Identification of Asparagine-linked Oligosaccharides Involved in Tumor Cell Adhesion to Laminin and Type IV Collagen

JAMES W. DENNIS,* C. A. WALLER, and VOLKER SCHIRRMACHER
*Cancer Research Laboratories, Department of Pathology, Queen's University, Kingston, Ontario, Canada K7L 3N6; and Institut für Immunobiologie und Genetik, Heidelberg, Federal Republic of Germany

ABSTRACT MDW4, a wheat germ agglutinin-resistant nonmetastatic mutant of the highly metastatic murine tumor cell line called MDAY-D2 has previously been shown to attach to fibronectin and type IV collagen, whereas MDAY-D2 and phenotypic revertants of MDW4 attached poorly to these substrates. The increased adhesiveness of the mutant cells appeared to be closely related to a lesion in cell surface carbohydrate structures. In an effort to identify the carbohydrates involved in cell attachment, glycopeptides isolated from mutant and wild-type cells as well as from purified glycoproteins were tested for their ability to inhibit the attachment of MDW4 cells to plastic surfaces coated with fibronectin, laminin, or type IV collagen. The addition of mannose-terminating glycopeptide to the adhesion assay inhibited MDW4 cell attachment to type IV collagen. In contrast, a sialylated poly *N*-acetyllactosamine-containing glycopeptide, isolated from wheat germ agglutinin-sensitive MDAY-D2 cells but absent in MDW4 cells, inhibited MDW4 attachment to laminin. None of the glycopeptides used in this study inhibited attachment of MDW4 cells to fibronectin-coated plastic. Peptide *N*-glycosidase treatment of the cells to remove surface asparagine-linked oligosaccharides inhibited MDW4 adhesion to type IV collagen, but not to laminin, and the same treatment of the wheat germ agglutinin-sensitive cells enhanced attachment to laminin. Tumor cell attachment to, and detachment from, the sublaminal matrix protein laminin and type IV collagen are thought to be important events in the metastatic process. Our results indicate that tumor cell attachment to these proteins may be partially modulated by the expression of specific oligosaccharide structures associated with the cell surface.

Cell adhesion to extracellular matrix (ECM)[1] is necessary for most nonlymphoid cells to proliferate and differentiate (1, 2). Many of the isolated structural components of ECM have been shown to mediate cell attachment in vitro, and these include collagen types I–V, fibronectin, laminin, chondronectin, entactin, and proteoglycans (1). The composition of ECMs depends on the type of cell producing the matrix. For example, fibroblasts synthesize and preferentially attach to fibronectin and types I and III collagen; the endothelial cell ECM that lines blood vessel walls contains laminin and type IV collagen (1). The large number of adhesion-mediating components in ECM, and the fact that some components have multiple binding sites has complicated the analysis of cell–substratum adhesion (1, 3). For example, fibronectin has binding sites for collagen, fibrin, heparin, polyamines, and the cell surface. Laminin binds to type IV collagen, heparin sulfate, and a cell surface receptor. To date the only cell surface receptor for attachment to vascular ECM to be isolated is that of laminin (4, 5). The laminin receptor may be particularly important to the metastatic phenotype because the ability of tumor cells to attach to laminin plus type IV collagen-coated surfaces in vitro has been shown to correlate with the lung-colonizing potential of the cells when they are administered intravenously (6).

Before attachment to ECM and invasion at a secondary site, the metastatic tumor cell escapes from the primary site and passes through the vascular ECM to enter the circulation. This process may be facilitated by the decrease in tumor cell adhesiveness to ECM proteins that often accompanies malignant transformation. Malignant cells often have reduced cy-

[1] *Abbreviations used in this paper:* Asn, asparagine; Con A, concanavalin A; ECM, extracellular matrix; Gal, galactose; GlcNAc, *N*-acetylglucosamine; IMDM, Iscove's modified Dulbecco's medium; Man, mannose; WGA^r, wheat germ agglutinin-resistant; WGA^s, wheat germ agglutinin-sensitive.

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 OCTOBER 1984 1416–1423

toskeletal organization and focal contacts, which may partially explain their less adhesive properties (7). It has also been suggested that the commonly observed increase in sialylation and size of the asparagine (Asn)-linked oligosaccharides on malignant cells may play a role in tumor cell non-adhesiveness and consequently metastasis (8, 9). Recent studies on a number of mutant cell lines have provided evidence that changes in cell surface carbohydrates can affect cell attachment to isolated ECM proteins. Ricin-resistant baby hamster kidney cells (10) and wheat germ agglutinin-resistant (WGA^r) murine tumor cells (8) having lesions in the carbohydrate structure of their glycoconjugates also showed changes in adhesion to fibronectin and collagens. AD-6, a mutant of 3T3 fibroblasts which was selected for increased detachability from plastic surfaces, was subsequently found to have a defect in oligosaccharide biosynthesis (11). Adhesion of melanoma cells (12) and fibroblasts (13) to fibronectin-coated plastic is reduced by treating the cells with tunicamycin, an inhibitor of Asn-linked oligosaccharide synthesis.

There are a number of ways in which altered oligosaccharide structure may affect the normal function of glycoproteins involved in cell attachment. For example, inhibition of Asn-linked oligosaccharide synthesis by tunicamycin has been shown to reduce the activity of the insulin receptor (14), alter the conformation and inhibit cell surface expression of the G protein in vesicular stomatitis virus-infected cells (15), and to inhibit Fc-related functions of IgG2b monoclonal antibodies without changing the hapten specific binding activity (16). In addition, the oligosaccharide portion of the glycoprotein can mediate a biological function by binding to a lectinlike receptor, an example of this being the delivery of lysosomal enzymes to lysomes via the mannose-6-P binding protein in the endoplasmic reticulum of mammalian cells (17).

Attachment of cells by membrane oligosaccharides to surfaces coated with lectins (18) glycosyltransferases (19) and glycosidases (18) has been shown to induce cell spreading similar to cells plated on ECM- or fibronectin-coated surfaces. Therefore, we have examined the possibility that some ECM molecules have lectinlike activities capable of binding cell surface oligosaccharides, thereby mediating attachment. Asn-linked glycopeptides have been isolated from MDW4, a WGA^r mutant and from the metastatic parental tumor line MDA-D2 (20). The WGA^r mutant attached more avidly to fibronectin- and type IV collagen-coated surfaces (8) and was shown to have a premature truncation of the antennae on the poly-*N*-acetyllactosamine-containing oligosaccharides found in the wild-type cells (20). Glycopeptides from mutant and wild-type cells as well as from standard glycoproteins were used in an attempt to inhibit MDW4 cell attachment to laminin, fibronectin, and type IV collagen, the major glycoprotein components of vascular ECM encountered by metastatic tumor cells. The results indicate that type IV collagen may bind to high mannose (Man) strucures on the cell surface, and that a poly-*N*-acetyllactosamine-containing Asn-linked oligosaccharide found on the metastatic wheat germ agglutinin-sensitive (WGA^s) cells inhibits cell attachment to laminin. Tumor cell detachment from vascular ECM at the primary tumor sites and subsequent attachment to a structurally similar ECM at secondary sites may be facilitated by modulation of specific cell surface oligosaccharide structures.

