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Isolation and Partial Chemical Characterization of Cell-Surface Glycopeptides from AS-30D Rat Hepatoma Which Possess Binding Sites for Wheat Germ Agglutinin and Concanavalin A†

David F. Smith,‡ Giovanni Neri,§ and Earl F. Walborg, Jr.*

ABSTRACT: AS-30D rat ascites hepatoma cells were agglutinable by wheat germ agglutinin (WGA), but only weakly agglutinable by high concentrations of concanavalin A (Con A). Treatment of the intact cells with papain rendered them highly agglutinable by both lectins and released a glycopeptide fraction from the cell surface. The cell-surface glycopeptide fraction inhibited the cytoagglutination of AS-30D cells by WGA, indicating that this fraction contained components that possessed the minimum requirements of cell-surface binding sites for WGA. The cell-surface glycopeptide fraction exhibited both WGA- and Con A-hemagglutination inhibitory activity. Partial resolution of the glycopeptides by gel filtration and ion-exchange chromatography resulted in the isolation of a sialoglycopeptide fraction, which represented 12% of the weight of the papain-labile, cell-surface glycopeptides and possessed the major portion of the WGA-binding activity. This glycopeptide fraction exhibited specific hemagglutination inhibitory activities which reflected the cytoagglutination properties of the intact AS-30D cell. Purification of Con A-binding activity from the papain-labile, cell-surface glycopeptides resulted in the isolation of a sialoglycopeptide fraction which possessed only Con A-hemagglutination inhibitory activity.

Chemical alterations at the cell periphery have been implicated in many of the manifestations of the cancer cell which are responsible for its altered social behavior (Abercrombie and Ambrose, 1962; Wallach, 1968). The phytoagglutinins, Con A[1] and WGA, have become valuable tools in the detection of altered oligosaccharide moieties present at the tumor cell periphery (Burger and Goldberg, 1967; Inbar and Sachs, 1969a). Transformed cells, in contrast to their normal counterparts, express binding sites at their cell periphery which are responsible for their cytoagglutination by Con A and WGA (Aub *et al.*, 1965; Inbar and Sachs, 1969a). With the exception of embryonic (Moscona, 1971; Noonan and Burger, 1971) and mitotic cells (Burger, 1973), nontransformed cells become agglutinable only after treatment with proteases (Burger, 1969; Inbar and Sachs, 1969a).

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[1] Abbreviations used are: WGA, wheat germ agglutinin; Con A, concanavalin A.

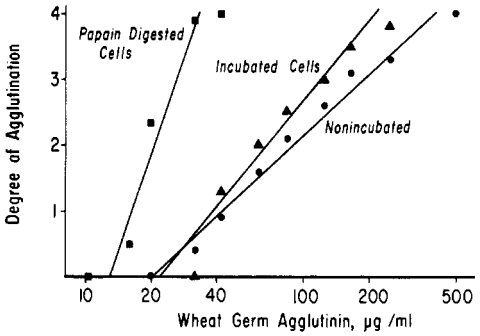

FIGURE 1: Cytoagglutination of AS-30D tumor cells by WGA. Agglutination assays were performed using Plasmacrit flocculation plates. Each well contained 1.5×10^5 washed cells in a volume of 0.05 ml. The concentration of WGA is expressed in μg/ml. The degree of agglutination was determined microscopically, and each point represents the average of at least two separate assays.

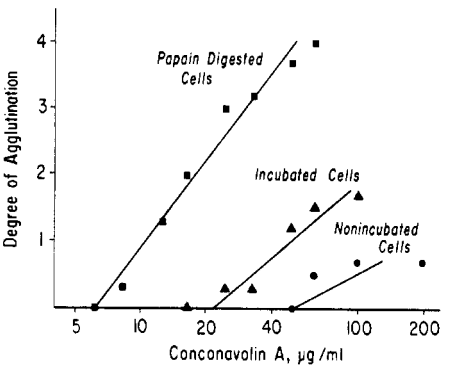

FIGURE 2: Cytoagglutination of AS-30D tumor cells by Con A. Agglutination assays were performed using Plasmacrit flocculation plates. Each well contained 1.5×10^5 washed cells in a volume of 0.05 ml. The concentration of Con A is expressed as μg/ml. The degree of agglutination was determined microscopically, and each point represents the average of at least two separate assays.

The basis of the differential agglutinability of normal and transformed cells has been investigated primarily at the cellular or membrane level. These investigations include the specific binding of labeled phytoagglutinins (Fox *et al.*, 1971; Cronin *et al.*, 1970; Inbar and Sachs, 1969b; Ozanne and Sambrook, 1971; Cline and Livingston, 1971) and studies of the cell-surface topography of agglutinin-binding sites of normal and transformed cells (Nicholson, 1971). These studies have provided an experimental basis for a number of theories to explain the differential agglutinability of normal and transformed cells, *e.g.*, the exposure of cryptic agglutinin-binding sites (Burger, 1969; Inbar and Sachs, 1969b) and altered topographical distribution of the agglutinin-binding sites (Nicholson, 1971; Inbar *et al.*, 1972).

