Carbohydrate Research, 111 (1983) 257–271
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

CLONES FROM CULTURED, B16 MOUSE-MELANOMA CELLS RESISTANT TO WHEAT-GERM AGGLUTININ AND WITH ALTERED PRODUCTION OF MUCIN-TYPE GLYCOPROTEINS*

LI-HSUEH LIN, JEFFREY L. STERN, AND EUGENE A. DAVIDSON†
Department of Biological Chemistry and the Specialized Cancer Research Center, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033 (U.S.A.)
(Received December 22nd, 1981; accepted in revised form, March 8th, 1982)

ABSTRACT

Several clones resistant to wheat-germ-agglutinin toxicity were isolated from B16 mouse-melanoma cells. The resistance may be explained in part by fewer binding sites for the agglutinin on the variant lines; the total, cellular sialic acid content appeared somewhat reduced. Analysis of cell glycoproteins indicated a decreased content of sialic acid in one glycoconjugate that binds to wheat-germ agglutinin and has O-linked oligosaccharides.

INTRODUCTION

In a previous study from this laboratory[1], we have described the isolation of a mucin-type sialoglycopeptide from both the cells and culture medium of B16 mouse-melanoma cells. This product was not detected in normal mouse-iris melanocytes[2]. A key feature of the sialoglycopeptide was its ability to bind to a wheat-germ-agglutinin (WGA) affinity column. Although it has been generally assumed that the specific ligand for wheat-germ agglutinin is *N*-acetylglucosamine [or its homologous β-D-(1→4) oligosaccharides][3], the B16 glycopeptide does not contain this sugar, and its interaction with the agglutinin is exclusively dependent on the number and spatial architecture of the sialyl residues[1,4]. WGA itself is toxic for the B16 mouse melanoma cells and, although the mechanism of cytotoxicity is unknown, binding at the cell surface seems likely to be a necessary first step. Since the parent glycoprotein from which the mucin-type sialoglycopeptide is derived[5] must be associated with the cell surface, at least in a transitory fashion, it was of interest to examine WGA-resistant cells for biochemical, morphological, and functional properties. This paper describes the isolation and preliminary characterization of several WGA-resistant clones of the B16 mouse melanoma, and some properties of two selected variants.

*Supported, in part, by Grant CA-15483 from the National Cancer Institute, National Institutes of Health.
†To whom correspondence and reprint requests should be addressed.

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EXPERIMENTAL

Materials. — Pronase CB and neuraminidase (*Vibrio cholerae*) were obtained from Calbiochem-Behring Corp. (San Diego, CA 92112); bovine serum albumin and Sepharose from Sigma Chemical Co., (St. Louis, MO 63178); D-[^{3}H]glucosamine hydrochloride and sodium [^{125}I]iodide from New England Nuclear (Boston, MA 02118); F-12 medium and antibiotic solution from Flow Laboratories, Inc. (McLean, VA 22102); and cyanogen bromide was purchased from Eastman Kodak Co., Rochester, NY 14650.

Wheat-germ agglutinin was isolated from crude wheat-germ according to the procedure of Nagata *et al.*[6,7], except that, after the second ammonium sulfate fractionation, an affinity column of ovalbumin–Sepharose was used for purification[8]. Conjugation of the lectin to Sepharose 4B was done essentially as described by March *et al.*[9]. The Sepharose 4B was activated by adding cyanogen bromide, dissolved in acetonitrile, to beads suspended in a solution of 2M sodium carbonate. The remaining active groups were blocked by shaking a suspension of the beads in 0.1M ethanolamine hydrochloride, for 4 h at 25°. The product was washed successively with 2M sodium chloride, 6M guanidine hydrochloride, and 0.05M Trishydrochloride (2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride, pH 8.0), prior to preparing a 0.6 × 8-cm column. Analysis indicated a concentration of 2 mg of WGA per mL of Sepharose 4B. The oligosaccharides were isolated from fetuin by the procedure of Spiro and Bhoyroo[10].

Cell culture. — An "amelanotic" clone of the B16 mouse-melanoma cell-line was routinely grown, in Falcon tissue culture T-flasks (75 mm^2), in F-12–1% BSA medium supplemented by the addition[11] of 2mM L-glutamine, 0.11% sodium hydrogencarbonate, penicillin (10 units/mL), and streptomycin (10 μg/mL). These cells still produced measurable quantities of melanin and, in contrast to the black color of the parent B16 line, appeared light tan in the aggregate, as either cell pellets or solid tumor. Cells were subcultured at confluency by suspension with 0.02% ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) in calcium- and magnesium-free, phosphate-buffered, saline solution. Cell counts were obtained by use of a hemacytometer, and the cell viability was determined by Trypan Blue exclusion. WGA-resistant cell lines were selected from this population and routinely maintained in the same fashion.

The toxicity of wheat-germ agglutinin for B16 melanoma cells was estimated by the following general technique: cells (1–4 × 10^4) were seeded onto Petri dishes (60-mm diam.) or into microwells in regular medium, and incubated overnight in order to permit adhesion to the substrate. The medium was removed and replaced with a medium containing WGA at concentrations ranging from 1 to 40 μg/mL. The dishes were incubated at 37° and inspected after decantation of the medium at 12, 48, and 96 h. The number of cells per dish was counted after staining with 1% Methylene Blue in 70% 2-propanol. Less than 1% of the parent cells remained viable

at an agglutinin concentration of 3 μg/mL, and survival at doses of 5 μg/mL and above was 10^{-4} or less; about 1% of such cells retained WGA resistance.

