Carbohydrate Research, 151 (1986) 37–50
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

CHARACTERISTICS OF TWO WHEAT GERM AGGLUTININ-RESISTANT VARIANTS OF B16 MOUSE MELANOMA CELLS WITH REDUCED TUMORIGENICITY*,†

PURNIMA D. PINNADUWAGE, VEERASINGHAM P. BHAVANANDAN, 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 27th, 1985; accepted for publication, January 1st, 1986)

ABSTRACT

Two variants of B16 mouse melanoma cells, selected for their resistance to toxic levels of wheat germ agglutinin isolectin 1 (WGA-1) in serum-free medium, showed by chromosome analysis that they are still mouse cell-lines, continue to produce melanin, and are less tumorigenic in mice than the parent B16 cells. The variants showed a marked decrease in cell agglutination with the wheat germ lectin and a slight increase in cell agglutination with concanavalin A. The binding of ^{125}I-labeled wheat germ agglutinin to the two variant lines was likewise decreased over a 10^3-fold range of lectin concentrations. Terminal sialyl residues were critical in WGA-1 binding to the wild-type cells. The binding data indicated a decrease in high-affinity binding as well as a decrease in the total number of binding sites in the variants. Polyacrylamide gel electrophoresis, followed by affinity staining with ^{125}I-wheat germ agglutinin, showed alterations in the wheat germ agglutinin-binding glycoproteins in the variants compared to those of the parent cell line. However, lactoperoxidase-catalyzed iodination revealed a similar cell-surface protein pattern among the three cell lines. Radioactive glycoproteins secreted or shed by the three cell lines grown in the presence of [^{3}H]glucosamine in serum-free medium were fractionated on the basis of their interaction with WGA-Sepharose (2 mg/mL). The WGA-bound glycoproteins from the two variants had molecular weights of 92,000, 56,000, and 42,000. None of these components was detected in the parent cell-line. A major WGA-binding glycoprotein, which accounted for 37% of the total [^{3}H]glucosamine incorporated, was isolated from the spent medium of the parent mouse melanoma cell-line. This glycoprotein was apparently absent in the WGA-1-resistant variants.

*Dedicated to Roger W. Jeanloz.
†Supported in part by Grant CA-15483 from the National Cancer Institute.

0008-6215/86/$ 03.50

INTRODUCTION

The cell surface plays an important role when a cell shifts from local to metastatic growth[1,2]. While glycoproteins, glycolipids, and glycosaminoglycans are important surface components of all cell types, little is understood about the function of the complex oligosaccharide chains covalently attached to membrane proteins and lipids. Such processes as differentiation and metastasis have been theorized to involve "signals" mediated through lectin-like cell surface molecules that bind to specific classes of carbohydrate structures on membranes of other cells. Changes in the array of either the lectin-like membrane components or the surface glycoconjugates therefore could alter the properties of the cells involved. It has been reported that oncogenically "transformed" cells are generally more readily agglutinable than normal cells, and this is attributed to differences in diffusibility of lectin receptors in the plane of the membrane. For this reason, ligand-mediated agglutinability has been regarded as a criterion for the transformed state[3,4]

Initial attempts to isolate lectin-resistant variants were conducted in the early 1970s. Since then, several laboratories have used cytotoxic plant lectins as selective agents for obtaining tissue-culture cells having altered membrane oligosaccharide units[5–10]. Specific alterations in the biosynthesis of glycoproteins or glycosphingolipids have been found in several such variants[5,11–13]. Thus, WGA-resistant Chinese hamster ovary cells and mouse melanoma variants have sugar deletions limited to sialic acids[5,12–14]. Since the lack of terminal sialyl residues results in an increase in the amount of terminally exposed galactose residues, these variants bind ricin more readily and are therefore more sensitive to ricin than the parent cell-lines. Pea lectin-resistant mouse lymphoma variants are known to have deletions limited to fucose residues[15,16].

The B16 mouse melanoma cell-line is an excellent model for the study of malignant melanomas because of its ultrastructural, biochemical, and immunological similarities to human malignant melanoma[17]. WGA*-resistant B16 mouse melanoma variants with altered surface properties, including cell adhesion and metastasis, have been described. These variants were shown to have decreased tumorgenic and metastatic properties when injected into mice[8,10,14].

This paper describes the isolation and some biological properties of two, less-tumorgenic variants of B16 mouse melanoma cells which are resistant to concentrations of wheat germ agglutinin isolectin 1 that are normally toxic.

---

*The abreviations used are BSA, bovine serum albumin; PBS, phosphate buffered saline; WGA, wheat germ agglutinin; WGA-I, wheat germ agglutinin-isolectin I; GlcNAc, 2-acetamido-2-deoxy-D-glucose; NeuAc, *N*-acetylneuraminic acid; Con-A, concanavalin A; PPO, 2,5-diphenyloxazole.

MATERIALS AND METHODS

Cell culture. — Media. The cell-culture medium components and fetal calf serum were purchased from Flow Laboratories (Rockville, MD). Modified Ham's F12 medium with glutamine was obtained as a dry powder (Lot 10421004, Catalog no. 10-421-22) and reconstituted in distilled, deionized water. Bovine serum albumin (1 g per 100 mL) (Sigma Chemical Company, St. Louis, MO) was added to the F12 medium and the solution filtered through Nalgene filter units (Catalog no. 120-0020) immediately after dissolving[18]. The filtrate, designated serum-free medium, was supplemented in all cases with penicillin (10 units/mL), streptomycin (10 μg/mL), and 0.11% $NaHCO_3$.