The chemical nature of Con A- and WGA-binding sites present at the periphery of tumor cells has received only limited investigation. Hakomori *et al.* (1967) have described a glycolipid isolated from human adenocarcinomas which possessed WGA hapten-like inhibitory activity; however, conclusive proof of its cell-surface location has not been established. Burger (1968) has described the release of a cell-surface, particulate component which demonstrated hapten-like inhibitory activity for WGA. This component has more recently been shown to contain glycoprotein (Jansons and Burger, 1973).

The demonstration that glycopeptides, released from the periphery of intact human erythrocytes, possessed receptor sites for a variety of hemagglutinating agents (Winzler, 1969; Marchesi *et al.*, 1972; Kornfeld and Kornfeld, 1970; Kornfeld *et al.*, 1971) suggested that cell-surface glycopeptides of tumor cells may be involved in the cytoagglutination of transformed cells by WGA and Con A.

Cell-surface glycopeptide fractions have been isolated by treatment of intact tumor cells with proteolytic enzymes such as trypsin (Langley and Ambrose, 1964; Codington *et al.*, 1970; Buck *et al.*, 1970) and papain (Walborg *et al.*, 1969; Smith and Walborg, 1972a). Wray and Walborg (1971) have demonstrated that cell-surface sialoglycopeptides, released from Novikoff tumor cells by incubation with papain, possessed the ability to inhibit the cytoagglutination of Novikoff tumor cells by WGA and Con A.

The research reported herein was performed to ascertain if a correlation existed between the WGA- and Con A-binding activities of glycopeptides isolated from the tumor cell surface and the cytoagglutination properties of the intact tumor cell. The AS-30D rat ascites hepatoma (Smith *et al.*, 1970), which is agglutinable by WGA but only weakly agglutinable by high concentrations of Con A, was utilized in these investigations.

Experimental Methods and Results

Cytoagglutination of AS-30D Tumor Cells by WGA and Con A. Cytoagglutination assays utilized AS-30D cells from transplant generations 148–151. Intact cells, collected as previously described (Smith and Walborg, 1972a), and cells incubated under identical conditions without papain were assayed for their agglutinability by WGA and Con A. Cells were washed three times by centrifugation at 40*g* for 5 min with calcium- and magnesium-free phosphate-buffered saline, pH 7.5 (PBS), prepared as described by Cronin *et al.* (1970). After the final wash, the cells were diluted to a final concentration of 3×10^6 cells/ml. The preparation of WGA and Con A, utilized for cytoagglutination, and the cytoagglutination inhibition assay have been described (Wray and Walborg, 1971).

Intact AS-30D tumor cells and tumor cells incubated without papain were present as small clusters of cells (Smith and Walborg, 1972a). The degree of agglutination was based on the presence of agglutinated clusters of cells, the size of the cell clumps, and the number of nonagglutinated clusters present, according to the following criteria: +1, at least two clumps of three–five small clusters in the majority of fields viewed; +2, clumps of five–ten clusters predominating in the majority of fields viewed; +3, clumps of 10–50 clusters predominating in the majority of fields viewed with smaller clumps or nonaggregated clusters still present; +4, clumps of >50 clusters with only a few smaller clumps visible. Since papain treatment resulted in single-cell suspensions of AS-30D tumor cells, the degree of cytoagglutination of these cells was determined according to the criteria described by Wray and Walborg (1971).

While washed, intact cells were agglutinated by WGA (half-maximal agglutination being attained at a WGA concentration of 90 μg/ml), the same cells never attained a similar degree of agglutination with Con A, even at concentrations as high as 200 μg/ml (see Figures 1 and 2). At Con A concentrations of 200 μg/ml, a cytotoxic effect (Shoham *et al.*, 1970) was observed, and the low degree of agglutination was difficult to distinguish from clumping of damaged cells.

Incubation of cells with papain for 40 min resulted in an increase in the agglutinability of cells by WGA as evidenced by

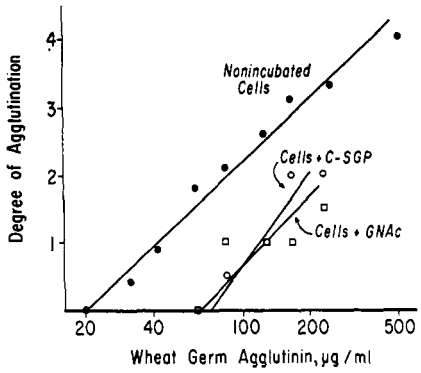

FIGURE 3: Inhibition of WGA cytoagglutination of AS-30D tumor cells. Agglutination assays were performed using Plasmacrit flocculation plates. Each well contained 1.5×10^5 washed cells in a volume of 0.05 ml. The degree of agglutination was determined microscopically, and each point represents the average of at least two separate assays. The concentration of WGA is expressed in μg/ml. Agglutination was tested in the presence of 10 mM 2-acetamido-2-deoxy-D-glucose (GNAc) and 40 μg/ml of C-SGP-III.

