Wallach, D. F. H. Cellular Membranes and Tumor Behavior: A New Hypothesis. Proc. Natl. Acad. Sci. U. S., *61:* 868–874, 1968.
25. Warren, L. Thiobarbituric Acid Assay of Sialic Acids. J. Biol. Chem., *234:* 1971–1975, 1959.
26. Winzler, R. J., Harris, E. D., Pekas, D. J., Johnson, C. A., and Weber, P. Studies on Glycopeptides Released by Trypsin from Intact Human Erythrocytes. Biochemistry, *6:* 2195–2202, 1967.