Complete serum medium is defined as minimum essential medium, with Eagle's salt solution supplemented with non-essential amino acids (each 0.1mM), sodium pyruvate (mM), twice the recommended concentration of vitamins, L-glutamine (2mM), heat-inactivated (1 h, 60°) fetal calf serum (10%), penicillin (10 units/mL), and streptomycin (10 μ/mL). Wheat germ agglutinin was isolated from rat wheat germ obtained from local health food stores according to the method of Nagata *et al.*, except that a GlcNAc-AH–Sepharose affinity column was used for the final purification[19]. The Isolectin 1 from wheat germ agglutinin was isolated by chromatography on column of SP-Sephadex C-50 according to the method of Rice and Etzler[20] and Allen *et al.*[21].

Isolation of lectin-resistant variants. — The B16 mouse melanoma cells were retrieved from storage under liquid nitrogen and first grown in complete serum medium in 75-cm^2 Falcon tissue-culture flasks. After 24 h, the medium was replaced with serum-free medium. Cells that had been passed at least twice in the latter were used in the selection procedure.

Isolation of the WGA I-resistant B16 melanoma cells was carried out by the following general procedure: 5×10^5 cells were incubated overnight in serum-free medium in 25-cm^2 Falcon tissue-culture flasks in a 5% CO_2 atmosphere to permit adhesion to the substrate. The medium was then replaced with serum-free medium containing various amounts of WGA (1–50 μg/mL). As all of the cells died at lectin concentrations >5 μg/mL, a 4–5 μg/mL lectin concentrations was chosen for the selection study.

Set 1. The cells were kept for 14 days in serum-free medium containing 4 μg/mL of WGA I. Visual observation at that time indicated that most cells were dead. The medium was then replaced with complete serum medium. After 6 days, the surviving cells were subjected to a second lectin treatment utilizing 5 μg/mL of lectin in serum-free medium. After 7 days, the cells were cultured for 30 days in serum-free medium containing 4.5 μg/mL of lectin before further growth.

Set 2. The cells were grown as just described, except that 5 μg/mL of WGA I was used for the initial selection. The surviving cells were then allowed to grow in serum-free medium rather than complete serum medium before the second lectin treatment.

During the selection study, medium was replaced every 2–3 days. Ten flasks were used in each set and only the second flask from Set 1 and the third flask from Set 2 had any surviving cells. The surviving cells from Set 1 (called Wga R2) and from Set 2 (called Wga R3) were routinely grown in complete serum medium.

Chromosome analysis. — Chromosome analyses of the B16, Wga R2, and Wga R3 cells were performed by the Division of Genetics at this institution.

Tumor-forming capacity and metastasis of the cells in mice. — The tumor-forming capacity of the several cell-types was tested as follows: Eight each, five-week-old, male C57BL/6J mice obtained from Jackson Laboratories (Bar Harbor, ME) were injected subcutaneously with 1×10^5 cells of parent B16, Wga R2, or Wga R3 cells in 0.1-mL sterile PBS. All mice were observed over a period of time for tumor formation and survival.

Metastasis to the lungs was examined by dissection and observation under a light microscope. As all three cell-lines make melanin, dark melanin spots on the lungs indicated metastasis.

Agglutination studies. — Cells were harvested by removing the medium, washing the cell layer with Ca/Mg-free PBS three times, and treating for 8 min at 37° with 0.02% EDTA in Ca/Mg-free PBS. The cells were washed with PBS four times by resuspension and centrifugation and finally suspended at 2×10^6 cells/mL in PBS. Lectin solutions in PBS were serially diluted, starting at a concentration of 50 μg/mL. The lectin solution (0.1 mL) in a plastic agglutination plate was incubated for 30 min at room temperature with 0.1 mL of the cell suspension. Controls without lectins and with lectins plus inhibitor saccharides (*N*-acetyl-D-glucosamine for WGA, methyl α-D-mannoside for Con-A, and lactose for ricin) were included in order to test the specificity of the agglutination.

Iodination of wheat germ agglutinin. — Chloramine-T (2 μg/mL, 10 μL) and 500 μCi of Na ^{125}I (Amersham Corp., Arlington Heights, IL) were added to 1.5 mg of wheat germ agglutinin in 50 μL of PBS in a stoppered tube. The reaction was allowed to continue for 3 min at room temperature with constant mixing, whereupon a 25-μL aliquot of tyrosine solution (0.3 mg/mL) was added to stop the reaction. The mixture in 100 μL of PBS was layered onto a PD-10 column (Pharmacia) (10 mL) that had been prewashed with 50 mL of 1% BSA in PBS and re-equilibrated with PBS. The column was eluted with PBS and 0.5-mL fractions were collected and analyzed for radioactivity. The void-volume fractions were pooled and dialyzed against PBS to recover iodinated WGA.

Binding studies. — Assay of binding of ^{125}I-labeled WGA to cells was carried out in plastic test tubes that had been presoaked for 4–5 h in 5 mL of 1% BSA in PBS. The mixture contained in 250 μL of PBS: 250 μg of BSA, 3×10^5 cells, and 0.1–100 μg of ^{125}I-labeled WGA. After one h of incubation at room temperature with constant shaking, the cells were washed three times with PBS and the radioactivity (^{125}I) bound to the cells was measured in a Beckman Model 6000 gamma counter.