MATERIALS AND METHODS

Materials: Type IV collagen and laminin were purified from the murine EHS sarcoma and kindly provided by R. Timpl (Max-Planck-Institut für Biochemie, München, Federal Republic of Germany) (21, 22). Serum fibronectin was purchased from Bethesda Research Laboratories (Gaithersburg, MD). Human transferrin, conalbumin type I, essentially fatty acid-free, fraction V BSA, and almond emulsin β-glucosidase type II were obtained from Sigma Chemical Co. (St. Louis, MO). Bio-Gel P-2 and P-6 came from Bio-Rad Laboratories (Mississauga, Ontario, Canada); DE52 cellulose from Whatman Chemical Separation, Inc. (Clifton, NJ); concanavalin A–Sepharose-4B, DEAE Sephacel and Sephadex G25 from Pharmacia Fine Chemicals (Dorval, Quebec, Canada); and Reacti Gel (25DF) from Pierce Chemical Co. (Rockford, IL). Terasaki Microtest plates were purchased from Falcon Plastics (Canlab, Toronto, Ontario, Canada).

Tumor Cells: The origin and properties of the highly metastatic DBA/2 strain tumor called MDAY-D2 has been described in detail (23, 24). The WGA^r mutant MDW4 was isolated from a mutagenized population of MDAY-D2 cells by selection in growth medium containing WGA^r as previously described (25, 26). MDW4-24a is a phenotypic revertant of MDW4 isolated from a metastasis found in DBA/2 mouse which had been injected subcutaneously with MDW4 cells (25). The nonmetastatic MDW4 cells spontaneously hybridize in situ with a bone marrow-derived host cell thereby regaining the "wild type" (i.e., MDAY-D2) lectin-resistant and the metastatic phenotypes (26).

Tumor cells were grown in suspension in RPMI-1640 tissue culture medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 mM glutamine, (Grand Island Biological Co., Grand Islands, NY). Tumor cells used for attachment experiments were cultured 3–10 d before the experiment in Iscove's modified Dulbecco's medium (IMDM) supplemented with 5 μg/ml human transferrin, 1.5 μM ethanolamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mg/ml essentially lipid-free BSA which had been detoxified on charcoal. The tumor cells divided at near normal rates and could be passaged in the serum-free medium for extended periods of time (i.e., > 1 mo). The cells were tested for mycoplasma by staining DNA with 4′,6′-diamino-2-phenylidole and found to be free of contamination during the course of these experiments.

For lectin sensitivity tests, the cells at 5×10^5/ml in fetal calf serum supplemented medium were plated in Linbro wells with concentrations of lectin ranging from 0 to 100 μg/ml. After 5 d the cells grown in absence of lectin had reached maximal density and the cell number in each concentration of lectin was determined.

Glycopeptides: The total cellular glycopeptides isolated from ~5×10^9 tumor cells of each MDAY-D2 and MDW4 were characterized as documented in reference 20 and used in the present study. Briefly, lipids were removed by extraction with chloroform/methanol (2:1) (vol/vol) and the residue was exhaustively digested in 1% (wt/wt) pronase followed by precipitation of glycosaminoglycans, DNA, and RNA by the addition of cetylpyridinium chloride to 4 mM. The glycopeptides were desalted on a Bio-Gel P-6 column then separated on Whatman DE52 cellulose column to obtain neutral and sialylated species. Each fraction separated on DE52 cellulose was desalted and applied to a concanavalin A-Sepharose-4B (Con A/Sepharose) column. The neutral and Con A-bound glycopeptides from WGA^r and WGA^s cells were shown to be similar by proton nuclear magnetic resonance analysis. These glycopeptides isolated during the study documented in reference 20 appeared by proton nuclear magnetic resonance analysis to be a mixture of ~40% Man_9, 15% Man_8, 15% Man_7, 25% Man_6, and 15% Man_5 (structure 5 in Fig. 1) (20).

Complex glycoproteins are abbreviated according to the sugar residues at the nonreducing ends, the Manα1–6 arm being named first; *S* denotes sialic acid, *G* is galactose (Gal), *Gn* is *N*-acetylglucosamine (GlcNAc), and *M* is Mannose. There was essentially no neutral glycopeptides in MDAY-D2 excluded by the Con A/Sepharose column but ~25% of the MDW4 glycopeptides were separated in this way and shown to be triantennary complex terminating in *N*-acetylglucosamine (*GnGnGn*, structure 3 in Fig. 1). The sialylated poly-*N*-acetyllactosamine-containing glycopeptide from MDAY-D2 cells bound to DE52 cellulose and was excluded by Con A/Sepharose. The structure was also present in MDW4 versus host cell hybrid cells removed from the metastasis of MDW4-injected mice (e.g., MDW4-24a). The presence of α2–3 linked sialic acid (SA) and the GlcNAcβ1–3 Gal linkage were confirmed by proton nuclear magnetic resonance analysis (*SSS*, structure 1 in Fig. 1) (20).

Glycopeptides from 1 g of conalbumin and 1 g of human transferrin were isolated as follows. The glycoprotein was dissolved in 10 ml of 0.1 M Tris, pH 7.9, 1 mM $CaCl_2$, 0.01 mM NaN_3, 100 mg pronase, and 5 drops of toluene blue and incubated at 37°C for 3 d. After correcting the pH to 7.9 with 1 M HCl, 50 mg of pronase was added on days 2 and 3. The sample was applied to a Sepharose G25 column (2.5 × 90 cm) and eluted with water. The pronase digestion and desalting were repeated. The conalbumin glycopeptide is a bisected triantennary complex terminating in GlcNAc as shown in structure 4 in Fig. 1 (27). It was retained by the G25 column and eluted in a symmetric peak. The human transferrin glycopeptide has previously been shown to be mostly sialylated biantennary SAα2–6Galβ1–4GlcNAcβ1–2Manα1–6 (SAα2–6Galβ1–4GlcNAcβ1–2Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc–Asn (i.e., *SS*)