Isolation of resistant cells. — The resistant cells were selected for their ability to grow in the presence of toxic concentrations of WGA. Usually $1 \cdot 10^5$ cells were plated onto Petri dishes (60-mm diam.), incubated overnight to allow adhesion, and then incubated in the presence of 5 μg/mL of lectin in complete growth-medium (F-12–1% BSA). After an incubation period of 2–3 weeks, the plates were inspected, and the very few surviving cells located by light-microscopy and individually cloned. The cells were then kept in WGA-containing medium at a lectin concentration of 10 μg/mL for 2 months prior to subculturing in nonselective medium. From several independent selections, four clones were isolated without any mutagen treatment. Four clones were isolated also from cells preincubated for 18 h at 37° with ethyl methanesulfonate at a concentration of 150 μg/mL. Under these conditions, the apparent frequency of variants surviving in the presence of WGA was calculated to be in the range of 1 in 10^6 (about one surviving cell per 10 Petri dishes); the mutagen treatment resulted in a two-fold increase in resistant clones. It is likely that the particular isolates examined were already present prior to lectin addition. However, they may still be stable variants since they retained resistance to WGA after extensive subculturing.

Clones 3 and 18, selected from the spontaneous (or pre-existing) group, were chosen for additional studies. These clones showed a growth behavior similar to that of the parent cell line (doubling time and confluent density). In general, the resistant clones could not be distinguished from each other, or from the parent line, on a morphological or size basis, but appeared to produce less melanin than did the parent cells; one clone seemed more melanotic. The WGA toxicity was regularly monitored over a period of one year after routine serial passage of the cells in F-12–1% bovine serum albumin (BSA). The cells were examined for Trypan Blue exclusion and morphology after incubation for 2 h with several concentrations of WGA. The parent B16 cells were detached from the substrate and unable to exclude Trypan Blue at a concentration of 2 μg/mL; at a 5 μg/mL and all higher concentrations, the cells were totally disrupted. However, with the resistant clones (3 and 18), the cells were not visibly affected at a concentration of 25 μg/mL and showed only a slight swelling, and were still viable at a concentration of 40 μg/mL of the lectin. The doubling times of clones 3 and 18 were not significantly altered from that of the parent cells at a lectin concentration of 10 μg/mL (data not shown).

Glycoprotein synthesis and properties. — In order to assess complex-saccharide synthesis, the cells were cultured, for 48 h prior to harvest, in a medium containing 10 μCi of D-[^{3}H]glucosamine hydrochloride/mL. The glycopeptides were isolated from the labeled cells, as well as from the spent medium as previously reported[2]. After Pronase digestion for 72 h at 37°, the glycopeptides were dialyzed for 24 h against 0.9% sodium chloride, and then for several days against de-ionized water at 4°. The nondialyzable glycopeptide fraction was subjected to affinity chromatography on a column (0.6 × 8 cm) of WGA–Sepharose containing 2 mg of lectin/mL

of packed volume. The column was washed with 50mM Tris buffer, pH 8.0, and the WGA-bound glycopeptide eluted with 0.1M *N*-acetyl-D-glucosamine in the same buffer. The eluate was collected in 1-mL fractions, and aliquots were taken for measurement of radioactivity. The WGA-bound fractions were combined for further characterization. In general, experiments were performed in triplicate. This procedure focuses on the *O*-glycosyl-linked glycoprotein of these cells[5], because the proteolytic digestion, followed by dialysis results in the loss of glycopeptides derived from *N*-linked glycoproteins. About 11% of the radioactivity incorporated into macromolecules was found in the nondialyzable fraction.

The sialic acid content of the glycopeptide was estimated, as percent of the total ^{3}H-label, by digestion with *Vibrio cholerae* neuraminidase. The reaction was performed in 0.1M sodium acetate buffer, pH 5.6, containing mM calcium chloride, with 0.1–1.0 unit of enzyme in a total digestion volume of 200 μL; incubation was carried out for 48 h at 37°, with one addition of enzyme at 24 h. The released sugar was separated from the residual glycopeptide by exclusion chromatography in 0.01M pyridinium acetate, pH 5.0, on a column (0.9 × 63.5 cm) of Bio-Gel P-2. The radioactivity content of the monosaccharide was determined, and its identity confirmed by paper chromatography[12].

In a time-course study of the effect of neuraminidase on the WGA-bound glycopeptide, the enzyme was incubated with the substrate for the specified time, the reaction stopped by boiling for 1 min, and the mixture fractionated on a Bio-Gel P-2 column as just described. The fraction eluted at the void volume and presumably representing the glycopeptide was collected and chromatographed on a WGA-Sepharose column.

Alkaline borohydride treatment of the glycopeptides was performed under nitrogen, for 72 h at 37°, in sealed tubes in the dark with 0.1M sodium hydroxide–M sodium borohydride. The mixture was cooled in an ice bath, and the excess of borohydride eliminated by careful addition of M acetic acid to pH 6–7. The solution was made neutral, chromatographed on a column (0.9 × 104 cm) of Bio-Gel P-6 in 0.1M pyridinium acetate, pH 5.0.