Control experiments were performed by incubation the ^{125}I-WGA with BSA

in the absence of cells and measuring the radioactivity that remained nonspecifically bound to the tubes after washing three times with PBS. The specificity of ^{125}I WGA-binding to the cells was assayed by including M *N*-acetylglucosamine in the mixture.

It has been suggested that WGA binding to cell surfaces involves a variety of sites, most of which contain sialyl residues[10,22]. Therefore, a WGA-binding study was carried on cells treated for 5 h at 37° with *Vibrio cholerae* sialidase.

Iodination of surface proteins. — To investigate the differences in total cell-surface proteins between the parent cell-line and the two lectin-resistant variants, lactoperoxidase-catalyzed iodination was performed as described by Hynes[23].

Cells were grown to confluence in 75-cm^2 Falcon tissue-culture flasks, harvested, washed 3 times with PBS, and resuspended in 200 μL of PBS containing 5mM D-glucose. Carrier-free Na^{125}I (500 μCi) was added, followed by lactoperoxidase and glucose oxidase to final concentrations of 20 μg/mL and 0.1 units/mL, respectively. The reaction was allowed to continue in a stoppered tube for 10 min at room temperature with occasional swirling. The reaction was stopped by adding phosphate-buffered iodide (PBS with NaCl replaced by NaI) solution containing 2mM benzylsulfonyl fluoride to inhibit proteases. The cells were washed 4 times with PBI, dissolved in buffer containing 2% SDS, and subjected to SDS-PAGE.

Electrophoretic studies. — Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed according to the methods described by Laemmli[24] and Hass and Kennett[25] in 1.5-mm thick, 7% polyacrylamide running gels with 4% stacking gels. The molecular-weight markers (Bio-Rad, Richmond CA) were phosphorylase B (92,000), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,000), and lysozyme (14,000). Following electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue R-250 in 50:50:1 MeOH–H_2O–AcOH and destained in the same solution without the dye. After drying the gel on Whatman 3MM filter paper, the ^{125}I-labeled components were made visible by autoradiography using Kodak X-O-MAT AR film. The ^{3}H-glycoproteins on the destained gels were detected by treatment with Me_2SO–PPO and fluorography. After gel electrophoresis of the total membrane proteins, the ^{125}I-WGA-binding glycoproteins were identified as described by Burridge[26].

The "crude membrane fraction" was obtained according to the method described by Briles *et al.*[5]. All operations were carried out at 4°. Briefly, the cells were washed three times with PBS, suspended in 4–5 volumes of the same buffer, and disrupted in a Dounce homogenizer. The cell lysates were centrifuged at 500 r.p.m. for 15 min to remove the nuclei and then at 38,000*g* for 1.5 h to obtain the "crude membrane" pellet. The pellet was dissolved in SDS–sample buffer and subjected to SDS-PAGE. Each lane had 25–30 μg of protein. Protein was determined as described by Lowry *et al.*[27].

^{3}H-Labeling of complex saccharides. — Cells in logarithmic growth were incubated for 3–5 days in 75-cm^2 Falcon tissue-culture flasks with serum-free medium

containing ^{3}H-glucosamine (10 μCi/mL) (Amersham Corp., Arlington Heights, IL). The spent medium was collected, centrifuged to remove cells, dialyzed against distilled H_2O for 3 days, and lyophilized.

The possibility that [^{3}H]glucosamine might indirectly interact with BSA was ruled out by a control experiment wherein BSA and [^{3}H]glucosamine were incubated in the absence of cells. After 5 days, the spent medium had only 0.01% nondialyzable radioactivity, which passed through the WGA–Sepharose column with no specific binding.

The ^{3}H-labeled cells were harvested by scraping with a sterile spatula or by treatment with EDTA. One portion was directly dissolved in buffer containing 2% SDS and subjected to SDS-PAGE and fluorography. Another portion was extracted with cold 2:1 $CHCl_3$–CH_3OH to remove lipids and centrifuged at 2200 r.p.m. The pellet was then re-extracted overnight at 4° with 50mM Tris buffer, pH 8.0, containing 1% NP-40, 0.2mM $PhCH_2SO_2F$, and 10mM benzamidine. The extract was centrifuged for 30 min at 40,000*g* and the pellet was re-extracted overnight at 4° with the same buffer. The supernatants were combined, dialyzed against distilled H_2O for 48 h, and lyophilized. The lyophilized material was treated overnight at 4° with 80% cold ethanol to remove the NP-40 and centrifuged at 2300 r.p.m. for 1.5 h. The pellet was dissolved in 2% SDS–sample buffer and subjected to SDS-PAGE and fluorography.

Ion-exchange chromatography. — DEAE-Sephacel in 0.5M NaCl, containing 25% ethanol (Sigma Chemical Co. St. Louis, MO) was packed in a column (1 × 10 cm), washed, and equilibrated in 10mM Tris buffer, pH 8.0. The samples, also in 10mM Tris-HCl buffer, were applied to the column. After washing the column with 3–4 volumes of 10mM Tris–HCl, pH 8.0, the bound material was eluted with a linear gradient of 0–0.5M NaCl in 10mM Tris–HCl, pH 8.0.