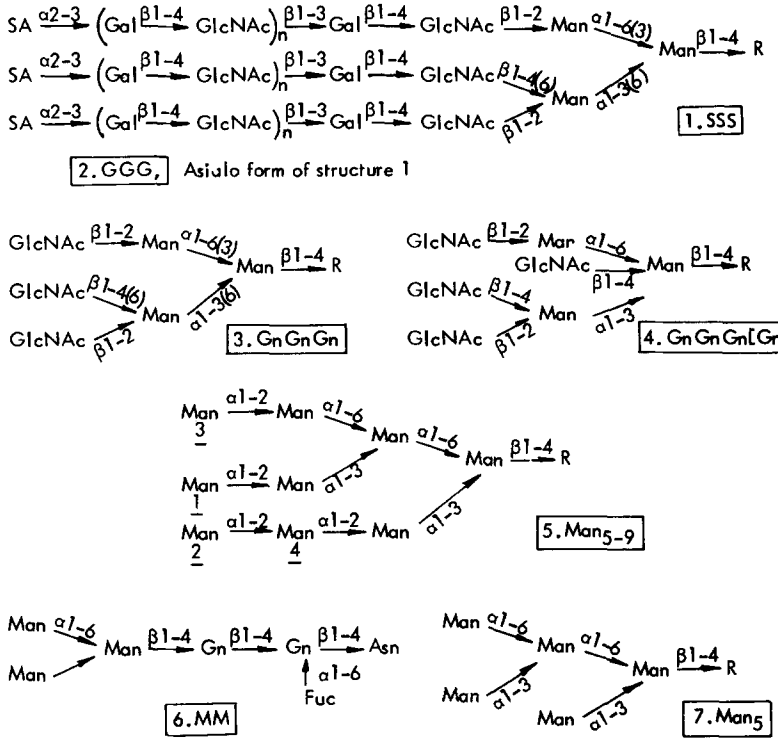

FIGURE 1 Structures of the Asn-linked glycopeptides tested as inhibitors of cell attachment in Fig. 3. Structure 1 (*SSS*) is the sialylated poly-*N*-acetyllactosamine-containing glycopeptide isolated from MDAY-D2 and is also present in the WGA^s MDW4 versus host cell hybrid lines removed from a metastasis of an MDW4-injected mouse (e.g., MDW4-24a). Structure 2 is the asialo form of glycopeptide 1 (*GGG*) (20). Structure 3 is the mutant complex glycopeptide isolated from MDW4 cells (*GnGnGn*). Structure 4 is the conalbumin glycopeptide (*GnGnGn[Gn]*) (27). Structure 5 represents the high-mannose glycopeptide (Man_{5-9}) isolated from both MDAY-D2 and MDW4 cells. Assuming that only the postulated intermediates of high-mannose processing were present (50), analysis of the proton nuclear magnetic resonance spectra indicated that the sample contained ~40% Man_9, 15% Man_8, 15% Man_7, 25% Man_6, and 15% Man_5. Man_5 to Man_9 are the structures resulting from the sequential removal of mannose residues numbered *1–4*. Structure 6 is the core glycopeptide *MM* isolated from human myeloma IgG (51). Structure 7 is Man_5 isolated from ovalbumin (52). *R* represents GlcNAcβ1–4GlcNAcβ1–Asn.

(28). In our hands, human transferrin oligosaccharides released from the protein by peptide *N*-glycosidase and separated by ion-exchange high-performance liquid chromatography revealed 4% neutral, 38% monosialylated, 53% disialylated, and 5% trisialylated species (data not shown). The *SS* glycopeptide species was separated on a DEAE Sephacel column (1 × 20 cm) equilibrated in 5 mM Tris, pH 7.0, and eluted with a 0–50 mM NaCl gradient in the same buffer. Glycopeptides were quantitated by the hexose assay using mannose as the standard (29). Sialic acid was removed from glycopeptides by incubating samples in 0.1 M HCl at 80°C for 1 h.

Dr. H. Schachter (Hospital for Sick Children, Toronto, Canada) kindly provided *MM* (structure 6 in Fig. 1) isolated from human myeloma IgG and Man_5 (structure 7 in Fig. 1) from ovalbumin glycopeptide E.

Cell Attachment Assay: Replicate wells in Terasaki Microtest plates were coated with 5 μg essentially fatty acid-free, fraction V BSA and concentrations of substrate ranging from 0.25 to 250 ng. The BSA and substrates were allowed to dry for 3 h followed by the addition of 5 μl of glycopeptide inhibitors in IMDM. Tumor cells grown for 3–10 d in IMDM plus supplements were washed twice in ice-cold IMDM, adjusted to 10^5/ml, and 5 μl was added to each well. The plates were maintained at 0°C in a 5% CO_2 atmosphere for 30 min, then inverted for 30 min. Cells adhering to the substrate and those falling to the base of the droplet were counted and the mean percent adhering calculated from triplicates (8).

Almond Emulsin Glycosidase Digestion: Peptide *N*-glycosidase was purified from 10 g of crude almond emulsin β-glucosidase as previously described (30). The enzyme was purified ~1,000-fold and migrated as one major band after reduction with 2-mercaptoethanol on SDS polyacrylamide gels. Tumor cells from serum-free medium were washed in IMDM, 0.1 mg/ml BSA, and 1.5 U of peptide *N*-glycosidase was added to 5–10 × 10^5 cells in 1 ml of IMDM, 0.1 mg/ml BSA. After 1 h at 37°C, the cells were washed twice in cold IMDM and applied to substrate coated microtest plates.

To monitor the removal of oligosaccharides from the cell surface, sialic acid residues were labeled using the $NaIO_4$/Na[^{3}H] BH_4 method (31). MDW4-24a cells at 5 × 10^5/ml in PBS were made 2 mM $NaIO_4$ and incubated at 0°C for 20 min. After washing, 1 mCi of Na[^{3}H] BH_4 was added and incubated for a further 25 min at room temperature. The cells were washed in PBS and incubated with peptide *N*-glycosidase, as described above. After 1 h the cells were washed in PBS at 4°C, and the chloroform/methanol (2:1 vol/vol) insoluble residue was dissolved in 0.25 M NaOH and radioactivity was measured.

RESULTS

Tumor Cell Attachment

In an earlier study, tumor cells were grown in RPMI-1640 supplemented with fetal calf serum and transferred to serum-free medium for use in the attachment assay (8). Serum contains fibronectin as well as other attachment factors that may bind to the cells and interfere with the attachment assays. Therefore a serum-free medium that can support long-term growth of the MDAY-D2 cells and MDAY-D2 variants was developed. The Iscove's-based serum-free medium supplemented with transferrin, ethanolamine, and BSA as described in Materials and Methods supported cell growth for months in tissue culture at a rate similar to that of serum-containing medium. Tumor cells grown in serum-free medium attached to laminin, fibronectin, and type IV collagen in the same manner as cells from serum with one exception. WGA^s (i.e., MDAY-D2 and MDW4-24a) cells from serum-containing medium attached to laminin (8), but the same cells grown >3 d in serum-free medium did not attach to laminin. The results in Fig. 2 show that for cells grown in serum-free medium, the nonmetastatic WGA^r mutant (MDW4) was more adhesive than the WGA^s MDW4-24a cells on fibronectin, laminin, and type IV collagen. The parental line MDAY-D2 had an attachment phenotype identical to that of the MDW4 versus host cell hybrid (i.e., MDW4-24a) (8). Because MDW4 and MDW4-24a were near tetraploid lines and therefore larger cells than MDAY-D2, they were compared in the experiments on inhibition of attachment described here.