Sialic acid content. — The sialic acid content of the cells was estimated by measuring the neuraminidase-sensitive or acid-labile sialic acid. The former procedure utilized 5×10^7 cells, incubated with shaking for 4 h at 37° with neuraminidase (5 units) in 0.05M phosphate buffer containing 5mM calcium chloride and 75mM sodium chloride, pH 5.8. The cells were removed by centrifugation, washed with the aforementioned buffer (1 mL), and the combined supernatant and wash solutions assayed for sialic acid[13]. The cell pellet was solubilized in 0.1% sodium dodecyl sulfate and the protein content determined by a dye-binding procedure[14]. Alternatively, 5×10^7 cells were suspended in 0.1M sulfuric acid (1 mL), and the suspension was kept for 45 min at 80°. Following centrifugation, an aliquot was removed for sialic acid determination[13].

The neuraminidase-accessible sialic acid declined somewhat in the WGA-

TABLE I

SIALIC ACID CONTENT OF CELL LINES[a]

Cell line	*Sialic acid determined after treatment with*	
	Neuraminidase	*Acid*
B16	14.3 ± 3.6	17.1 ± 4.4
Clone 3	12.6 ± 2.9	15.0 ± 3.1
Clone 18	11.8 ± 3.2	13.6 ± 3.2

[a]Sialic acid content expressed as μg per 5×10^7 cells.

resistant clones. Similar results were obtained for the sialic acid released by acid. Data are summarized in Table I.

Binding to wheat-germ agglutinin. — WGA was radiolabeled with [^{14}C]formaldehyde by a reductive alkylation method utilizing a modification of the procedure described by Winkelhake[15]. WGA was prepared at a concentration of 5.0 μg/mL in 0.2M borate buffer, pH 8.6, and [^{14}C]formaldehyde (51.6 μL, 10 μCi) was added to 2.0 mL of the solution; this represented a three-fold molar excess based on the lysine content of the agglutinin. The reaction was allowed to proceed for 5 min at 4° with continuous agitation by a stream of nitrogen. At the end of 5 min, 3% sodium borohydride (15 μL) was added under nitrogen with rapid mixing. The resulting solution was kept for 5 min at 4°, and an additional 5 min at 25°. The reaction was terminated by the addition of M Tris buffer, pH 6.0, to a final concentration of 0.3M. The labeled WGA was dialyzed for 24 h against 50mM Tris buffer, and then against de-ionized water. The protein solution was examined for homogeneity by poly(acrylamide) gel electrophoresis in the presence of sodium dodecyl sulfate, and for ligand properties by agglutination activity. The gel electrophoresis was performed in 6% poly(acylamide) gels at a constant voltage (70 V) for 2 h and 40 min in 0.01M phosphate–0.13M sodium chloride, pH 7.2 (PBS). The gels were removed and sliced into 2-mM sections. Each slice was incubated at 60° with 60% perchloric acid (0.2 mL) and 30% hydrogen peroxide (0.4 mL).

When the gel was dissolved, scintillation cocktail (Aquasol-2) was added, and the radioactivity measured in a liquid-scintillation counter. The data indicated that only one component was present. The mobility of the radiolabeled protein was the same as that of unlabeled WGA in the gel electrophoresis system, as well as in exclusion chromatography on Sephadex G-100. Amino acid analysis of a comparable product of alkylation with unlabeled formaldehyde indicated that ~2/3 of the lysine residues had been alkylated. The specific activity of the isolated lectin was 5.15×10^6 c.p.m./mg; the agglutination titer of the labeled WGA for human red-blood cells was not affected by the labeling process, an indication that affinity for sialoglycoconjugates (*e.g.*, glycophorin) was unchanged.

The ^{14}C-labeled WGA was used in binding experiments as follows: the aggluti-

nin was diluted with PBS to give final concentrations ranging from 0.1 to 100 μg/mL. After growth as described, cells were harvested, washed twice with PBS, and suspended in calcium- and magnesium-free PBS (1.1 mL). An aliquot (20 μL) of the labeled lectin was added to 10^6 cells, and the suspension incubated for 20 min at 25° with occasional shaking. Triplicate sets were run in the absence and presence of 0.1M *N*-acetylglucosamine in PBS. The cells were centrifuged, washed thrice with PBS, and dissolved in 2% sodium dodecyl sulfate (1 mL), and an aliquot was taken for measurement of radioactivity. The value of total binding less the value in the presence of *N*-acetylglucosamine (nonspecific binding), which was uniformly less than 15% of the maximal, total-bound value, was taken as the specific binding. Recovery controls showed no loss of lectin due to nonspecific adsorption over the concentration range employed.

Tumorigenicity. — Each of 7–10 C57BL/6j mice was inoculated subcutaneously with 10^4, 10^5, or 10^6 parent or WGA-resistant, Clone-3 and -18 cells in PBS (0.2 mL); this experiment was performed twice. All mice were observed over a two-month period for tumor incidence and survival.

Cell-surface proteins. — Cell-surface proteins were labeled with iodine-125 by lactoperoxidase-catalyzed iodination using a modification of the method described by Hynes[16]. Cells were grown to confluency in 450-mL T flasks, harvested, washed three times with PBS, and resuspended in PBS containing 5mM D-glucose (2 mL). Carrier-free sodium [^{125}I]iodide (400 μCi) was added, followed by lactoperoxidase and D-glucose oxidase to final concentrations of 20 μg and 0.1 units/mL, respectively. The reaction was allowed to proceed in a stoppered tube for 10 min at 25° with occasional swirling. The iodination was stopped by the addition of a five-fold excess of PBS containing 2mM (phenylmethyl)sulfonyl fluoride. The cells were centrifuged off, washed with PBI (PBS where sodium iodide had replaced sodium chloride), and extracted with 0.5M lithium diiodosalicylate–0.1% Triton X-100 (1 mL). This extractant was shown to be more effective than Triton alone and to solubilize ~90% of the radioactivity from the cell pellet. The solubilized, iodinated, surface proteins were passed through a WGA-Sepharose column and resolved into WGA-nonbound and WGA-bound fractions. These were separately concentrated and examined by exclusion chromatography on Sepharose 4B. The WGA-bound glycoprotein fraction from control and Clone-18 cells was isolated and digested with Pronase under the same conditions as those utilized for metabolically labeled material[2]. The Pronase digest was dialyzed and fractionated on WGA-Sepharose. The WGA-bound fractions (eluted with *N*-acetylglucosamine) were further chromatographed on a DEAE-cellulose column (0.9 × 36 cm), being eluted with a linear gradient (0.01 to 1.0M) of pyridinium acetate, pH 5.0.