Affinity chromatography. — The albumin present in the dialyzed, spent culture medium was removed by passage through an Affigel Blue column (BioRad, Richmond, CA) (50–100 mesh, 11 mg/mL capacity) equilibrated in PBS. The effluent was dialyzed against distilled water to remove salt and lyophilized to recover ^{3}H-labeled material. Coupling of the purified WGA-I to Sepharose 4B gel was carried out after activation by cyanogen bromide as described by March *et al.*[28]. Gel filtration on Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) was performed in 50mM Tris–HCl buffer, pH 8.0; the column size was 0.9 × 60 cm.

The recovery of radioactivity from the columns was >85%, unless otherwise specified. Fractions of 1 mL were collected and, in preparative runs, an aliquot was used to determine the radioactivity. The distribution of radioactivity among the peaks is reported as percentages of the total recovered.

RESULTS

The three cell-lines (B16, Wga R_2, and Wga R_3) all make melanin, as the

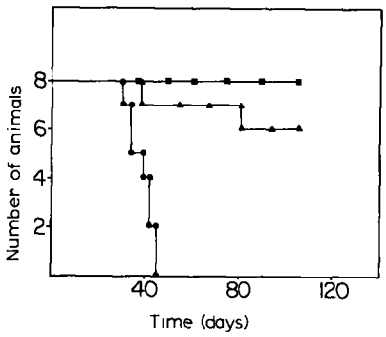

Fig. 1. Survival curve of the mice injected with B16, Wga R_2, and Wga R_3 cells. Eight mice in each set were injected subcutaneously with 10^5 cells in 0.1 mL of sterile PBS. B16 injected mice (●), Wga R_2 injected mice (▲), and Wga R_3 injected mice (■).

growth medium became characteristically dark brown–black within 4–5 days. Chromosome analysis showed that the two variants, Wga R_2 and Wga R_3, are mouse cells-lines, confirming that they were derived from the parent mouse cell-line and did not arise from an artifact.

The survival curve (Fig. 1) shows that all mice injected with the parent cells died within 44 days, whereas the mice injected with the two variant cells showed substantially longer survival. All those treated with the Wga R_3 cells were still alive after 105 days. It was not possible to check for metastasis in the mice injected with B16 cells as they apparently died of the primary tumor. The Wga R_2- and Wga R_3-injected mice did not show any metastases to the lungs after 105 days. When the Wga R_3-injected mice were challenged with parent B16 cells, 50% of the mice survived more than 50 days.

TABLE I

AGGLUTINATION OF B16, Wga R_2, AND Wga R_3 CELLS BY WHEAT GERM AGGLUTININ[a]

Lectin concentration (μg/mL)	*Degree of agglutination*		
	B16	*Wga R_2*	*Wga R_3*
25.0	+++	+++	+++
12.5	+++	++	++
6.3	+++	++	++
3.2	+++	+(+)	+
1.6	++	+	+
0.8	+(+)	–	–
0.4	–	–	–
0.2	–	–	–
0.1	–	–	–

[a]Cells (2×10^5) were incubated with the solutions of wheat germ lectin, serially diluted starting at a concentration of 25 μg/mL. Agglutination was scored as follows +++ Highly agglutinated, ++ Moderately agglutinated, + Slightly agglutinated, and – Not agglutinated.

TABLE II

CELL AGGLUTINATION IN THE PRESENCE OF RICIN, PEANUT LECTIN, OR CON-A[a]

Lectin concentration	*Peanut lectin*			*Ricin*			*Con-A*		
(μg/mL)	*B16*	*Wga R_2*	*Wga R_3*	*B16*	*Wga R_2*	*Wga R_3*	*B16*	*Wga R_2*	*Wga R_3*
25	+(+)	++	+(+)	+++	+++	+++	++	+++	+++
12,5	+	+	+	+++	+++	+++	++	+++	+++
6.25	(+)	(+)	(+)	+++	+++	+++	+++	++	++
3.2	(+)	(+)	(+)	+++	+++	+++	+(+)	++	++
1.6	(+)	(+)	(+)	++	+++	+++	+(+)	++	++
0.8	–	–	–	+(+)	++	++	(+)	+	+
0.4	–	–	–	+(+)	++	++	(+)	(+)	+
0.2	–	–	–	+	+	+	–	–	(+)
0.1	–	–	–	–	+	+	–	–	–

[a]Cells (2×10^5) were incubated with the lectin solutions listed above serially diluted starting at a concentration of 25 μg/mL. Agglutination was scored as follows: +++ Highly agglutinated, ++ Moderately agglutinated, + Slightly agglutinated, and – Not agglutinated.

Comparisons of cell-surface and media materials. — The results of the agglutination experiments with the B16, Wga R_2, and Wga R_3 cells are given in Table I. At high WGA concentrations, all three cell types showed agglutination, whereas, at low lectin concentrations, the Wga R_2 and Wga R_3 cells showed a clear decrease in cell agglutination, indicating changes in the lectin receptors on the surface of the variant cells.