MDW4 has previously been shown to have three to four times less neuraminidase-releasable cell surface sialic acid compared with MDAY-D2 and the MDW4 versus host cell hybrid cells and, in addition, the removal of sialic acid from WGA^s cells with neuraminidase enhanced cell attachment to type IV collagen and fibronectin (8). These results led to the suggestion that an asialo-glycoconjugate may be involved in MDW4 adhesion. A more detailed examination of the Asn-linked oligosaccharide in mutant and WGA^s cells indicated that complex oligosaccharides in the mutant were also deficient in Gal and terminated in GlcNac (Fig. 1) (20). The mutant was also resistant to the Gal-binding lectin leucoagglutinin (32) and hypersensitive to the GlcNAc-binding lectin from *Bandeirea simplificifolia* seed (33) in agreement with

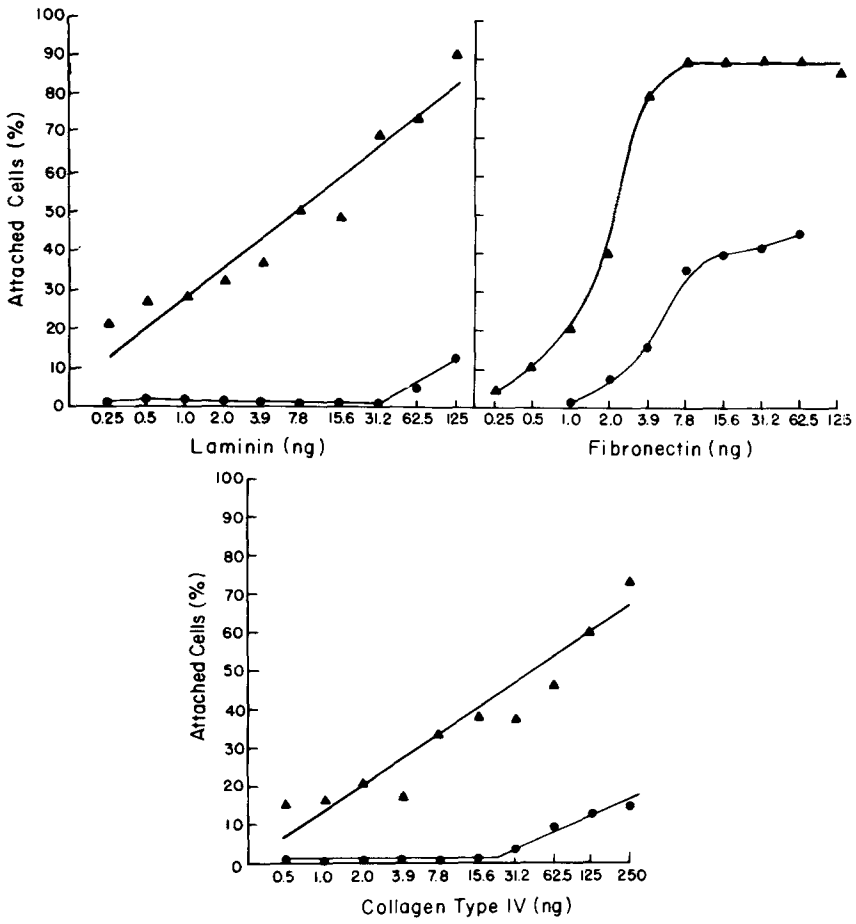

FIGURE 2 Tumor cell attachment (performed as described in Materials and Methods) as a function of increasing substrate concentration. ▲, attachment of the WGA^r mutant MDW4; ●, attachment of a WGA^s MDW4 versus host cell hybrid removed from a metastasis of an MDW4-injected mouse (i.e., MDW4-24a) to laminin, fibronectin, and type IV collagen was performed as described in Materials and Methods.

the observed changes in oligosaccharide structure and the specificities of these lectins (Table I). In an earlier study on lectin binding, MDW4 was found to have an increase in cell surface Con A-binding sites (34), which probably accounts for the increased sensitivity of MDW4 cells to Con A in tissue culture (Table I).

To summarize, the WGA^s cells (i.e., MDAY-D2 and MDW4 versus host cell hybrids) had sialylated poly-*N*-acetyllactosamine-containing complex (structure 1 in Fig. 1) which has been replaced in the mutant by GlcNAc terminating complex consisting of a mixture of ~33% triantennary with GlcNAc 2, 4 on the Manα1-3 and 66% triantennary with the GlcNAc 2, 6 on the Manα1-6 (structure 3 in Fig. 1) (20). The mutant also has an apparent increase in Con A-binding oligosaccharides on the cell surface, possibly of the high mannose type (structure 5 in Fig. 1). The GlcNAc terminating complex isolated from the mutant as well as the Con A-binding high mannose-type glycopeptides appeared to be reasonable candidates for oligosaccharides that might be involved in the increase adhesion of MDW4 cells to substratum.

TABLE I

Lectin Sensitivity, Metastasis, and Adhesion Characteristics of the Tumor Lines

Cell line	Lectin sensitivity (D10)*				Metastasis‡	Substratum adhesion§
	WGA	PHA(l)	BS_{II}	Con A		
	$\mu g/ml$					
MDAY-D2	7	7	>100	33	high	low
MDW4	>50	>50	6	14	non	high
MDW4-24a	7	6	>100	40	high	low

Abbreviations: BS_{II}, *Bandeirea simplifolia* lectin II; PHA(l), leucoagglutinin lectin.

* The lectin sensitivities were determined as described in Material and Methods. The concentration of lectin reducing the cell number to 10% of the control after 5 d of tissue culture is the D10 value.

‡ MDW4 cells grew at the subcutaneous site of injection and only the progeny of hybrids between MDW4 and a bone marrow-derived host cell were found in the metastases (e.g., MDW4-24a). The hybrids had regained both the lectin resistance profile and the high metastatic capacity of the parental line MDAY-D2 (23, 26).

§ MDW4 cells attach more avidly than MDAY-D2 and MDW4-24a to fibronectin, laminin, and type IV collagen in vitro.

Glycopeptide Inhibition of MDW4 Cell Attachment

In experiments testing the glycopeptide inhibition of cell attachment, microtest wells were coated with a concentration of substrate determined on the same day to give 50–70% cell attachment. The glycopeptides were added at a concentration of 0.2 nmol/10-μl well (i.e., 20 μM), approximately three orders of magnitude more than the glycopeptide concentration present in the 500 cells added to each well. The results in Fig. 3 show that *SSS* inhibited MDW4 cell attachment to laminin but not to fibronectin or type IV collagen. *GGG*, the same glycopeptide with sialic acid removed also inhibited attachment but the mutant glycopeptide *GnGnGn* showed no activity. Glycopeptides with the same terminal residues as those on the inhibitory structures, namely *SS* and *GG* from human transferrin did not inhibit cell attachment to laminin (Fig. 4). A titration of the inhibitors in Fig. 4 showed that the concentration of *SSS* and *GGG* required for 50% inhibition