RESULTS

The nondialyzable glycopeptides obtained from labeled cells and from spent medium after Pronase digestion were examined on a WGA-affinity column. A typical

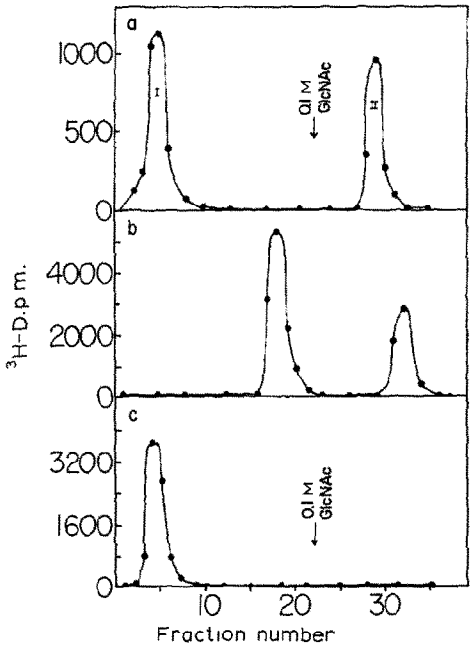

Fig. 1. Characterization of [^{3}H]glycopeptide obtained from WGA-resistant, Clone-3 cells after Pronase digestion: (a) affinity chromatography on WGA-Sepharose; (b) chromatography of [^{3}H]-glycopeptides on Bio-Gel P-2 in 0.1M pyridinium acetate, pH 5.0, after digestion with neuraminidase; and (c) rechromatography of Peak I (a) on WGA-Sepharose.

chromatography-profile, for cell-associated glycopeptides, from a WGA-Sepharose column is shown in Fig. 1a. Peak II, eluted with 0.1M *N*-acetylglucosamine in Tris buffer, represents the WGA-bound fraction. The percentage of radioactivity, in this fraction obtained from the parent B16 cells and two WGA-resistant clones, is given in Table II. The WGA-bound glycopeptide fraction contained ~10% of the ^{3}H radio-

TABLE II

AFFINITY[a] FOR A WGA-SEPHAROSE COLUMN AND SIALIC ACID CONTENT[b] OF [^{3}H]GLYCOPEPTIDE OBTAINED FROM DIFFERENT CELL LINES

Cell line	*Affinity of*		
	Cell	*Medium*	*Sialic acid content*
B16 melanoma	34.2	9.5	46.0
Clone 3	34.7	8.6	35.0
Clone 18	11.4	21.6	23.9

[a]As percent of total radioactivity. [b]The content is given as the percent of radioactivity for sialic acid as compared to the sum of radioactivities for glucosamine, galactosamine, and sialic acid; data are for the cell-associated fraction.

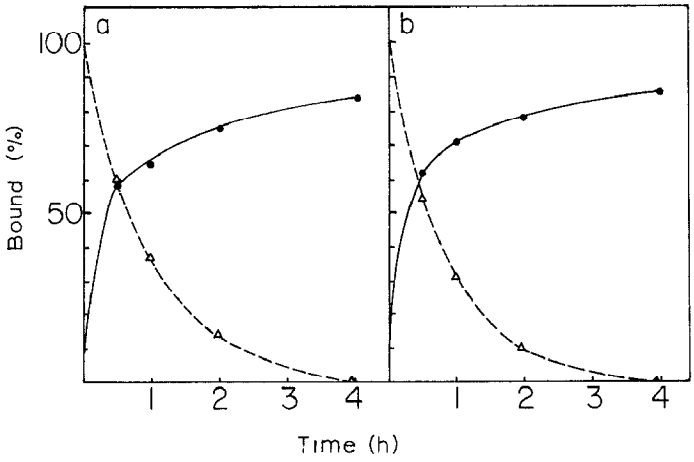

Fig. 2. Neuraminidase digestion of WGA-bound material from B16 (a) and Clone-18 cells (b). The enzyme digestion was performed for the specific times as indicated. The partially desialylated glycopeptide was chromatographed on a WGA-Sepharose column to determine the percentage of binding: (△) bound fraction; and (●) sialic acid (%) released.

activity present in the total glycopeptide fraction. The amount of material bound to WGA was essentially the same for the B16 cells and Clone 3, whereas a significantly reduced amount of label was found in this fraction from Clone 18 (11.4 *vs.* 34.2%). However, more WGA-bound material was present in the spent medium of the L18 cells. This material was not characterized extensively, but it did contain O-linked oligosaccharides. Otherwise, ~10% of the nondialyzable radioactivity from the spent medium was bound to WGA; recoveries from all affinity-chromatography experiments were between 85 and 100%.