It has been shown previously that the variants of melanoma cells selected for resistance to WGA contain modified surface glycoproteins that constituted poten-

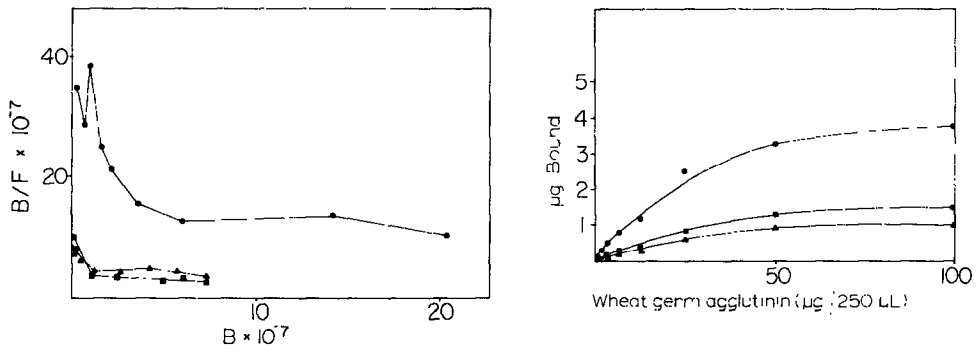

Fig. 2. The direct binding curve of ^{125}I-WGA to B16, Wga R_2, and Wga R_3 cells (right panel). The mixture contained in 250 μL of PBS 250 μg of BSA, 3×10^5 cells, and 0.1–100 μg of ^{125}I-WGA. Each point represents the average of duplicate samples, corrected for non-specific binding. The left panel is a Scatchard plot of the ^{125}I-WGA binding to the parent B16 cells and the variants (Wga R_2 and Wga R_3). B: molecules bound per cell; F: molar concentration of free lectin in solution. B16 cells (●), Wga R_2 cells (▲), and Wga R_3 cells (■).

tial WGA-receptor sites[7,10,12,22,29]. Cell agglutination with Con-A, ricin, and peanut lectin was examined on the assumption that, if the decreased binding of WGA to the variants was due to a loss of sialyl residues, increased agglutination might be exhibited with these lectins. The results (Table II) do not indicate any difference in cell agglutination with ricin and peanut lectins, although, in the presence of Con-A, the two variants showed a slight increase in cell agglutination compared to the parent cells.

Binding studies with ^{125}I-labeled WGA showed that both Wga R_2 and Wga R_3 cells do not bind WGA as well as the parent B16 cell line (Fig. 2). The results indicated that Wga R_2 and Wga R_3 cells have only one-third of the WGA-binding sites compared to B16 cells. Non-specific binding increased with increasing lectin concentration, but was always <3% of total binding.

The role of terminal sialyl residues in WGA binding was tested with neuraminidase-treated cells. The results showed that removal of sialic acid decreased WGA binding by 60–68%, agreeing with earlier observations[10,12,22] that WGA binding to cell surfaces involves a variety of sites, most but not all of which contain sialyl residues. Binding data analyzed by the method of Scatchard[30] indicated a multiphasic curve for the B16 cells, whereas the Wga R_2 and Wga R_3 cells showed biphasic curves (Fig. 2). The complexity of the curves makes it difficult to calculate meaningful association constants.

An autoradiogram of cell-surface components labeled by lactoperoxidase-catalyzed ^{125}I-iodination revealed a similar labeling pattern for all three cell lines.

Fig. 3 shows the autoradiogram obtained for electrophoretically separated, total cellular proteins stained with radioiodinated WGA. All three cell-lines contain several WGA-binding cellular proteins in the molecular weight range of 24,000–80,000. The fastest moving band in the two variants is apparently missing in the B16 cellular proteins.

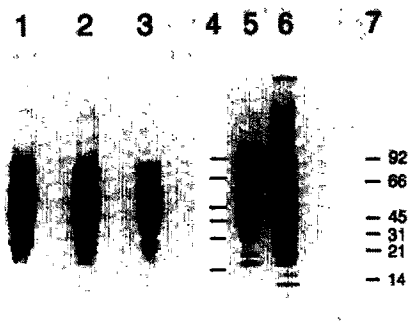

Fig. 3. Autoradiogram of total cellular proteins stained with ^{125}I-WGA after gel electrophoresis in the presence of SDS. Each sample contained ~25 μg of protein. Lanes 1–3 2 weeks exposure, lanes 5, 6: 1 week exposure, lanes 4, 7, molecular-weight standards. Standards are the same as in Fig. 4. 1, Wga R_3 cells; 2 and 6, Wga R_2 cells; 3 and 5, B16 cells; 4 and 7, molecular-weight markers.

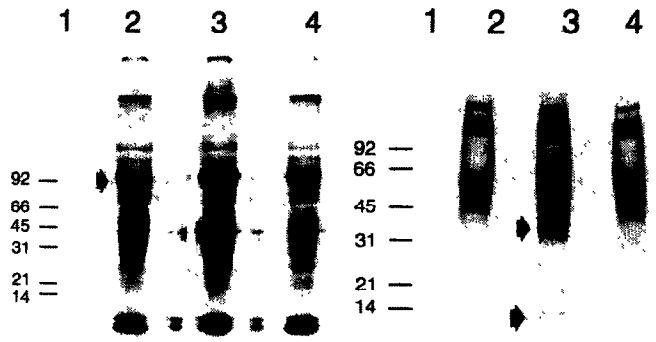

Fig. 4. (A) Fluorograph of [^{3}H]glucosamine-labeled cells in the presence of SDS. Each lane had ~200,000 d.p.m. The reference proteins are phosphorylase B (92,000), BSA (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,000), and lysozyme (14,000). Lane 1, molecular-weight standards, lane 2, B16 cells; lane 3, Wga R_2 cells; and lane 4, Wga R_3 cells.