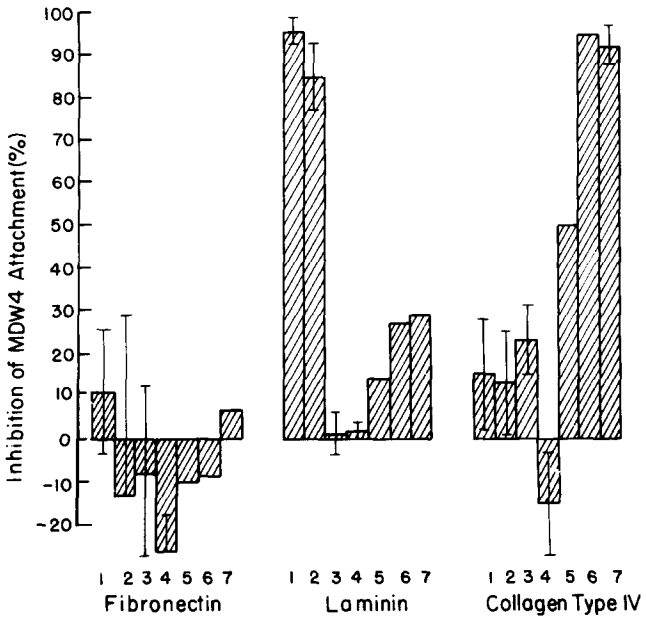

FIGURE 3 Inhibition of MDW4 cell attachment to fibronectin, laminin, and type IV collagen by the Asn-linked glycopeptide shown in Fig. 2. Microtest wells were coated with 5 μg of BSA and either 7.8 ng of laminin, 3.9 ng fibronectin, or 3.9 ng type IV collagen and glycopeptides were added at a concentration of 20 μM. Attachment of MDW4 cells to the respective substrates in the presence and absence of glycopeptides was used to calculate inhibition. The results presented with standard error bars are the mean inhibition values of three to six experiments performed in triplicate, the others are the mean of two experiments performed in triplicate.

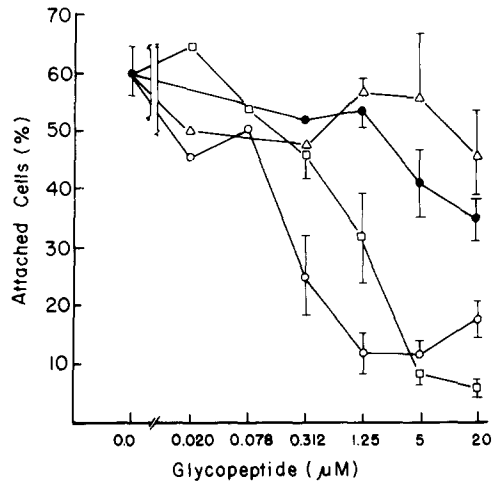

FIGURE 4 Inhibition of MDW4 cell attachment to laminin as a function of glycopeptide concentration. Microtest plate wells were coated with 15.6 ng of laminin plus 5 μg of BSA. The glycopeptides used were human transferin *GG* (△); human transferrin *SS* (●); wild-type cellular *GGG* (○); and wild-type cellular *SSS* (□). Each point is the mean of three replicates and error bars represent the standard error.

was ~1.5 and 0.3 M, respectively. *SSS* did not appear to inhibit attachment by binding avidly to laminin as preincubation of laminin on the microtest plates with *SSS* then washing did not inhibit MDW4 cell attachment (data not shown).

MM, Man_5, and the mixture Man_{5-9} inhibited MDW4 cell attachment to type IV collagen but not to fibronectin (Fig. 3). Half-maximal inhibition of cell attachment for *MM* and Man_5 was observed at 0.3 and 1 μM, respectively, and the mixture Man_{5-9} was less active with 50% inhibition at ~10 μM (Fig. 5). This would be expected if the Man_5 component of Man_{5-9} (i.e., 15%) were the major inhibitor in the mixture. None of the complex structures, 1, 2, 3, or 4 shown in Fig. 1 inhibited MDW4 attachment to type IV collagen (Fig. 3).

Attachment of Peptide N-Glycosidase-treated Cells

Asn-linked oligosaccharides on the intact cell surface have been shown to be susceptible to cleavage by peptide *N*-glycosidase (35). The enzyme releases intact complex and high mannose oligosaccharides from the protein leaving the ε-amino of the Asn on the reducing end. Both MDW4 and MDW4-24a cells were treated with peptide *N*-glycosidase and compared to mock-treated cells for attachment to type IV collagen and laminin (Fig. 6). Enzyme-treated MDW4 cells were less adhesive on type IV collagen as would be expected if the mechanism of type IV collagen binding required the recognition of cell surface high mannose structures. There appears to be less high Man structures on the WGA^s cells (34) and this may account for their nonadhesive behavior on type IV collagen (Fig. 2). As would be predicted from this line of reasoning, MDW4-24a attachment to type IV collagen was not affected by peptide *N*-glycosidase treatment.

Although treatment of MDW4 cells inhibited attachment to type IV collagen, no effect was seen for attachment to laminin suggesting that Asn-linked oligosaccharides were not mediating cell attachment to laminin. MDW4-24a cells became more adhesive to laminin after peptide *N*-glycosidase treatment (Fig. 6). This result is consistent with the idea that WGA^s cells can attach to laminin provided that an inhibiting Asn-linked oligosaccharide is removed, presumably the *SSS* structure. The enzyme treatment did not restore the attachment phenotype of MDW4-24a on laminin to that of MDW4 possibly due to the incomplete removal of *SSS* from the cell surface (Fig. 6, *inset*). The removal of the oligosaccharide

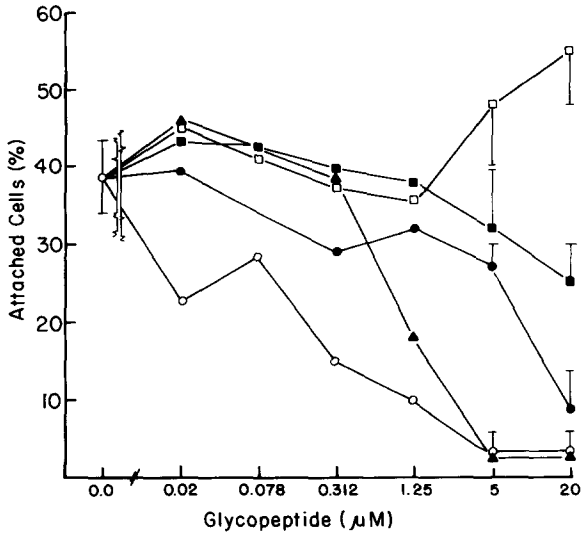

FIGURE 5 Inhibition of MDW4 cell attachment to type IV collagen as a function of glycopeptide concentration. Microtest plate wells were coated with 4 ng of type IV collagen plus 5 μg of BSA. The glycopeptides were conalbumin, *GnGnGn(Gn)* (□); MDW4, *GnGnGn* (■); Man_{5-9} (●); ovalbumin Man_5 (▲); and IgG, *MM* (○).