The sialic acid content of the cell-associated, WGA-bound glycopeptide was estimated after digestion with neuraminidase for 48 h, and chromatography on a Bio-Gel P-2 column (Fig. 1b). The product in the second peak corresponds in mobility to that of authentic *N*-acetylneuraminic acid.

Neuraminidase treatment of the WGA-bound glycopeptides from the parent B16 cells resulted in the release of 46% of the radioactivity as sialic acid; the remaining radioactivity was found as galactosamine; glucosamine was not detected. Similar analyses for resistant Clones 3 and 18 gave proportions of 35.0 and 23.9% of the label in sialic acid, respectively. The residual label in both these cases was exclusively in galactosamine.

The relationship between the sialic acid of the bound glycopeptide and the WGA affinity was examined further by a kinetic study with neuraminidase. The results shown in Fig. 2 clearly demonstrate the importance of sialic acid for WGA-binding for the associated glycopeptide from the parent-B16 and Clone-18 cells. The labeled sialic acid released by acid hydrolysis with 25mM sulfuric acid for 1 h at 80° was taken as 100% for each sample. During the first 0.5 h, the enzyme released >50% of the sialic acid from both glycopeptides, and ~1/2 of the labeled glyco-

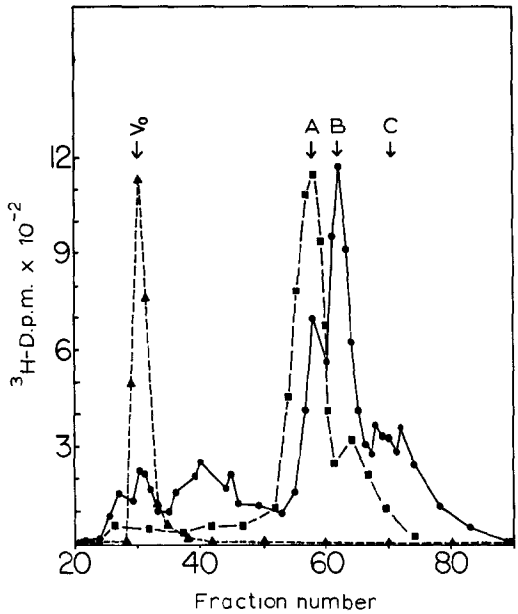

Fig. 3. WGA-bound sialoglycopeptide from Clone 18 cells, chromatographed on a Bio-Gel P-6 column: (▲—▲) before and (●—●) after sodium borohydride treatment; and (■—■) product from parent B16 cells after sodium borohydride treatment. Markers are as follows: (A) and (B) are a tetra- and a tri-saccharide, respectively, obtained from fetuin, and (C) is *N*-acetylneuraminic acid; Vo, void volume.

peptide was still retained on a WGA-Sepharose column. However, when $<15\%$ of sialic acid remained in the glycopeptide, the WGA affinity was completely abolished. After incubation for 24 h, the release of sialic acid was $\sim 100\%$. The asialoglycopeptide was excluded from Bio-Gel P-6, suggesting that several oligosaccharide chains were clustered on the polypeptide, a result confirmed by more detailed studies of WGA-binding properties[1].

Incubation of the WGA-bound glycopeptide from Clone 18 cells with alkaline sodium borohydride[2,17] resulted in $>95\%$ of elimination of the sugar residues from the peptide. Exclusion chromatography of the resultant products on Bio-Gel P-6 (Fig. 3) revealed major products having mobilities comparable to those of standard tri- and tetra-saccharides; a relatively small proportion was present as higher-molecular-weight products. The relationship between chromatographic mobility and size has been previously described[2]. This size distribution is in contrast to that of the products from the parent-B16 clone, the WGA-bound glycopeptide fraction of which contains primarily tetrasaccharide chains having two sialyl groups[2]. Nonetheless, the affinity of the variant glycopeptide for the lectin suggests that its overall structure (frequency of sugar substitution) is similar to that of the parent cell, and comparable to the class I glycopeptides previously described[2]; in WGA-resistant Clone 18, the tetrasaccharide component of this material is markedly reduced with an increase in

TABLE III

TUMOR-FORMING ABILITY OF B16 MELANOMA CELLS AND WGA-RESISTANT CLONE 3 AND 18[a]

Cell dose	*Weeks*					
	2		*4*		*8*	
	B16	*Clone 3*	*B16*	*Clone 3*	*B16*	*Clone 3*
Experiment I						
10^4	0/10	0/10	1/10	0/10	4/10	4/10
10^5	2/9	3/10	4/9	8/10	9/9	10/10
10^6	8/10	6/10	9/9	10/10	3 Deaths[b]	2 Deaths[b]
Experiment II						
	B16	*Clone 18*	*B16*	*Clone 18*	*B16*	*Clone 18*
10^4	0/7	0/8	1/7	0/8	2/7	0/8
10^5	2/8	0/7	6/8	1/7	6/8	2/7

[a]The cells were prepared and injected as described in the text. The C57B1/6j mice were observed for 2 months; data are presented as the number of animals with palpable tumors per number of animals injected. [b]No survivors in either group after 4 additional weeks.

trisaccharide; in addition, there is a decrease in the absolute amount of ^{3}H-label in cell-associated sialic acid (Table II).

The structure of the trisaccharide was examined by chromatography and shown to be heterogeneous (*i.e.*, both linear and branched isomers were present), although the linear product (NeuAc→Gal→GalNAc) was preponderant.

