Evidence that the Major Cell Surface Glycoprotein of the TA3-Ha Carcinoma Contains the *Vicia graminea* Receptor Sites†

John F. Codington,* Amiel G. Cooper, Michael C. Brown, and Roger W. Jeanloz

ABSTRACT: The *Vicia graminea* lectin receptor of the nonstrain-specific TA3-Ha mammary carcinoma ascites cell of the strain A mouse was shown to be predominantly or exclusively on a large mucin-type surface glycoprotein. TA3-Ha cells adsorbed the lectin in amounts equivalent to 5–9 mg of this glycoprotein/10^9 cells, which was 100–400 times greater than by the strain-specific TA3-St cell, employed as a control. Release of sialic acid by incubation with neuraminidase increased the adsorptivity of the TA3-Ha cell three- to fourfold and of the TA3-St cell six- to tenfold. Proteolysis of TA3-Ha cells released into the supernatant solutions approximately the same amount of inhibitory activity, equivalent to approximately 5 mg of the glycoprotein, and only 10% of the original adsorptivity remained on the cells. By contrast, TA3-St cells released no detectable inhibitory activity into the medium when subjected to similar proteolysis, even after neuraminidase treatment. Upon fractionation of released material on gel columns, high-molecular weight material and activity were found in the same fractions, but purified samples differed significantly in specific activity and carbohydrate composition. Heterogeneity in the carbohydrate moieties of the macromolecules was further demonstrated by incubation of these samples with neuraminidase, which enhanced their inhibitory activities from two- to tenfold.

Two ascites sublines, TA3-Ha and TA3-St, of the mouse mammary adenocarcinoma TA3 are of interest because of their different biological properties and surface membrane compositions. The TA3-Ha, but not the TA3-St, subline is lethal for allogeneic strains of mice, and this difference may be related to the presence at the surface of the Ha subline of a considerable amount of a large-molecular weight glycoprotein (Codington *et al.*, 1973). This glycoprotein can be removed from viable Ha cells by proteolysis and can be recovered in macromolecular form from the supernatant solution after trypsin or papain treatment (Codington *et al.*, 1972, 1974). The bulk of this material is present in the excluded fraction from a Bio-Gel P-100 column and has been designated glycoprotein fraction I (GPF-I) (Codington *et al.*, 1972).

Inhibition of the agglutination of human erythrocytes of N blood group specificity by the lectin of *Vicia graminea* seeds is a sensitive method for the detection of molecules bearing the receptor for this lectin (Springer *et al.*, 1972; Cooper *et al.*, 1974). The measurement of this material in the ascites fluid and serum of syngeneic mice bearing TA3-Ha ascites cells was recently reported by Cooper *et al.* (1974). The receptor for the *V. graminea* lectin is probably the 3-*O*-(2-acetamido-β-D-galactopyranosyl)-α-D-galactopyranosyl-L-serine (or threonine) glycopeptide moiety (Uhlenbruck and Dahr, 1971).

It was of interest to ascertain whether the receptor sites for the *V. graminea* lectin that are present in the soluble substances of the body fluids are located predominantly or exclusively on the high-molecular weight glycoprotein (GPF-I) that has been isolated previously from the surface of the Ha cell. Thus, we have measured the capacity of viable Ha cells to adsorb the *V. graminea* lectin before and after proteolysis and have determined the lectin-inhibiting activity of various purified fractions. In addition, we have compared the capacity of Ha and St cells to bind the lectin both before and after neuraminidase treatment.

Materials and Methods

TA3 Ascites Cells. The strain-specific cell, TA3-St, and the nonstrain-specific cell, TA3-Ha, were developed from the 16th and 34th transplantation, respectively, of the same solid tumor, the TA3 mammary carcinoma, which had arisen spontaneously in a strain A/HeHa female mouse (Hauschka *et al.*, 1971). Both sublines have been maintained in the ascites form by serial transplantations in the syngeneic strain A mouse. The history and characteristics of both sublines have been described by Hauschka *et al.* (1971). In this investigation cells were harvested on day 7 after intraperitoneal inoculations of 2×10^6 TA3-St cells or 8×10^4 TA3-Ha cells in either male A/HeHa mice (obtained from West Seneca Laboratory, Buffalo, N.Y.) or male A/HeJ mice (purchased from Jackson Laboratory, Bar Harbor, Maine).

Enzymes. Twice-crystallized TPCK-trypsin[1] (EC 3.4.21.4) and papain (EC 3.4.22.2, 11 units/mg) were ob-

† From the Laboratory for Carbohydrate Research, Departments of Biological Chemistry and Medicine, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts 02114 (J.F.C. and R.W.J.), and the Department of Pathology, Tufts University School of Medicine, Boston, Massachusetts 02111 (A.G.C. and M.C.B). *Received August 20, 1974.* This is publication No. 658 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School and Massachusetts General Hospital. This investigation was supported by Research Grants CA-08418 and CA-14420 from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service. One of us (A.G.C.) is a recipient of a U.S. Public Health Service Career Development Award CA-70680.