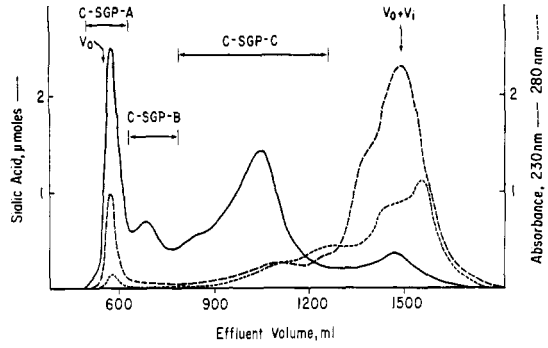

FIGURE 4: Gel filtration of cell-surface glycopeptides on Sephadex G-50. A cell-surface sialoglycopeptide fraction (SGP) from 300 ml of packed cells was applied in a 20-ml volume to a 5.5 × 80 cm column of Sephadex G-50. The column was eluted with 0.1 N acetic acid at a flow rate of 60 ml/hr at 23°. The fraction size was 15 ml.

the 4.5-fold decrease in WGA concentration necessary to produce half-maximal agglutination. Cells incubated with papain became highly agglutinable by Con A, half-maximal agglutination being attained at a Con A concentration of 18 μg/ml. Although cells incubated in the absence of enzyme demonstrated a slight increase in agglutinability, the major portion of the increase in agglutinability could be attributed to the effect of protease treatment. After a threshold concentration of agglutinin was attained, the degree of agglutinability increased linearly as a function of the log of agglutinin concentration until maximal agglutination (+4) was reached.

Preparation of the Cell-Surface Sialoglycopeptide Fraction. A cell-surface sialoglycopeptide fraction was prepared from cells obtained from the 116th to 124th transplantation generations of AS-30D rat[2] ascites hepatoma. The cell suspension, containing 4×10^7 cells/ml, was incubated for 40 min at 37° in the presence of papain at a concentration of 3 units/ml of cell suspension. These incubation conditions yielded maximal release of cell-surface sialoglycopeptides, since a tenfold increase in papain concentration did not significantly increase the amount of sialic acid released per milliliter of cells. The supernatant fluid obtained from papain-treated cells was collected and partially purified by acidification with trichloroacetic acid and dialysis by the method described by Walborg *et al.* (1969), as modified by Smith and Walborg (1972a). Gel filtration of this cell-surface sialoglycopeptide fraction (SGP) on Sephadex G-50 resulted in the isolation of a crude sialoglycopeptide fraction (C-SGP). The isolation and partial chemical characterization of C-SGP from the 116th transplantation generation of AS-30D tumor cells (C-SGP-III) have been reported (Smith and Walborg, 1972a).

Inhibition of Cytoagglutination of AS-30D Tumor Cells. Cytoagglutination inhibition assays were performed as described by Wray and Walborg (1971) and the degree of agglutination was scored as described for intact AS-30D tumor cells. C-SGP-III and 2-acetamido-2-deoxy-D-glucose were tested as inhibitors of cytoagglutination of intact cells. Assays were performed at least in duplicate so that an average could be calculated when the same degree of agglutination was observed for more than one concentration of agglutinin.

As illustrated in Figure 3, C-SGP-III was a potent inhibitor of cytoagglutination by WGA. In the presence of C-SGP (40 μg/ml) the concentration of WGA necessary to produce half-maximal agglutination increased from 90 to >200 μg/ml, and the threshold concentration of WGA necessary to produce agglutination increased from 20 to 70 μg/ml. The degree of inhibition of cytoagglutination by C-SGP was concentration dependent. C-SGP at a concentration of 100 μg/ml completely abolished cytoagglutination by WGA, even at a WGA concentration as high as 500 μg/ml. Cytoagglutination could also be inhibited by 2-acetamido-2-deoxy-D-glucose, a known inhibitor of WGA cytoagglutination (Burger, 1969). As illustrated in Figure 3, the threshold concentration of WGA increased from 20 to 62 μg/ml in the presence of 2-acetamido-2-deoxy-D-glucose at a concentration of 10 mM. Cytoagglutination by WGA at a concentration of 500 μg/ml was completely abolished by 20 mM 2-acetamido-2-deoxy-D-glucose.

Resolution of SGP into Component Glycopeptide Fractions. When submitted to gel filtration on Sephadex G-50 (Pharmacia Laboratories, Inc., Piscataway, N. J.), eluted with 0.1 N acetic acid, SGP was resolved into three distinct sialic acid containing components, as shown in Figure 4. The fractions (designated C-SGP-A, C-SGP-B, and C-SGP-C) were pooled, neutralized, dialyzed against deionized water, and lyophilized. For all gel filtration experiments, V_o (volume outside the gel matrix) was determined by measuring the effluent volume of Blue Dextran 2000 (Pharmacia) and $V_o + V_i$ (sum of the volumes inside and outside the gel matrix) was determined by measuring the effluent volume of Cl^-, as detected by precipitation with $AgNO_3$.