Tumorigenicity was tested for the B16 line and for the WGA-resistant Clones 3 and 18; the results are summarized in Table III. The tumorigenicity of the parent B16 cells is marked, and that of Clone 3 is essentially identical. As a rule, a palpable

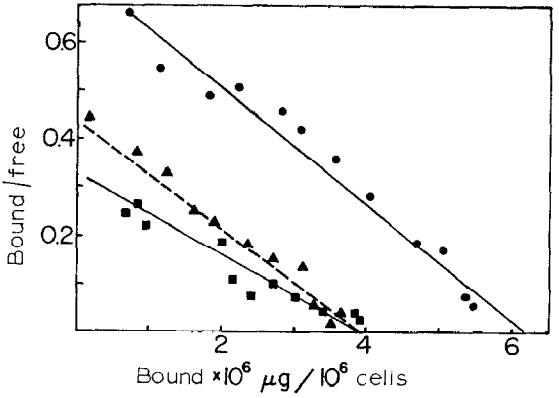

Fig. 4. Scatchard plot of WGA-binding data. Binding experiments were performed as described in the text: (●—●) B16 melanoma, (▲—▲) Clone 3, and (■—■) Clone 18. Points were equally weighted, and the slope calculated for minimal standard-deviation.

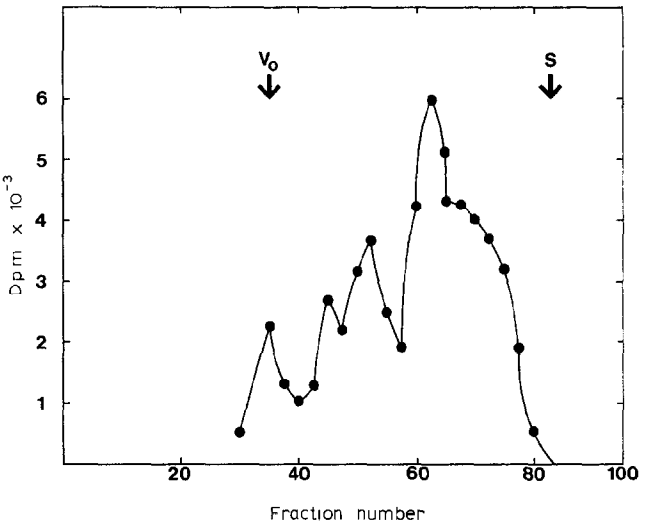

Fig. 5. Chromatography in a column (1.5 × 50 cm) of Sepharose 4B of WGA-bound glycoproteins from Clone 18, B16 mouse-melanoma cells. The eluting buffer was 50 mM Tris · HCl, pH 8.0. The profiles for the parent B16 or Clone-3 cells were essentially the same. Fractions 56–70 contained the WGA-binding glycoprotein having *O*-linked saccharides, and were pooled for further study. Vo, void volume.

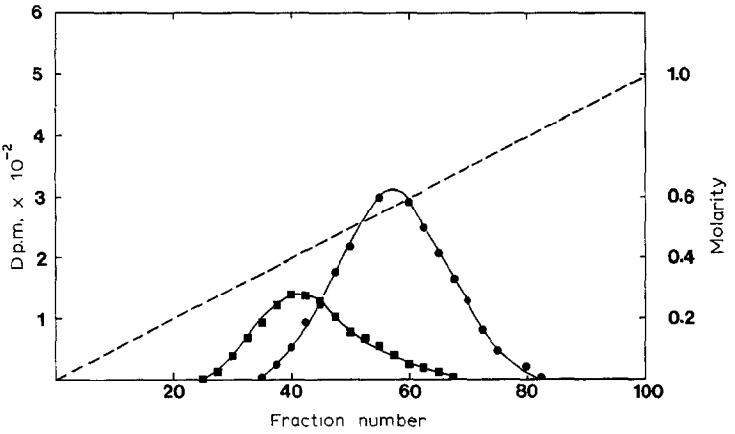

Fig. 6. Chromatography in a column (0.9 × 36 cm) of DEAE-cellulose of nondialyzable glycopeptides derived from WGA-bound glycoproteins by Pronase digestion: (●—●) B16 cells; and (■—■) Clone-18 cells.

tumor was observed within 2 weeks after inoculation with 10^5 or 10^6 cells. However, for Clone 18, tumor incidence and development were somewhat reduced from those observed for the parent B16 cells. Extended observation of animals inoculated with 10^5 cells showed that all developed tumors, but that those given L18 cells had a longer latency period. These results were reproduced in a second experiment but, as yet, insufficient numbers of animals have been tested to ascribe statistical significance to these results.

The binding of ^{14}C-WGA to the parent, B16 melanoma cells and the two WGA-resistant clones was evaluated; data[18] are presented in Fig. 4. The binding capacity was estimated for the B16 melanoma, Clone 3, and Clone 18 to be 6.2, 3.9, and 3.8 $\mu g/10^6$ cells, respectively. Although only a small number of variants have been examined, the binding sites on the resistant cells appear to be fewer but of comparable affinity to those of the parent cells.

The surface proteins of the B16 melanoma and Clones-3 and -18 cells were compared by examination of proteins susceptible to iodination. Poly(acrylamide) gel electrophoresis of iodinated WGA-bound proteins revealed no differences between control, B16-cell material and that derived from either Clone-3 or Clone-18 cells; 3 major and 2 minor bonds were observed for each. A more specific examination was made of the WGA-binding glycoprotein that contains O-linked sialyloligosaccharides. Partial purification on a Sepharose 4B column (1.2 × 50 cm) (Fig. 5) was followed by proteolytic degradation and ion-exchange chromatography. The patterns indicate a somewhat lower charge density for the Clone-18 glycopeptides (Fig. 6), a result consistent with the reduction in sialic acid content and shift from tetra- to tri-oligosaccharides released by treatment with alkaline borohydride.