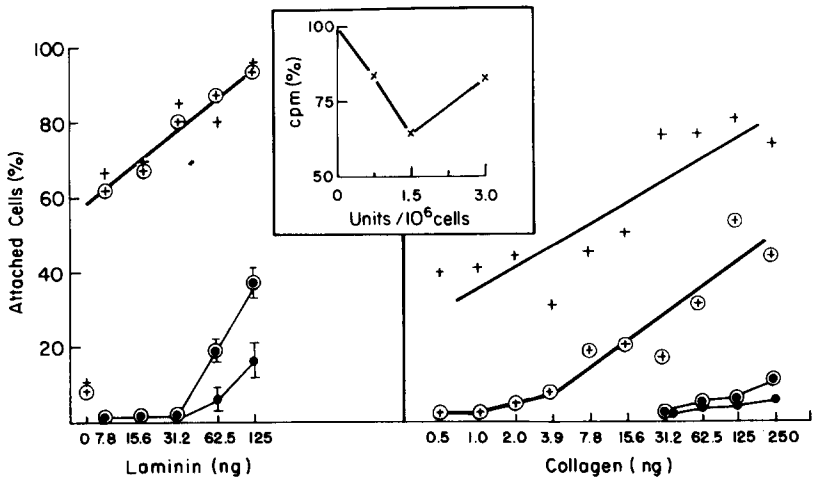

FIGURE 6 Attachment of peptide *N*-glycosidase treated tumor cells to laminin and type IV collagen. MDW4, ⊕ and MDW4-24a ◉ were incubated with peptide *N*-glycosidase (circled symbols), or without (uncircled symbols), and tested for adhesion as described in Materials and Methods. The *inset* demonstrates the partial removal of sialylated oligosaccharides from $NaIO_4$/Na-[^{3}H]BH_4-labeled MDW4-24a cells as a function of peptide *N*-glycosidase concentration. Cells tested in the attachment assays were treated with 1.5 U/10^6 cells at 37°C for 1 h.

from native glycoproteins by peptide *N*-glycosidase has been shown to vary depending on tertiary structure and accessibility of the oligosaccharides (30).

DISCUSSION

Attachment studies using lectin-resistant mutants with lesions in oligosaccharide synthesis (8, 10) and the use of the inhibitor tunicamycin (12, 13) have provided indirect evidence that Asn-linked oligosaccharide are required for various types of cell adhesion phenomenon. In this report, Asn-linked glycopeptides isolated from a WGA^r mutant adhesive on fibronectin, laminin, and type IV collagen, and from the nonattaching wild-type cells were tested for their ability to inhibit cell attachment.

MDW4 cell attachment to type IV collagen was inhibited by the mannose terminating glycopeptides *MM* and Man_5 and to a lesser degree by the mixture Man_{5-9}. MDW4 cells have more cell surface Con A-binding sites than the WGA^s cells (34). Because the major Con A-binding glycopeptides isolated from these cells were the high mannose structures (20), it is possible that inhibition of MDW4 attachment to type IV collagen by the Man terminating glycopeptides was due to competition with similar structures on the cell surface for an oligosaccharide-binding site in the collagen. Removal of Asn-linked oligosaccharides by almund emulsin peptide *N*-glycosidase treatment reduced MDW4 attachment to type IV collagen supporting the hypothesis that Asn-linked oligosaccharides (e.g., Man_5) may directly mediate cell attachment to type IV collagen. Man_{6-9} species are usually found inside the cell as processing intermediates in the production of complex, Man_5, and hybrid structures (36). Therefore, the finding that Man_5 glycopeptide was a more potent inhibitor than the mixture Man_{5-9} may be due to a preference of type IV collagen for the processed high mannose structure. A lectin activity on teratocarcinoma cells recognizing high mannose oligosaccharides has been reported to mediate homotypic cell adhesion (37). Because laminin and type IV collagen have been isolated from teratocarcinoma ECM (38), it is possible that the carbohydrate binding component detected in this study was type IV collagen.

In that the major change in Asn-linked oligosaccharides in the mutant cells was the acquisition of *GnGnGn* (20), it was suspected that this structure may be important for the increased adhesion of the cells to one or more ECM proteins. However, the mutant *GnGnGn* structure did not inhibit attachment to laminin, fibronectin, or type IV collagen at the concentrations tested. This does not eliminate the possibility that the presence of *GnGnGn* or absence of the wild-type structure affects the expression or activity of a glycoprotein involved in cell attachment. Rather than the presence of a new mutant oligosaccharide, the absence of the wild type *SSS* appeared to be the cause of increased MDW4 attachment to laminin. *SSS* is absent from MDW4 cells yet its addition to the attachment assay inhibited MDW4 cell attachment to laminin, possibly due to steric interference at the laminin receptor-laminin binding site. Laminin receptors on MDW4-24a cells may be glycosylated with the *SSS* structure consequently blocking laminin binding or the proximity of *SSS* on other cell surface glycoproteins may be sufficient to block cell attachment to laminin. MDW4-24a cells showed increased attachment to laminin after they had been treated with peptide *N*-glycosidase presumably due to the partial removal of *SSS* from the cell surface.

A number of other structurally related glycopeptides did not inhibit cell attachment to laminin. *SS*, *GG*, and the triantennary *GnGnGn* were not inhibitory, indicating that recognition of *SSS* and *GGG* required more of the structure than terminal monosaccharides, and that triantennary substitution of the core was also insufficient. It is possible that the repeating Gal β1–4GlcNAc in the antenna of *SSS* was functionally important for inhibiting cell attachment to laminin. Mobile cells encountering laminin in vascular ECM such as erythrocytes (39) and the K562 leukemia (40) have been shown to have the large Asn-linked poly-*N*-acetyllactosamine-containing glycopeptide. In addition, embryonal carcinoma cells appear to have a similar structure that disappears during in vitro differentiation (41). Because laminin appears at an early stage in embryogenesis (42), the presence of the poly-*N*-acetyllactosamine-containing oligosaccharide could play a

role in embryonic cell detachment and movement.

MDW4 cell attachment to fibronectin was not inhibited by the Asn-linked glycopeptides used in this study. It has been suggested that ganglioside GTlb mediates cell attachment to fibronectin (43); however, GTlb also inhibited cell attachment to gelatin and lectins indicating a lack of specificity (18). Lesions in the glucosaminoglycans, glycolipids, and *O*-linked oligosaccharides of MDW4 are being investigated as possible candidates for the increased adhesion of MDW4 to fibronectin.