The ultraviolet (uv) absorbance of column effluents was measured in 1-cm cuvets utilizing the Gilford Model 220 spectrophotometer. Sialic acid was quantitated using the method of Warren (1959). *N*-Acetylneuraminic acid, obtained from Pierce Chemical Co., Rockford, Ill., was used as a standard. Prior to sialic acid analysis, aliquots of column fractions were dried and hydrolyzed in 0.1 N H_2SO_4 for 1 hr at 80°. Sugar concentrations are expressed as micromoles/fraction. Recovery of sialic acid after gel filtration and ion-exchange chromatography was quantitative.

C-SGP-A, C-SGP-B, and C-SGP-C were digested separately with Pronase using conditions described by Wray and Walborg (1971). Each of the Pronase digests of C-SGP-A,

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

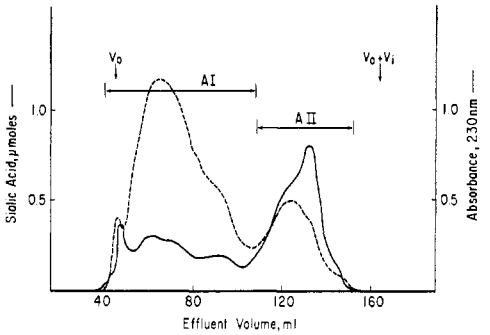

FIGURE 5: Gel filtration of P-SGP-A on Sephadex G-200. P-SGP-A (85 mg) was applied in a 2.0-ml volume to a 1.5 × 80 cm column of Sephadex G-200. The column was eluted with 0.1 N acetic acid at a flow rate of 4.5 ml/hr at 23°. The fraction size was 2.0 ml.

C-SGP-B, and C-SGP-C was submitted to gel filtration on columns (3 × 50 cm) of Sephadex G-25, using water as eluent at a flow rate of 20 ml/hr at 23°. Each of the sialic acid containing fractions, eluted at the V_o of the columns, was pooled, lyophilized, and designated P-SGP-A, P-SGP-B, and P-SGP-C, respectively.

Fractionation of Pronase-Digested Glycopeptides, P-SGP-A and P-SGP-C. Gel filtration of P-SGP-A on Sephadex G-200 resolved two major glycopeptide fractions, as shown in Figure 5. The fractions (designated AI and AII) were pooled, neutralized, dialyzed against deionized water, and lyophilized.

Ion-exchange chromatography of fraction AI was performed on DEAE-cellulose (Whatman, Microgranular DE32, 1.0 mequiv/g of dry exchanger, obtained from H. Reeve Angel and Co., Ltd., London), which had been precycled through acid and base and equilibrated in starting buffer. The results of this chromatography are shown in Figure 6. Lyophilization of the fractions eluted prior to the initiation of the gradient revealed the presence of material, designated DAI-1. Although this material contained no sialic acid, it was rich in neutral sugar as determined by the method of Dubois *et al.* (1956). The sialoglycopeptide fraction was designated DAI-2, and a third fraction, DAI-3, was obtained by eluting the column with 2.4 M pyridine–acetic acid buffer, pH 5.3, after gradient elution.

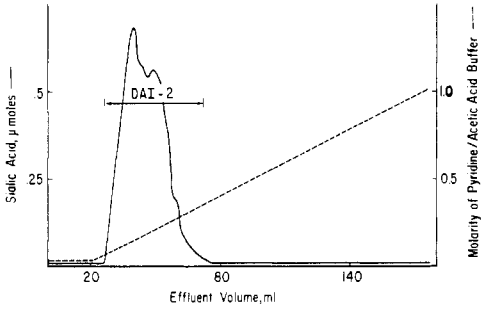

FIGURE 6: Ion-exchange chromatography of AI. The sample (42 mg) was dissolved in 2 ml of 0.002 M pyridine–acetic acid buffer, pH 5.3, and applied to a 1 × 10 cm column of DEAE-cellulose. Column elution was accomplished by sequential application of the following buffers: (a) 20 ml of 0.002 M pyridine–acetic acid buffer, pH 5.3; (b) a linear gradient of 75 ml of 0.002 M pyridine–acetic acid buffer, pH 5.3, *vs.* 75 ml of 1.2 M pyridine–acetic acid buffer, pH 5.3. The concentration of each buffer was expressed as the final molarity of pyridine. All buffers were adjusted to pH 5.3 with acetic acid. Fractions (2.0 ml) were collected at a flow rate of 10 ml/hr at 23°, and the concentration of buffer was determined conductometrically.