DISCUSSION

The toxicity mechanisms of most lectins are not known. The lectin from *Ricinus communis* (RCA_{II}) was proposed to act *via* a two-step process in which the lectin is first bound to the cell surface, and then transported inside where it acts directly to inhibit protein synthesis[19,20]. This is apparently not the case for WGA. Isselbacher[21] and Greene *et al.*[22] reported that WGA can inhibit amino acid transport in cultured cells, suggesting that the lectin may exert its effect by altering plasma-membrane functions.

Lustig *et al.*[23] studied the mechanism of WGA-mediated cytolysis of murine-mastocytoma cells, and proposed a step-wise process including: (a) specific binding of the agglutinin, (b) cap formation, (c) membrane-permeability changes, (d) osmotic swelling, and (e) lysis of cells. These experiments demonstrated a complete correlation between capping and cytolysis. One facet of lectin cytotoxicity thus depends on the greater mobility of the membrane and on the topographical organization of cell-surface proteins or other receptor molecules.

WGA is well known for its ability to agglutinate various types of animal cells, in particular malignant, normal dividing, and normal, protease-treated cells[24,25]. The agglutination mechanism may be due to the tetravalent nature of WGA, since Nagata and Burger[6] demonstrated that WGA is composed of 2 subunits and that each protomer binds saccharide ligands with equal affinity at two different locations. A relationship between WGA binding and cell sialic acid content has been previously suggested[26–28]. Specific biochemical lesions that have been described in resistant variants include deficient glycosyltransferases resulting in noncompleted sugar chains[29]. Although the data presented in this report (Scatchard plots) are consistent

with binding sites of comparable affinity, other groups have reported more than one class of WGA-binding site for certain cell-lines[26,28]. It seems clear, however, that the major loci of WGA interaction with any cell surface are the sialyl residues of sialylglycoproteins. A known lack of an *N*-acetylglucosaminyltransferase is likely to result in the failure to complete some complex oligosaccharide chains having a terminal sialyl group[29,30,31]. The approximately linear Scatchard plots obtained in this study only imply sites of comparable affinity and not identical sites.

The different complementation groups that have been identified for lectin-resistant mutants of Chinese-hamster ovary cells[32] clearly indicate that several discrete alterations in cellular metabolism can give rise to the particular phenotype observed in this study. A common feature of those that have been examined in some detail is a decrease of surface sialic acid.

The resistance of the cell lines described in the present experiments can be partially explained by the presence of fewer lectin-binding sites than are on the parent cell. However, the mechanism of resistance is still not clear and, although the sialic acid level was reduced in the variant lines, the specific molecule(s) involved has not been fully identified. Glycosyltransferase levels were not studied, and it is possible that a defect in a specific sialyltransferase may be responsible for the observed changes, but other mechanisms (*e.g.*, altered ectosialidase levels) may apply, including specific polypeptide changes. Compared to the B16 cells, the WGA-resistant Clone 3 appears to have a comparable amount of the mucin-type, WGA-binding glycopeptide, whereas Clone 18 appears to produce a modified structure having a reduced sialic acid content. Since differences in nonaccessible, cell-surface proteins were not detected by this technique, and alterations in dialyzable glycopeptides not documented, specific conclusions regarding surface-protein components cannot be drawn. In addition, the lectin–cell interaction need not be solely dependent on glycoproteins, since glycolipids with appropriate oligosaccharide units (gangliosides) might also interact with WGA, especially where oriented in clusters in the plasma-membrane bilayer.

The facile elimination of the saccharide groups, and the near absence of glucosamine in the nondialyzable, WGA-bound glycopeptide clearly identified the bulk of this fraction as having O-linked carbohydrate groups. The size of the eliminated oligosaccharide alditols is consistent with the observed reduction in sialic acid content, but cannot be interpreted as identifying this mucin-type glycoprotein as the sole (or primary) surface macromolecule interacting with WGA, or as the exclusive locus of reduced sialic acid content.

WGA-resistant cell lines have been studied elsewhere[26,28,29,31,33–37]. A comparison of metastasizing and nonmetastasizing B16 lines indicated an alteration in the saccharide structure (reduced content of sialic acid, increased content of fucose) of a multibranched, N-linked glycoprotein associated with lowered metastatic properties. One such WGA-resistant line had an apparent loss of outer-tier sugars, and a reduction of high-affinity-binding sites[30,34]. It has been suggested that WGA binding to cell surfaces involves a variety of sites, not all of which may contain sialic acid. In addition, data have been presented indicating that discrete WGA-resistant lines

of CHO cells express different kinds of sialyl residues. The overall conclusions indicated by our results are consonant with the latter possibility.

Thus, it seems likely that there are several macromolecular candidates for surface, WGA-binding loci of B16 cells. It is possible that affecting the biosynthesis of any one of them will modulate the events following lectin attachment to a nontoxic level. Based on our studies and reports from others, we conclude that several sites are present with comparable WGA affinity, and that a threshold value exists below which lectin attachment does not result in cell death.