The cellular environment can have a profound effect both qualitatively and quantitatively on the cell surface oligosaccharide structures. Confluent cells compared with cells in log-phase growth have increased high mannose to complex ratios (44). A recent study comparing tumor cells growing at a subcutaneous site with tumor cells in the liver demonstrated changes in the WGA-binding glycoproteins of the plasma membrane due apparently to the tissue environment since transplanting the tumor cells from the subcutaneous site to liver or the reverse was accompanied by a rapid reverse in the glycoprotein pattern (45). Therefore, differences in tissue environments which influence cell surface oligosaccharides expression may allow metastatic tumor cells to be relatively nonadherent to ECM proteins at the primary and, subsequently, attach at the secondary site to similar ECM proteins. Modulation of adhesion by altering oligosaccharide structure has been observed in embryonic tissue. Removal of sialic acid from oligosaccharides on retinal cell–cell adhesion molecules accompanies development from embryonic to adult forms and from inactive to active homotypic binding (45).

Yogeeswaran and Salk (9) have shown that the metastatic capacity of a range of murine tumor cell lines is positively correlated with the degree of sialylation of available galactose and *N*-acetylgalactosamine residues on the cell surface glycoconjugates. These results suggest that the more completed (i.e., sialylated) the complex oligosaccharides, the more metastatic and autonomous from host growth controls the tumor cell becomes. A number of under-sialylated WGA^r mutants isolated from the B16 melanoma (47), RAW-117-H10 lymphosarcoma (48) and MDAY-D2 (25) have been found to be less metastatic than the respective parental cell line. For example, MDW4 has been shown to be more susceptible to natural killer cells, although the mutant has not acquired new tumor antigens (49), and is more adhesive to laminin, fibronectin, and type IV collagen. Both natural killer sensitivity and cell adhesion appear to be related to the expression of incomplete complex oligosaccharides in the mutant (Dennis, J. W., manuscript submitted for publication). Therefore, it is tempting to speculate that the completion of the complex oligosaccharide masks or suppresses recognition of underlying oligosaccharide structures and consequently liberates the metastatic tumor cell from certain growth-controlling factors such as cell adhesion to ECM protein and natural killer cell lysis.

We thank Dr. T. Timpl for his kindly supplying type IV collagen and laminin and Dr. H. Schachter for supplying *MM* and Man_5. We also thank B. Fluhrer and B. Muller for their expert secretarial help.

This work was supported in part by research grants from the National Cancer Institute of Canada and the Medical Research Council of Canada to Dr. Dennis.

Received for publication 29 March 1984, and in revised form 2 July 1984.

REFERENCES

1. Alitalo, K., and A. Vaheri. 1982. Pericellular matrix in malignant transformation. *Adv. Cancer Res.* 37:111–158.
2. Kleinman, H. K., G. R. Martin, and P. H. Fisher. 1979. Ganglioside inhibition of fibronectin-mediated cell adhesion to collagen. *Proc. Natl. Acad. Sci. USA.* 76:3367–3371.
3. Aplin, J. D., and R. C. Hughes. 1982. Complex carbohydrates of the extracellular matrix structures, interactions and biological roles. *Biochim. Biophys. Acta.* 694:375–418.
4. Malinoff, H. L., and M. S. Wicha. 1983. Isolation of a cell surface receptor protein for laminin from murine fibrosarcoma cell. *J. Cell. Biol.* 96:1475–1479.
5. Terranova, V. P., C. N. Rao, T. Kalebic, I. M. Margulies, and L. A. Liotta. 1983. Laminin receptor on human breast carcinoma cells. *Proc. Natl. Acad. Sci. USA.* 80:444–448.
6. Terranova, V. P., R. A. Liotta, R. G. Russo, and G. R. Martin. 1982. Role of laminin in the attachment and metastasis of murine tumor cells. *Cancer Res.* 42:2265–2269.
7. Raz, A., and B. Geiger. 1982. Organization of cell-substrate contacts and membrane-associated cytoskeleton in tumor cell variants exhibiting different metastatic capacities. *Cancer Res.* 42:5183–5190.
8. Dennis, J., C. Waller, R. Timpl, and V. Schirrmacher. 1982. Surface sialic acid reduces attachment of metastatic tumor cells to collagen type IV and fibronectin. *Nature (Lond.).* 300:274–276.
9. Yogeeswaran, G., and P. L. Salk. 1981. Metastatic potential is positively correlated with cell surface sialylation of cultured murine tumor cell lines. *Science (Wash. DC).* 212:1514–1516.
10. Pena, S. D. J., and R. C. Hughes. 1978. Fibronectin-plasma membrane interactions in the adhesion and spreading of hamster fibroblasts. *Nature (Lond.).* 276:80–83.
11. Pouyssegur, J., M. Willingham, and I. Pastan. 1977. Role of cell surface carbohydrates and proteins in cell behaviour: studies on the biochemical reversion of an N-acetylglucosamine-deficient fibroblast mutant. *Proc. Natl. Acad. Sci. USA.* 74:243–247.
12. Nicolson, G. L., T. Irimura, R. Gonzalez, and E. Ruoslahti. 1981. The role of fibronectin in adhesion of metastatic melanoma cells to endothelial cells and their basal lamina. *Exp. Cell Res.* 135:461–465.
13. Butters, T. D., V. Devalia, J. A. Alpin, and R. C. Hughes. 1980. Inhibition of fibronectin mediated adhesion of human fibroblasts to substratum: effects of tumicamycin and some cell surface modifying reagents. *J. Cell Sci.* 44:33–58.
14. Reed, B. C., G. V. Ronnett, and M. D. Lane. 1981. Role of glycosylation and protein synthesis in insulin receptor metabolism by 3T3-L1 mouse adipocytes. *Proc. Natl. Acad. Sci. USA.* 78:2908–2912.
15. Gibson, R., S. Kornfeld, and S. Schlesinger. 1980. A role for oligosaccharides in glycoprotein biosynthesis. *Trends Biochem. Sci.* 5:290–293.
16. Nose, M., and H. Wigzell. 1983. Biological significance of carbohydrate chains on monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 80:6632–6636.
17. Fisher, H. D., A. Gonzalez-Noriega, W. S. Sly, and D. J. Morre. 1980. Phosphomannosyl-enzyme receptor in rat liver. *J. Biol. Chem.* 255:9608–9615.
18. Rauvala, H., W. G. Carter, and S. Hakomori. 1981. Studies on cell adhesion and recognition I. *J. Cell Biol.* 88:127–137.
19. Rauvala, H., J. Prieels, and J. Finne. 1983. Cell adhesion mediated by a purified fucosyltransferase. *Proc. Natl. Acad. Sci. USA.* 80:3991–3995.
20. Dennis, J., J. Carver, and H. Schachter. 1984. Asn-linked oligosaccharides in murine tumor cells: comparison of a WGA^r non-metastatic mutant and a related WGA^s metastatic line. *J. Cell Biol.* 99:1034–1044.
21. Timpl, R., H. Wiedemann, V. VanDelden, H. Furthmayr, and K. Kuhn. 1981. A network model for the organization of type IV collagen molecules in basement membranes. *Eur. J. Biochem.* 120:203–211.
22. Timpl, R., H. Rhode, P. G. Robey, S. I. Rennard, J. Foidart, and G. R. Martin. 1979. Laminin—a glycoprotein from basement membranes. *J. Biol. Chem.* 254:9933–9937.
23. Kerbel, R. S., J. W. Dennis, A. E. Lagarde, and P. Frost. 1982. Tumor progression in metastasis: an experimental approach using lectin resistant tumor variants. *Cancer Metastasis Rev.* 1: 99–140.
24. Kerbel, R. S., M. Florian, M. S. Man, J. Dennis, and I. F. C. McKenzie. 1980. Carcinogenicity of tumor cell populations: origin of a putative H-2 isoantigenic loss variant tumor. *J. Natl. Cancer Inst.* 64:1221–1230.
25. Dennis, J., T. P. Donaghue, M. Florian and R. S. Kerbel. 1981. Apparent reversion of stable *in vitro* genetic markers detected in tumor cells from spontaneous metastases. *Nature (Lond.)* 292: 242–245.
26. Kerbel, R. S., A. E. Lagarde, J. W. Dennis, and T. P. Donaghue. 1983. Spontaneous fusion *in vivo* between normal host and tumors cells: possible contribution to tumor progression and metastasis studied with a lectin resistant mutant tumor. *Mol. Cell. Biol.* 3:523–538.
27. Dorland, L., J. Haverkamp, J. F. G. Vliegenthart, G. Spik, B. Fournet, and J. Montreuil. 1979. Investigation by 360-MHZ ^{1}H-nuclear magnetic-resonance spectroscopy and methylation analysis of the single glycan chain in chicken ovotransferin. *Eur. J. Biochem.* 100:569–574.
28. Dorland, L., T. Haverkamp, B. L. Schut, J. F. Vliegenthart, G. Spik, G. Strecker, B. Fournet, and J. Montreuil. 1977. The structure of the asialo-carbohydrate units on human serotransferin as proven by 360-MHZ proton magnetic resonance spectroscopy. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 77:15–19.
29. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28:350–356.
30. Tarentino, A. L., and T. H. Plummer, Jr. 1982. Oligosaccharide accessibility to peptide *N*-glycosidase as promoted by protein-unfolding reagents. *J. Biol. Chem.* 257:10776–10780.
31. Gahmberg, C. G., and L. C. Anderson. 1977. Selective radioactive labeling of cell surface sialoglycoproteins by periodate-tritiated borohydrate. *J. Biochem.* 252:5888–5894.
32. Cummings, R. D., and S. Kornfeld. 1982. Characterization of structural determinants required for the high affinity interaction of asparagin-linked oligosaccharides with immunobilized "*phaseolus vulgaris*" leukoagglutinating and erythroagglutinating lectins. *J. Biol. Chem.* 257:11230–11234.
33. Ebisu, S., P. N. Shankar Iyer, and I. J. Goldstein. 1978. Equilibrium dialysis and carbohydrate-binding studies on the 2-acetamido-2-deoxy-D-glucopyranosyl-binding lectin from *Bandiraea simplicifolia* seeds. *Carbohydr. Res.* 61:129–138.
34. Dennis, J. W., and R. S. Kerbel. 1981. Characterization of a deficiency in fucose metabolism in lectin-resistant variants of a murine tumor showing altered tumorigenic and metastatic capacity *in vivo*. *Cancer Res.* 41:98–104.
35. Takahachi, N. 1983. Glycopeptidases releasing Asn-linked oligosaccharides from glycopeptides. *Seikogaku.* 55:1204–1211.
36. Berger, E. G., E. Buddecke, J. P. Kamerling, A. Kobata, J. C. Paulson, and J. F. G.