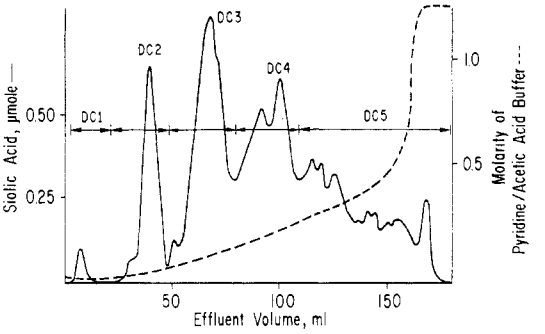

FIGURE 7: Ion-exchange chromatography of P-SGP-C. The sample (62 mg) was dissolved in 2 ml of 0.002 M pyridine–acetic acid buffer, pH 5.3, and applied to a 1 × 10 cm column of DEAE-cellulose. Column elution was accomplished by sequential application of the following buffers: (a) 20 ml of 0.002 M pyridine–acetic acid buffer, pH 5.3; (b) a concave gradient of 116 ml of 0.002 M pyridine–acetic acid buffer, pH 5.3, *vs.* 27 ml of 1.2 M pyridine–acetic acid buffer, pH 5.3; and (c) 20 ml of 0.6 M pyridine–acetic acid buffer, pH 5.3. The concentration of each buffer was expressed as the final concentration of pyridine. All buffers were adjusted to pH 5.3 with acetic acid. The mixing chamber was a 5.5 × 14 cm gradient chamber, and the reservoir was a 2.1 × 11 cm gradient chamber obtained from Glenco Scientific Inc., Houston, Texas (Catalog Nos. 3130-B-300 and 3130-A-50, respectively). Fractions (2.0 ml) were collected at a flow rate of 10 ml/hr at 23°, and the concentration of buffer was determined conductometrically.

Ion-exchange chromatography of P-SGP-C on Whatman DE-32 resulted in its resolution into five components as illustrated in Figure 7. These fractions were pooled, lyophilized, and designated DC1, DC2, DC3, DC4, and DC5. The fractionation of SGP is summarized in Figure 8.

Hemagglutination and Hemagglutination Inhibition Assays. Wheat germ agglutinin was prepared by the procedure of Nagata and Burger (1972). The active fraction from DEAE-cellulose was utilized.

Con A (3 × crystallized) suspended in 30% saturated $(NH_4)_2SO_4$ (Miles Laboratories, Inc., Elkhart, Ind.) was dialyzed against three changes of deionized water. The crystallized protein was collected by centrifugation and dissolved

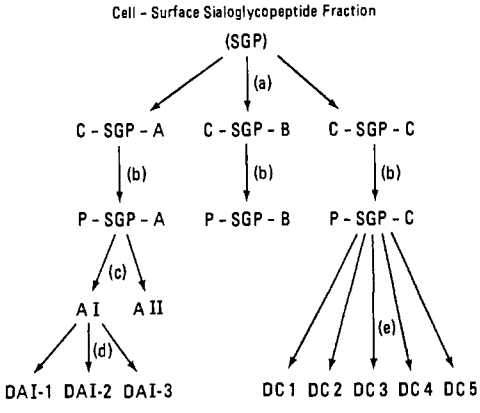

FIGURE 8: Fractionation of cell-surface sialoglycopeptide (SGP). (a) Gel filtration on Sephadex G-50 in 0.1 N acetic acid; (b) Pronase digestion, gel filtration on Sephadex G-25 in deionized water; (c) gel filtration on Sephadex G-200 in 0.1 N acetic acid; (d) ion-exchange chromatography on DEAE-cellulose eluted with a linear gradient of 0.002 M *vs.* 1.2 M pyridine–acetic acid buffer, pH 5.3; (e) ion-exhange chromatography on DEAE-cellulose eluted with a concave gradient of 0.002 M *vs.* 1.2 M pyridine–acetic acid buffer, pH 5.3.

TABLE I: Specific Con A- and WGA-Hemagglutination Inhibitory Activities of Compounds Known to Interact with Con A and WGA.

Compounds	Sp Con A Inhib Act.		Sp WGA Inhib Act.	
	HAIU[a]/mg	HAIU[a]/μmol	HAIU[a]/mg	HAIU[a]/μmol
Methyl α-D-mannopyranoside		300		
p-Nitrophenyl α-D-mannopyranoside		300		
Methyl α-D-glucopyranoside		60		
Ovalbumin	130	5900[b]	<130	
Ovalbumin glycopeptide[c]	2300	5100[d]	<130	
Ovomucoid			470	13,500[e]
2-Acetamido-2-deoxy-D-glucose				30
p-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside				240

[a] Inhibitory activities are expressed as hemagglutination inhibitory units (HAIU). One unit of inhibitory activity is defined as the minimum amount of inhibitor required to completely inhibit 3 hemagglutination units of Con A or WGA. [b] Assuming a mol wt of 45,000 (Warner, 1954). [c] Ovalbumin glycopeptide was prepared by the method of Montgomery *et al.* (1965). [d] Assuming a mol wt of 2210 as determined by gel filtration on Sephadex G-50 (Bhatti and Clamp, 1968) and aspartic acid analysis assuming 1 mol of aspartic acid/mol of glycopeptide. [e] Assuming a mol wt of 28,800 (Chatterjee and Montgomery, 1962).

in 1 M NaCl adjusted to pH 8.0 with 5% $NaHCO_3$. Solubilized Con A was further purified by affinity chromatography on Sephadex G-50, as described by Agrawal and Goldstein (1965). The purified Con A, eluted from the column with glucose, was dialyzed against 1 M NaCl containing 5 mM $MgCl_2$ and stored at 4°.