(B) Fluorograph of proteins precipitated by 80% ethanol from NP-40 extracts of delipidated [^{3}H]glucosamine-labeled cells. Lane 1, molecular-weight markers; lane 2, Wga R_2 cells; lane 3, B16 cells; and lane 4, Wga R_3 cells.

Fig. 4 shows the fluorograph of the [^{3}H]glucosamine-labeled whole-cell extract following SDS-PAGE. The results suggest a similar total cellular glycoprotein pattern among all three cell-lines. One clear difference is that the band of molecular weight ~92,000 in the B16 cell line (Fig. 4A, lane 2) is shifted (or different) in the two variants (lane 3 and 4); some quantitative differences may be observed.

The fluorograph of the detergent-extracted and ethanol-precipitated, ^{3}H-labeled cell-components is shown in Fig. 4B. The fastest migrating band, from the B16 cell membranes, with an approximate molecular weight of 14,000 (lane 3) is missing or decreased in the two variants (lanes 2 and 4). The band having a molecular weight of ~38,000 in the B16 cells (lane 3) is also missing in the two variants.

As the culture medium contained 1% BSA, it was not practical to analyze directly for the total glycoproteins shed or secreted by the cells into the medium. Therefore, a portion of the dialyzed medium obtained from [^{3}H]glucosamine-labeled cultures was passed through an Affigel Blue column of required size (11 mg/mL capacity) to remove the albumin. The recovery of the radioactivity was 82–89%. This material from the three cell lines (B16, Wga R_2, and Wga R_3) was subjected to DEAE-Sephacel ion-exchange chromatography (Fig. 5). The B16 products showed the presence of charged components eluted with 0.13, 0.20, and 0.23M NaCl. Less charge heterogeneity was observed for the Wga R_2 or Wga R_3 cells, which showed the presence of one major component eluting with 0.13M NaCl.

The labeled glycoproteins secreted or shed were also fractionated into WGA-bound and -unbound, on the basis of their interaction with WGA–Sepharose.

Results of affinity chromatography of the secreted glycoproteins from the tumorigenic B16, B16-F_1 (low metastatic behavior), and B16-F_{10} (high metastatic

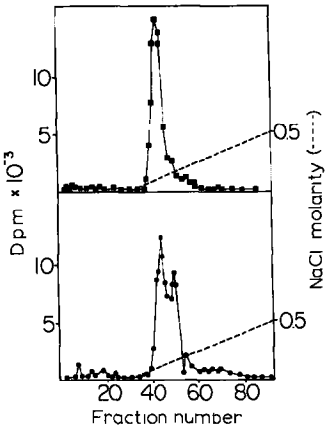

Fig. 5. Ion-exchange chromatography of ^{3}H-labeled material from the medium of parent B16 cells and the Wga R_2 and Wga R_3 variants, on DEAE-Sephacel (1 × 10 cm). The column (0.7 × 50 cm) was run in 10mM Tris–HCl buffer and eluted with a 0–0.5M NaCl gradient. Upper panel, Wga R_3 cells; lower panel, B16 cells.

behavior) cells and the two lectin-resistant variants, Wga R_2 and Wga R_3, on WGA-Sepharose (2 mg/mL density) are illustrated in Table III. The results show a striking difference in the specifically bound glycoprotein fractions from the parent and the two lectin-resistant variants. As shown in Table III, 37% of the radiolabel was specifically eluted from the B16 cell medium with 0.1M GlcNAc, whereas this proportion was only 10–12% for the Wga R_2 and Wga R_3. The specifically bound material was pooled, dialyzed, and lyophilized.

Fig. 6 shows the fluorograph of the WGA-bound material following SDS-PAGE. The results show the presence of one major, WGA-binding glycoprotein having an approximate mol. wt. of 50,000 in B16 spent medium. This glycoprotein appears completely absent in the lectin-resistant, less tumorigenic variants (lanes 1 and 2). The variants showed three major bands on SDS-PAGE, of approximate mol. wts. 92,000, 56,000, and 42,000, none of which was evident in the parent cell-line.

TABLE III

PERCENTAGE OF [^{3}H]GLUCOSAMINE-LABELED GLYCOPROTEINS RELEASED TO THE MEDIUM FROM B16, B16-F_1, AND B16-F_{10} CELLS, AND FROM VARIANTS Wga R_2, AND Wga R_3 SPECIFICALLY BOUND TO WGA–SEPHAROSE

Cell line	*% Bound*
B16	37.0
B16-F_1	21.0
B16-F_{10}	65.0
Wga R_2	11.9
Wga R_3	12.2

[a]^{3}H-Labeled material from the medium was applied to (2 mg/mL) WGA–Sepharose (0.7 × 15 cm) and the specifically bound glycoproteins were eluted with 0.1M GlcNAc

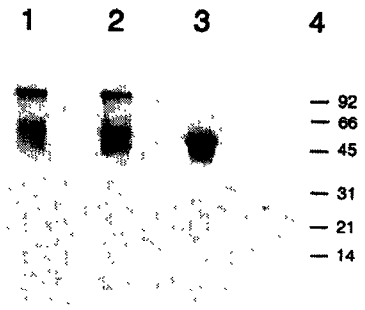

Fig. 6. Fluorograph of [^{3}H]glucosamine-labeled, WGA-bound glycoproteins from the medium of B16, Wga R_2, and Wga R_3 cells. Approximately 60,000 c.p.m. were applied in each lane. The reference proteins are phosphorylase B ()2,000), BSA (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,000), and lysozyme (14,000). Lane 1, Wga R_3 cell medium; lane 2, Wga R_2 cell medium; lane 3, B16 cell medium; lane 4, molecular-weight markers.