Vliegenthart. 1982. Structure, biosynthesis and function of glycoprotein glycans. *Experientia (Basal).* 38:1129–1258.
37. Grabel, L. B., S. D. Rosen, and G. R. Martin. 1979. Teratocarcinoma stem cells have a cell surface carbohydrate-binding component implicated in cell-cell adhesion. *Cell.* 17:477–484.
38. Ozawa, M., M. Sato, and T. Muramatsu. 1983. Basement membrane glycoprotein laminin is an agglutinin. *J. Biochem.* 94:479–483.
39. Fukuda, M., M. N. Fukuda, and S. Hakomori. 1979. Developmental changes and genetic defect in carbohydrate structure of band 3 glycoprotein of human erythrocyte membrane. *J. Biol. Chem.* 254:3700–3703.
40. Turco, S. J., J. S. Rush, and R. A. Laine. 1980. Presence of erythroglycan on human K562 chronic myelogenous leukemia-derived cells. *J. Biol. Chem.* 255:3266–3269.
41. Muramatsu, T., G. Gachelin, M. Damonneville, C. Delarbre, and F. Jacob. 1979. Cell surface carbohydrates of embryonal carcinoma cells: polysaccharidic side chains of F9 antigens and of receptors to two lectins FBP and PNA. *Cell.* 18:183–191.
42. Leivo, I., A. Vaheir, R. Timpl, and J. Wartioviara. 1980. Appearance and distribution of collagens and laminin in the early mouse embryo. *Dev. Biol.* 76:100–114.
43. Kleinman, H. K., G. R. Martin, and P. H. Fisher. 1979. Ganglioside inhibition of fibronectin-mediated cell adhesion to collagen. *Proc. Natl. Acad. Sci. USA.* 76:3367–3371.
44. Sasak, W., A. Herscovics, and A. Quaroni. 1982. Cell density-dependent changes in cell surface glycopeptides and in adhesion of cultured intestinal epithelial cells. *J. Biochem.* 201:359–366.
45. Chan, W. S., A. Jackson, and G. A. Turner. 1984. Differences in surface expression of WGA-binding proteins of cells from a lymphosarcoma and its liver metastases. *Br. J. Cancer.* 49:181–191.
46. Edelman, G. M. 1983. Cell adhesion molecules. *Science (Wash. DC).* 219:450–457.
47. Tao, T. W., and M. M. Burger. 1977. Nonmetastasizing variants selected from metastasizing melanoma cells. *Nature (Lond.)* 270:437–438.
48. Reading, C. L., P. N. Belloni, and G. L. Nicolson. 1980. Selection and *in vivo* properties of lectin attachment variants of malignant murine lymphosarcoma cell lines. *J. Natl. Cancer Inst.* 64:1241–1249.
49. Dennis, J. W., T. P. Donaghue, D. A. Carlow, and R. S. Kerbel. 1981. Demonstration of a correlation between tumor cell H-2 antigen content, immunogenicity and tumorigenicity using lectin-resistant tumor variants. *Cancer Res.* 41:4010–4019.
50. Kornfeld, S., E. Li, and I. Tabas. 1978. The synthesis of complex-type oligosaccharides. *J. Biol. Chem.* 253:7771–7778.
51. Narasimham, S., J. R. Wilson, E. Martin, and H. Schachter. 1979. A structural basis for four distinct elution profiles on Concanavalin-A-Sepharose affinity chromatography of glycopeptides. *Can. J. Biochem.* 57:83–96.
52. Harpaz, N., and H. Schachter. 1980. Control of glycoprotein synthesis. *J. Biol. Chem.* 255:4885–4893.