Dilutions (1:2, 1:3, 1:4, 1:6,...1:96) of each agglutinin stock solution, containing 0.4 mg/ml of Con A or WGA, were prepared using PBS. Aliquots (5 μl) of each dilution of Con A or WGA were placed in 12 separate wells of a microtest plate (No. 3034 Microtest Tissue Culture Plate, Falcon Plastics, Oxnard, Calif.). Five microliters of PBS were then added to each well. The solutions were permitted to stand at 23° for 1 hr prior to addition of erythrocytes.

Erythrocytes were obtained from guinea pigs (American Slicks, Wayfran Cavy Breeders, Sperry, Okla.) by heart puncture. A stock suspension of erythrocytes was prepared by dilution of the whole blood with Alsevers solution (1:1 v/v). For hemagglutination assay erythrocytes were washed (210*g*, 10 min) three times with PBS. Aliquots (5 μl) of a 1% (v/v) suspension of erythrocytes in PBS were added to each well of the microtest plate. After 30 min, agglutination was determined by observing the contents of each well using a substage microscope. The end points in the hemagglutination assays were defined as the highest dilution of WGA or Con A which demonstrated hemagglutination activity. One unit of hemagglutination activity was defined as the amount of agglutinin present at the end point.

Utilizing this assay system the WGA preparation possessed 17,000 hemagglutination units/mg, assuming an extinction coefficient ($E^{1\%}_{280nm}$) of 12; and the Con A preparation possessed 23,000 hemagglutination units/mg, calculated using an $E^{1\%}_{280nm}$ of 11.4 (Agrawal and Goldstein, 1967). To assay for hemagglutination inhibition, three hemagglutination units of WGA or Con A (5 μl of an agglutinin solution containing 600 hemagglutination units/ml) were added to 12 separate wells of a microtest plate. Materials to be tested for hemagglutination inhibitory activity were prepared as stock solutions in PBS at concentrations of 3 mg/ml or 25 mM. Dilutions (1:2, 1:3, 1:4, 1:6,...1:96) of each test solution were prepared using PBS. To each 3 hemagglutination units of agglutinin was added 5 μl of each dilution of test sample. After 1 hr at 23°, 5 μl of the erythrocyte suspension was added to each well and hemagglutination inhibition was determined after an additional 30 min. One unit of hemagglutination inhibitory activity was defined as the minimum amount of inhibitor necessary to completely inhibit 3 hemagglutinating units of Con A or WGA.

As shown in Table I, the specificities of Con A and WGA, determined by this hemagglutination inhibition assay, correlated well with their reported specificities (So and Goldstein, 1967; Burger, 1969; Burger and Goldberg, 1967). Hemagglutination by WGA was inhibited eight times more effectively with *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside than with 2-acetamido-2-deoxy-D-glucose, indicating the importance of the pyranose ring and/or the β configuration at the anomeric carbon atom. This assay also demonstrated that the Con A binding activity of ovalbumin could be quantitatively recovered in the ovalbumin glycopeptide. Ovalbumin has been shown to contain α-D-mannopyranosyl residues at nonreducing, terminal positions of its oligosaccharide moiety (Huang *et al.*, 1970).

Resolution of Cell-Surface Glycopeptides Possessing Con A- and WGA-Binding Activity. To determine if cell-surface sialoglycopeptides possessed the ability to interact with either WGA or Con A, the glycopeptide fractions were tested for their hemagglutination inhibitory activity. The distribution of WGA- and Con A-binding activities in C-SGP is reported in Table II. C-SGP-A, which contained 40% of the weight and 18% of the sialic acid of C-SGP, possessed the major portion of the WGA-binding activity. C-SGP-B, which contained 15% of the weight and 19% of the sialic acid of C-SGP, possessed no detectable WGA- or Con A-binding activity. C-SGP-C, the lowest molecular weight class of sialoglycopeptides, contained 45% of the weight and 62% of the sialic acid of C-SGP and possessed only Con A-binding activity.

Compositional Analysis of Glycopeptide Fraction DAI-2. The glycopeptide fraction (5 mg) was dried *in vacuo* over P_2O_5 at 100° in an Abderhalden-type vacuum drying apparatus, and the dry material weighed on a microanalytical balance to an accuracy of ±20 μg. This material was dis-

TABLE II: Purification of Wheat Germ Agglutinin and Concanavalin A Binding Activity from the Cell-Surface Glycopeptide Fraction.

Fraction	Yield (mg/100 mg of C-SGP)	Sp WGA and Con A Inhib Act.		Total WGA and Con A Inhib Act. per Fraction ($\times 10^{-3}$)	
		WGA (HAIU[a]/mg)	Con A (HAIU[a]/mg)	WGA (HAIU[a]/ Fraction)	Con A (HAIU[a]/ Fraction)
C-SGP	100	700	650	70	65
C-SGP-A	40	1400	450	56	18
P-SGP-A	33	1400	230	46	8
AI	18	2500	350	45	6
DAI-1	1	<70	650		1
DAI-2	12	2500	300	30	4
DAI-3	4	N.D.[b]	<70		
AII	15	<130	130		2
C-SGP-B	15	<130	<70		
P-SGP-B	12	<130	<70		
C-SGP-C	45	<70	400		18
P-SGP-C	35	<70	400		14
DC1	5	<70	1000		5
DC2	5	<70	1100		6
DC3	8	<70	200		2
DC4	7	<70	100		1
DC5	9	<70	<70		

[a] Inhibitory activities are expressed as hemagglutination inhibitory units (HAIU). One unit of inhibitory activity is defined as the minimum amount of inhibitor required to completely inhibit 3 hemagglutination units of Con A or WGA. [b] Not determined.

solved in deionized water, and aliquots were taken for analysis. Amino acid analysis was performed using the Beckman Model 120B amino acid analyzer following hydrolysis in constant boiling HCl at 110° under N_2.