[1] Abbreviations used are: TPCK-trypsin, L-1-tosylamido-2-phenylethyl chloromethyl ketone trypsin; PBS, phosphate-buffered saline; BSS, balanced salt solution.

tained from Worthington Biochemical Corp., Freehold, N.J. TPCK-trypsin was purified as previously described (Codington *et al.*, 1970); papain was used without purification. Neuraminidase (EC 3.2.1.18, from *Vibrio cholerae*, 500 units/ml) was purchased from Behring Diagnostics, Somerville, N.J.

Hemagglutination Inhibition Assays and Adsorptions of Vicia graminea Lectin. Erythrocytes of MN specificity from a human male, group O, Rh positive, donor were washed well with phosphate-buffered saline. *V. graminea* seeds were extracted with phosphate (0.01 M)-buffered saline (0.15 M) at neutral pH (PBS) as described by Cooper *et al.* (1974), and the extract was stored in small ampoules at −20°. The agglutination of erythrocytes by the lectin was measured in a continuous flow automated system, as previously described (Cooper *et al.*, 1974). A standard curve of inhibition was obtained by measuring the per cent inhibition of the lectin by serial amounts of fraction C (Slayter and Codington, 1973), obtained from GPF-I. Springer *et al.* (1972) reported that GPF-I was approximately as active as the most active, trypsin-cleaved glycopeptide fragment from the human N specific erythrocyte. Complete inhibition of hemagglutination was achieved by 5–10 μg/ml of GPF-I. It was found that 50–60 ng/ml of fraction C gave 50% inhibition of the standard lectin solution, and as little as 5 ng could cause measurable inhibition (Cooper *et al.*, 1974). In contrast, no inhibition of the *V. graminea* lectin was observed with as much as 2.5 mg of fetuin, bovine submaxillary mucin, or α_1-acid glycoprotein, either before or after removal of sialic acid (A. G. Cooper and M. C. Brown, unpublished results). In adsorption studies, serial numbers of washed, viable cells (or enzyme-treated viable cells) were removed by centrifugation, and the pellet was suspended in 0.1-ml aliquots of PBS–diluted lectin solution (Cooper *et al.*, 1974). After 60 min at 4°, the cells were removed by centrifugation, an aliquot of each supernatant solution was diluted tenfold with PBS, and the per cent of lectin adsorbed was measured. That number of cells of each type that adsorbed 50% of the lectin was equated with the amount of fraction C necessary to give the same per cent inhibition of the lectin solution. The *V. graminea* lectin-adsorbing capacity of each cell type was expressed as equivalents of fraction C (in mg) per 10^9 cells.

Incubation of Cells with Proteolytic Enzymes. Suspensions of 0.5–2.0 × 10^8 cells/ml in balanced salt solution (BSS, Dulbecco and Vogt, 1954) were mixed gently on a rotating apparatus (A. H. Thomas, Philadelphia, Pa.) at 4° in successive 20-min incubations, as previously described (Codington *et al.*, 1972, 1974), with TPCK-trypsin (18 μg/ml) or with papain (93 μg, 1.0 units/ml) and dithiothreitol (15 μg/ml). Cells were centrifuged at 80*g* for 10 min after each incubation, and the supernatant solutions were clarified by centrifugation at 37,000*g*, and lyophilized. The dried samples were weighed, and aliquots were taken for lectin-inhibiting activity and chemical measurements. The lectin-adsorbing capacity of the cells was measured before and after enzyme treatment.

Removal of Sialic Acid from Glycoproteins. Samples of glycoproteins were weighed, dissolved in BSS, and incubated with neuraminidase (25 units/ml) for 18 hr in the presence of 5 μl of toluene. Aliquots were tested for the sialic acid liberated (Warren, 1959) and for the inhibitory activity, as just described. The results were not influenced by small amounts of the enzyme. Sialic acid was removed from viable cells by incubation for 80 min at 25° with neuraminidase (200 units/ml). Control cells were incubated without neuraminidase. After removal of the cells by centrifugation, the sialic acid in the supernatant solutions was determined (Warren, 1959). After the cells had been washed several times, they were tested for viability (Trypan Blue exclusion) and counted. Neuraminidase-treated and control cells were then employed in adsorption experiments, as just described.

Table I: Adsorption of *Vicia graminea* Lectin by Intact Cells.

Cell Type	Adsorption of *Vicia graminea* Lectin[a]		
	Nontreated Cells	Neuraminidase-Treated Cells	Protease-Treated Cells
Ta3-Ha	5.0–9.0	15–21[b]	0.5
Ta3-St	0.02–0.05	0.2–0.3[c]	
Normal strain A spleen leukocytes	0.002		
Normal strain A erythrocytes	0.002		
Human Mn erythrocytes	0.02		

[a] Results expressed as milligram equivalents of the standard, GPF-I, fraction C, per 10^9 cells. [b] 76% of neuraminidase-susceptible sialic acid removed. [c] 82% of neuraminidase-susceptible sialic acid removed.