The relationship between various surface macromolecules and biological properties, such as tumorigenicity and metastatic or immunogenic potential, remains to be delineated. A recent report has described an inverse relationship between metastatic behavior and WGA resistance[33]. The presence of mucin-type glycoproteins in cancerous cells has been noted by several investigators[2,12,38–41]. The results reported herein provide support for the production of WGA-bound sialoglycopeptides as a tumor-associated characteristic.

REFERENCES

1 V. P. Bhavanandan and A. W. Katlic, *J. Biol. Chem.*, 254 (1979) 4000–4008.
2 V. P. Bhavanandan, J. Umemoto, J. Banks, and E. A. Davidson, *Biochemistry*, 16 (1977) 4426–4437.
3 N. Sharon and H. Lis, *Science*, 177 (1972) 949–959.
4 P. J. Greenaway and D. LeVine, *Nature New Biol.*, 241 (1973) 191–192.
5 V. Sheik Fareed, V. P. Bhavanandan, and E. A. Davidson, *Carbohydr. Res.*, 65 (1978) 73–83.
6 Y. Nagata and M. M. Burger, *J. Biol. Chem.*, 249 (1974) 3116–3122.
7 Y. Nagata, A. R. Goldberg, and M. M. Burger, *Methods Enzymol.*, 32 (1974) 611–615.
8 V. T. Marchesi, *Methods Enzymol.*, 28 (1972) 354–356.
9 S. C. March, I. Parikh, and P. Cuatrecasas, *Anal. Biochem.*, 60 (1974) 149–152.
10 R. G. Spiro and V. Bhoyroo, *J. Biol. Chem.*, 249 (1974) 5704–5717.
11 J. Banks, V. P. Bhavanandan, and E. A. Davidson, *Cancer Res.*, 37 (1977) 4336–4345.
12 J. B. Adams, *Biochem. J.*, 94 (1965) 368–377.
13 L. Warren, *J. Biol. Chem.*, 234 (1959) 1971–1975.
14 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.
15 J. L. Winkelhake, *J. Biol. Chem.*, 252 (1977) 1865–1868.
16 R. O. Hynes, *Proc. Natl. Acad. Sci. U.S.A.*, 70 (1973) 3170–3174.
17 D. M. Carlson, *J. Biol. Chem.*, 243 (1968) 616–626.
18 G. Scatchard, *Ann. N.Y. Acad. Sci.*, 51 (1949) 660–672.
19 S. Olsnes, *Naturwissenschaften*, 59 (1972) 497–502.
20 K. Refnes, S. Olsnes, and A. Pihl, *J. Biol. Chem.*, 249 (1974) 3557–3562.
21 K. J. Isselbacher, *Proc. Natl. Acad. Sci. U.S.A.*, 69 (1972) 585–589.
22 W. Greene, C. M. Parker, and C. W. Parker, *J. Biol. Chem.*, 251 (1976) 4017–4025.
23 S. Lustig, Y. Handel, and D. H. Pluznik, *Biochim. Biophys. Acta*, 498 (1977) 154–166.
24 M. M. Burger, *Proc. Natl. Acad. Sci. U.S.A.*, 62 (1969) 994–1003.
25 M. M. Burger and A. R. Goldberg, *Proc. Natl. Acad. Sci. U.S.A.*, 57 (1967) 359–366.
26 E. B. Briles, E. Li, and S. Kornfeld, *J. Biol. Chem.*, 252 (1977) 1107–1116.
27 H. A. Robertson, J. R. Etchison, J. S. Robertson, D. F. Summers, and P. Stanley, *Cell*, 13 (1978) 515–526.
28 P. Stanley and J. P. Carver, *Proc. Natl. Acad. Sci. U.S.A.*, 74 (1977) 5056–5059.
29 P. Stanley, V. Caillibot, and L. Siminovitch, *Cell*, 6 (1975) 121–128.
30 M. M. Burger, *Int. Cancer Congr. Abstr., 12th*, Symp. 13, (1978) 47.
31 T. W. Tao and M. M. Burger, *Nature (London)*, 270 (1977) 437–438.
32 P. Stanley and L. Siminovitch, *Somatic Cell Gen.*, 3 (1977) 391–405.

33 J. DENNIS, T. DONAGHUE, M. FLORIAN, AND R. S. KERBEL, *Nature (London)*, 292 (1981) 242–245.
34 J. FINNE, T.-W. TAO, AND M. M. BURGER, *Cancer Res.*, 40 (1980) 2580–2587.
35 J. E. JUMBLATT, T.-W. TAO, V. SCHLUP, J. FINNE, AND M. M. BURGER, *Biochem. Biophys. Res. Commun.*, 95 (1980) 111–117.
36 P. STANLEY, T. SUDO, AND J. P. CARVER, *J. Cell Biol.*, 85 (1980) 60–69.
37 G. YOGEESWARAN AND T.-W. TAO, *Biochem. Biophys. Res. Commun.*, 95 (1980) 1452–1460.
38 J. F. CODINGTON, K. B. LINSLEY, R. W. JEANLOZ, T. IRIMURA, AND T. OSAWA, *Carbohydr. Res.*, 40 (1975) 171–182.
39 J. F. CODINGTON, B. H. SANFORD, AND R. W. JEANLOZ, *Biochemistry*, 11 (1972) 2559–2564.
40 J. MOLNAR, R. A. LUTES, AND R. J. WINZLER, *Cancer Res.*, 25 (1965) 1438–1445.
41 J. MOLNAR, R. A. LUTES, AND R. J. WINZLER, *Cancer Res.*, 25 (1965) 1860–1866.