The amino acid analysis of DAI-2 is shown in Table III. Hydrolysis was performed for 24 and 72 hr to permit extrapolation of degraded amino acids to zero time using the formula of Hirs *et al.* (1954). The valine and isoleucine values were significantly higher in the 72-hr hydrolysate; therefore, only the highest values for these amino acids were utilized. Arginine and methionine were not detected in the hydrolysates.

Hexosamines were quantitated concurrently with amino acid analysis by the method of Walborg *et al.* (1963). Under these conditions considerable degradation of hexosamines occurred; therefore, the values were extrapolated to zero time using the formula of Hirs *et al.* (1954). These results are shown in Table III. When the hexosamine content of DAI-2 was determined, using milder hydrolysis conditions in which hexosamine degradation was not a major factor (4 N HCl, 8 hr, 100°, under N_2), the glucosamine and galactosamine contents were 0.893 and 0.349 μmol/mg of glycopeptide, respectively (Table III). These values were in good agreement with the extrapolated values obtained in conjunction with amino acid analysis.

Neutral sugars were quantitated using the ion-exchange chromatograghic procedure of Walborg and Kondo (1970). Hydrolysis was accomplished using strong cation exchange resin (Dowex 50W-X8 in the H^+ form) in 0.25 N H_2SO_4 for 48 hr, according to the procedure described by Kim *et al.* (1967), and neutralization of the filtered hydrolysate was performed as described by Walborg *et al.* (1965). The results of neutral sugar analysis are shown in Table IV. Total neutral sugar content was determined by the method of Dubois *et al.* (1956). D-Mannose, L-fucose, D-galactose, and D-glucose were used as standards, and a weighted extinction coefficient was calculated based on the relative amounts of each monosaccharide present, as resolved and quantitated by ion-exchange chromatography. The neutral sugar content obtained by the latter method represented a maximum value. Resolution and quantitation of neutral sugars indicated that DAI-2 contained 1.65 μmol of hexose/mg of glycopeptide while the phenol–sulfuric acid method indicated the presence of 1.60 μmol of hexose/mg. These values are in good agreement and indicate the effectiveness of the hydrolytic conditions utilized for neutral sugar analysis.

Sialic acid was determined as *N*-acetylneuraminic acid by the method of Warren (1959) following hydrolysis in 0.1 N H_2SO_4. The amino acid and carbohydrate compositions of DAI-2 are summarized in Table IV. The compositional analysis accounts for 84.3% of the dry weight of DAI-2.

Discussion

The cytoagglutination studies performed on AS-30D tumor cells indicated that this tumor system represented an interesting exception to the generalization that transformed cells are highly agglutinable by both WGA and Con A. The agglutinability of these cells by low concentrations of Con A only after papain digestion indicated that they possessed cryptic binding sites for Con A. Treatment of tumor cells with protease has been shown to result in either no loss (Pollack and Burger, 1969) or an increase (Pattillo *et al.*, 1971; Kapeller and Doljansky, 1972) in their cytoagglutinability by WGA. AS-30D cells also exhibited increased agglutinability by WGA following treatment with papain. The ability of 2-

TABLE III: Amino Acid and Hexosamine Composition of DAI-2.

Component	μmol/mg of Glycopeptide		
	24-hr Hydrolysis	72-hr Hydrolysis	Av or Extrap Value
Lysine	0.079	0.102	0.090
Histidine	0.024	0.036	0.030
Aspartic acid	0.312	0.318	0.315
Threonine	0.465	0.440	0.478
Serine	0.274	0.240	0.293
Glutamic acid	0.316	0.308	0.312
Proline	0.142	0.159	0.150
Glycine	0.143	0.140	0.142
Alanine	0.153	0.156	0.155
Half-cystine	0.087		0.087
Valine	0.186	0.210	0.210
Isoleucine	0.067	0.076	0.076
Leucine	0.100	0.099	0.100
Tyrosine	0.055	0.049	0.058
Phenylalanine	0.065	0.073	0.069
Glucosamine	0.719	0.684	0.928
Galactosamine	0.329	0.283	0.354

TABLE IV: Peptide and Carbohydrate Composition of DAI-2.