DISCUSSION

Wheat germ agglutinin is a cytotoxic plant lectin. Three major isolectins have been identified in WGA preparation[5,20,21], and amino acid substitutions have been found at the NeuAc binding sites of WGA I and WGA II[31]. It has been demonstrated that the isolectins I and II have different affinities for sialyl oligosaccharides[32]. Although both isolectins bind sialyl α-(2→3) isomers with higher affinity than the α-(2→6) form, WGA isolectin I binds all sialyl oligosaccharides more tightly than does WGA II.

Previous selection studies on the B16 cell line using WGA have been performed in the presence of serum[33,34]. As serum contains many components that bind to WGA, a serum-free medium was used in the experiments described here, thus allowing selection in the presence of relatively low concentrations of WGA[18]. The overall morphology of the three cell-lines appeared to be similar when viewed under a light microscope, although Wga R_2 and Wga R_3 showed slight aggregation.

The survival patterns of the mice injected with B16, Wga R_2, and Wga R_3 cells clearly indicate that the variant cells are much less tumorigenic. The mice injected with B16 cells developed large subcutaneous tumors, whereas the Wga R_2 and Wga R_3-injected mice only developed small, melanin-containing nodules. These results are in agreement with previous studies reporting decreased tumor incidence and metastasis in WGA-resistant variants of B16 cell line[8,10]. When the WGA R_3-injected mice were challenged with B16 cells, 50% of the mice survived more than 50 days, suggesting that the variant cells are immunogenic.

The agglutination studies using different lectins indicate that the B16 mouse melanoma cells as well as the two lectin-resistant variants have receptor sites on their surface for WGA, Con-A, and ricin. Cell agglutination in the presence of

different lectin concentrations indicated less WGA binding in the variants and presumably a change in cell-surface lectin receptors of the variants. The decreased WGA binding may not be due to a loss of terminal sialyl residues, but rather to a change in their spatial arrangement. It has also been shown that substitution at O-3 of GlcNAc by fucose inhibits interaction of WGA with this sugar[35]. High fucosyltransferase activity has been shown to be present in similar variant cell-lines[14,36], and thus the presence of additional fucose residues on surface glycoproteins might also result in decreased binding of lectin.

The cell-iodination experiments indicated a similar surface-protein pattern in all three cell lines. However, ^{125}I-WGA-binding studies showed that both the Wga R_2 and Wga R_3 cells bound WGA less well than the B16 cells. The binding sites on the former reach saturation at low concentrations of lectin, whereas the B16 cells require at least a three-fold greater lectin concentration to reach saturation. Scatchard plots showed that, in the case of Wga R_2 and Wga R_3, the decreased WGA binding is due to a decrease in binding affinity as well as a decrease in the total number of binding sites. Complex binding of WGA to Chinese hamster ovary cells and lectin-resistant variants has been reported[37].

The mechanism of WGA resistance in the variants is not clear but presumably involves some alteration in external, accessible saccharides; changes in glycosyltransferase activities have been reported for some lectin-resistant variants[36,38]. A comparison of metastasizing and non metastasizing B16 lines (B16F10 and B16F1) has indicated an alteration in the saccharide structure (decreased content of sialic acid and increased content of fucose) of multibranched, *N*-linked glycoproteins, associated with lowered metastatic properties[14,39–41]. It should be noted that such interactions need not be solely dependent on glycoproteins, as glycolipids with appropriate oligosaccharide units might also interact with WGA, especially when oriented in clusters in the plasma membrane bilayer[42].

The ^{3}H-labeled glycoproteins secreted or shed to the medium were fractionated into WGA-bound and WGA-unbound components on the basis of their interaction with WGA–Sepharose (2 mg/mL). The percentage of specifically bound glycoproteins varied widely among several B16 lines tested, ranging from 65% for B16-F_{10} cells to 10–12% in the two variants (Table III). The WGA-bound material secreted into the medium also showed differences. There is one major WGA-binding glycoprotein in the parent mouse melanoma cell-line having an approximate mol. wt. of 50,000, whereas the variants showed three major components with approximate mol. wts. of 92,000, 56,000, and 42,000. As the 0.1M GlcNAc-eluted fraction contained both labeled amino sugar and sialic acid, it was possible that the interaction with WGA was via either of these sugars. Treatment of the WGA-bound glycoproteins with sialidase abolished binding to WGA–Sepharose, indicating that sialic acid residues are primarily responsible for the interaction with WGA–Sepharose. This conclusion is consistent with the observation by other workers that WGA has a high affinity for clustered sialic acid residues on glycopeptides[22,43].

The change in the WGA-binding glycoproteins of the lectin-resistant variants may be related to the decrease in tumor-forming or metastasizing capacities. It is also possible that these two changes are independent of each other. At present, it is not known with certainty whether the differences in cell surface or secreted glycoproteins are directly relevant to the expressed malignant behavior of cells.