Component	μmol/mg of Glycopeptide	Residue Wt (%)
Peptide		26.8
Carbohydrate		
sialic acid	0.166	4.8[a]
glucosamine	0.928	18.8[b]
galactosamine	0.354	7.2[b]
mannose	0.409	6.6
fucose	0.202	3.3
galactose	0.784	12.7
glucose	0.252[c]	4.1
	Total	84.3

[a] Calculated as *N*-acetylneuraminic acid. [b] Calculated as *N*-acetylhexosamine. [c] Contamination of the sample by glucose-containing components derived from the cellulose ion exchanger cannot be excluded.

acetamido-2-deoxy-D-glucose to inhibit cytoagglutination of AS-30D tumor cells by WGA further substantiated previous data (Burger, 1969) indicating that this monosaccharide is a component of the cell-surface, WGA-binding site.

The ability of C-SGP to inhibit cytoagglutination by WGA and Con A indicated that this fraction contained components that possessed the minimum requirements of phytoagglutinin-binding sites, *i.e.*, localization at the cell periphery and ability to interact with Con A and WGA, as evidenced by its ability to inhibit hemagglutination by these phytoagglutinins.

The resolution of the Con A- and WGA-binding activities of C-SGP has been accomplished utilizing gel filtration and ion-exchange chromatography. When SGP was submitted to gel filtration on Sephadex G-50 eluted with 0.1 N acetic acid, rather than deionized water as previously described (Smith and Walborg, 1972a), it was resolved into three glycopeptide fractions: C-SGP-A, C-SGP-B, and C-SGP-C. Further resolution of the component glycopeptides of C-SGP-A on Sephadex G-200 indicated that this fraction contained sialoglycopeptides ranging in apparent molecular weights from 3000 to 200,000 or more; however, the ability of glycopeptides and glycoproteins to exhibit aberrantly high molecular weights on Sephadex has been well documented (Pepper and Jamieson, 1970). Similar molecular weight heterogeneity has been observed in glycopeptides obtained by protease digestion of intact platelets (Pepper and Jamieson, 1970), intact Novikoff hepatoma cells (Wray and Walborg, 1971), and mouse spleen cell membranes (Nathenson *et al.*, 1970).

Since essentially all of the WGA-binding activity resided in those glycopeptides excluded from Sephadex G-50 (P-SGP-A), this fraction was chosen for purification of WGA-binding glycopeptides.[3] Gel filtration on Sephadex G-200 and subsequent ion-exchange chromatography resulted in the isolation of a sialoglycopeptide fraction (DAI-2). This fraction, which represented 12% of the weight of C-SGP, possessed the major portion of the WGA-binding activity and low Con A-binding activity. DAI-2 consists of a family of glycopeptides possessing polypeptide chains which are highly substituted with heterosaccharide chains. This conclusion is based on the following chemical properties of DAI-2: (1) its high apparent molecular weight, as judged by its behavior on Sephadex G-200; (2) its compositional analysis, particularly the enrichment of the peptide moiety in those amino acids (Asp, Thr, and Ser) known to be involved in the linkage of carbohydrate to the peptide chain; and (3) its resistance to proteolysis.

The cell-surface sialoglycopeptide fraction from Novikoff ascites tumor cells was shown to contain several components having a molecular weight of <3300 which possessed Con A-, but no WGA-binding activity (Wray and Walborg, 1971). Similar glycopeptides (DC1–DC5), possessing only Con A-binding activity, were also isolated from the surface of AS-30D cells (a cell line which is weakly agglutinated by Con A), suggesting that these glycopeptides were either present at a density insufficient to produce agglutination or that their topographical distribution at the cell periphery precluded their involvement in cytoagglutination.

Control experiments (cells incubated without papain and papain incubated without cells) have indicated the presence in C-SGP of a neutral sugar-containing component, that contained no sialic acid (Smith and Walborg, 1972a). Therefore, the neutral sugar containing fractions of C-SGP, which were not retained on DEAE-cellulose columns eluted with low ionic strength buffer, may not represent components of the cell surface released by papain. Fraction DC1, which was not retained on DEAE-cellulose, was rich in neutral sugar, and contained <0.07 μmol of sialic acid/mg. Fraction DC2 was, therefore, the only sialoglycopeptide fraction in C-SGP which demonstrated an increase in specific Con A-binding activity. This fraction represented 5% of the total weight and 7% of the total sialic acid of C-SGP.

The glycopeptide components isolated from the AS-30D tumor cell surface may be considered proteolysis products of plasma membrane glycoproteins. Isolation of a cell-surface

[3] The specific WGA-hemagglutination inhibitory activities of C-SGP and DAI-2 reported earlier (Smith and Walborg, 1972b) were erroneously high due to the use of an unstable WGA preparation.

glycopeptide fraction (DAI-2), which possessed phytoagglutinin-binding activity that reflected the cytoagglutination properties of the intact AS-30D cell, suggests that plasma membrane glycoproteins may play an important role in the cytoagglutination of AS-30D tumor cells by WGA. These findings emphasize the necessity to investigate the phytoagglutinin-binding properties of protease-labile, cell-surface glycopeptides from other tumor cells with differing cytoagglutination properties and from normal cells. Comparison of the chemical properties and agglutinin-binding activities of such glycopeptides may provide information concerning the chemical alterations in cell-surface oligosaccharide moieties which are responsible for the agglutinability of malignantly transformed cells by Con A and WGA.

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