REFERENCES

1 G. L. NICHOLSON, *Biochim. Biophys. Acta,* 458 (1976) 1–72.
2 R. O. HYNES, *Biochim. Biophys. Acta,* 458 (1976) 73–107.
3 H. LIS AND N. SHARON, *Ann. Rev. Biochem.,* 42 (1973) 541–574.
4 H. LIS AND N. SHARON, *Science,* 177 (1972) 949–959.
5 E. B. BRILES, E. LI, AND S. KORNFELD *J. Biol. Chem.,* 252 (1977) 1107–1116.
6 C. GOTTLIEB AND S. KORNFELD, *J. Biol. Chem.,* 251 (1976) 7761–7768.
7 R. L. JULIANO AND P. STANLEY, *Biochim. Biophys. Acta,* 389 (1975) 401–406.
8 T. W. TAO AND M. M. BURGER *Nature,* 270 (1977) 437–438.
9 J. A. WRIGHT *Int. J. Biochem.,* 10 (1979) 951–956.
10 L. LIN, J. L. STERN, AND E. A. DAVIDSON, *Carbohydr. Res.,* 111 (1983) 257–271.
11 A. MAEGER, A. UNGITCHANUKIT, R. NAIRN, AND R. C. HUGHES, *Nature,* 257 (1975) 137–139.
12 P. STANLEY, T. SUDO, AND J. P. CARVER, *J. Cell Biol.,* 85 (1980) 60–69.
13 E. B. BRILES, E. LI, AND S. KORNFELD, *Fed. Proc. Fed. Am. Soc. Exp. Biol.,* 35 (1976) 1642.
14 J. FINNE, T. W. TAO, AND M. M. BURGER, *Cancer Res.,* 40 (1980) 2580–2587.
15 I. S. TROWBRIDGE, R. HYMAN, T. FERSON, AND C. MAZANSKAS, *Eur. J. Immunol.,* 8 (1978) 716–723.
16 M. L. REITMAN, I. S. TROWBRIDGE, AND S. KORNFELD, *J. Biol. Chem.,* 255 (1980) 9900–9906.
17 R. E. HAUSMAN AND A. A. MOSCONA, *Proc. Natl. Acad. Sci., U.S.A.,* 72 (1975) 916–920.
18 J. R. BANKS, V. P. BHAVANANDAN, AND E. A. DAVIDSON, *Cancer Res.,* 37 (1977) 4336–4345.
19 Y. NAGATA, A. R. GOLDBERG, AND M. M. BURGER, *Methods Enzymol.,* 32 (1974) 611–615.
20 R. H. RICE AND M. E. ETZLER, *Biochemistry,* 14 (1975) 4092–4099.
21 A. K. ALLEN, A. NEUBERGER, AND N. SHARON, *Biochem. J.,* 131 (1973) 155–162.
22 V. P. BHAVANANDA AND A. W. KATLIC, *J. Biol. Chem.,* 254 (1979) 4000–4008.
23 R. O. HYNES, *Proc. Natl. Acad. Sci. U.S.A.,* 70 (1973) 3170–3174.
24 U. K. LAEMMLI, *Nature,* 277 (1970) 680–685.
25 J. B. HASS AND R. H. KENNETT, in R. H. KENNETT, T. J. MCKEAR, AND K. B. BECHTOL (Eds.), *Monoclonal Antibodies,* Plenum Press, New York, 1979 pp. 407–411.
26 K. BURRIDGE, *Proc. Natl. Acad. Sci. U.S.A.,* 73 (1976) 4457–4461.
27 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. RANDALL, *J. Biol. Chem.,* 193 (1951) 265–275.
28 S. C. MARCH, I. PARIKH, AND P. CUATRECASAS, *Anal. Biochem.,* 60 (1974) 149–152.
29 J. E. JUMBLATT, T. TAO, V. SCHLUP, J. FINNE, AND M. M. BURGER, *Biochem. Biophys. Res. Commun.,* 95 (1980) 111–117.
30 G. SCATCHARD, *Ann. N.Y. Acad. Sci.,* 51 (1949) 660–672.
31 C. S. WRIGHT, *J. Mol. Biol.,* 145 (1981) 453–461.
32 K. A. KRONIS AND J. P. CARVER, *Biochemistry,* 21 (1981) 3050–3057.
33 T. W. TAO AND M. M. BURGER, *Int. J. Cancer,* 29 (1982) 425–430.
34 J. W. DENNIS AND R. S. KERBEL, *Cancer Res.,* 41 (1981) 98–104.
35 H. E. CARLSSON, J. LOONGREN, I. J. GOLDSTEIN, J. E. CHRISTNER, AND G. W. JOUDIAN, *FEBS Lett.,* 62 (1976) 38–40.
36 J. FINNE, M. M. BURGER, AND J. P. PRIEELS, *J. Cell Biol.,* 92 (1982) 277–282.
37 P. STANLEY AND J. P. CARVER, *J. Cell Biol.,* 79 (1978) 617–622.
38 S. NARASIMHAN, S. ALLEN, R. C. HUGHES, AND H. SCHACHTER, *Glycoconjugate J.,* 1 (1984) 51–61.
39 L. WARREN, A. CLAYTON, AND G. TUSZUNSKI, *Biochim. Biophys. Acta,* 516 (1978) 97–127.
40 G. YOGEESWARAN AND T. TAO, *Biochem. Biophys. Res. Commun.,* 95 (1980) 1452–1460.
41 G. YOGEESWARAN AND P. SALK, *Science,* 212 (1981) 1514–1516.
42 W. R. REDWOOD AND T. G. POLEFKA, *Biochim. Biophys. Acta,* 455 (1976) 631–643.
43 W. L. ADAIR AND S. KORNFELD, *J. Biol. Chem.,* 249 (1974) 4696–4704.