Column Fractionation of Protease-Released Glycoproteins. After incubation of TA3-Ha or TA3-St cells with proteolytic enzymes, the released material, dissolved in pyridine acetate buffer (pH 5.3, 0.05 M), was applied to a column of Bio-Gel P-4 and eluted with the same buffer, as previously described (Codington *et al.*, 1972). Sialic acid and protein were determined for each fraction, and pooled fractions were lyophilized and tested for inhibitory activity. Further fractionations of excluded material were performed on columns of Bio-Gels P-30 or P-100 (Codington *et al.*, 1972) or on Sepharose 4B (Slayter and Codington, 1973).

Results

Trypsin treatment of the nonstrain-specific TA3-Ha ascites cell was reported (Codington *et al.*, 1972) to release at least 1 mg/10^9 cells of glycoprotein fraction I (GPF-I). In recent experiments, however, as much as 2 mg of GPF-I has been isolated after fractionation on Bio-Gel P-100. This material was found to be highly inhibitory to the agglutination of N specific human erythrocytes by the lectin of *V. graminea* seeds (Springer *et al.*, 1972). It was expected, therefore, that this cell would possess at its surface a large number of receptor sites for this lectin. As shown in Table I, the TA3-Ha cell adsorbed an amount of lectin equivalent to 5–9 mg/10^9 cells of fraction C (Slayter and Codington, 1973), a fraction derived from GPF-I. This represented 100 to 400 times more lectin than was adsorbed by the strain-specific TA3-St subline of the same tumor, which adsorbed the equivalent of only 20–50 μg of fraction C/10^9 cells. More receptor sites were uncovered on the surface of each cell by the removal of sialic acid with neuraminidase (Table I), the six- to tenfold increase exhibited by the TA3-St cell

Table II: Material Released from 10^9 Viable Cells by Successive Incubations with Proteolytic Enzymes at 4°.

Cell	Enzyme Incubation	Bound Sialic Acid Released by Proteolysis (μg)	Released *V. graminea* Inhibition Activity[a] Untreated Material	Released *V. graminea* Inhibition Activity[a] Neuraminidase-treated Material
TA3-Ha[c]	(1) TPCK-trypsin	109	2220	4620
	(2) TPCK-trypsin	88	1610	2960
	(3) TPCK-trypsin	41	615	1400
	(Cell wash)	36	216	870
	(4) Papain	48	225	940
	(5) Papain	58	126	680
	(6) Papain	55	125	930
Total from six proteolyses		435 (75%)[b]	5137	12,400
TA3-St[d]	TPCK-trypsin	67 (25%)	<2.5	<2.5
	Papain	92 (34%)	<2.5	<2.5

[a] Results expressed as microgram equivalents of fraction C per 10^9 cells. [b] The values in parentheses are the per cent of the total surface sialic acid released by neuraminidase. [c] For the Ha cell, six successive proteolytic incubations were performed at 4°, and each supernatant solution was examined separately. The concentration of trypsin was 18 μg/ml, and papain, 93 μg/ml. [d] For the St cell, incubations with TPCK-trypsin (18 μg/ml, two 20-min incubations, 4°, pooled supernatant solutions) and papain (93 μg/ml, two 20-min incubations, 4°, pooled supernatant solutions) were performed as separate experiments.

Table III: Gel Filtration of the Material Cleaved from 10^9 Cells by TPCK-Trypsin.

Cell	Bio-Gel Column	Fraction[a]	Weight[b] of Fraction (μg)	*Vicia graminea* Inhibitory Activity (μg)
TA3-Ha	P-4	4A	2500	1250
	P-4	4B	360	<4
	P-4	4C	600	<7
	P-30	30A	1050	3250
	P-30	30B	120	74
	P-30	30C	37	13
	P-30	30D	270	4
	P-30	30E	190	3
	P-30	30F	200	<2
TA3-St	P-4	4A	1600	<1
	P-4	4B	500	<1
	P-4	4C	500	<1

[a] The fraction letters refer to successive pools of column fractions, as shown in Codington *et al.*, 1972. [b] Based on lyophilized weight.

being greater than the three- to fourfold increase of the TA3-Ha cell.

Since the human MN-specific erythrocyte used in these studies is readily agglutinated by the lectin, its relatively low adsorptivity per cell, approximately equivalent to that of the TA3-St cell (Table I), is noteworthy. The surface area of the human erythrocyte is less than 25% of that of the TA3-St ascites tumor cell (Altman and Dittmer, 1964; Friberg, 1972), however, and accordingly its adsorptivity per unit surface area would be more than fourfold that of the TA3-St cell. Syngeneic strain A normal red cells and spleen cells adsorbed only minute amounts of the lectin.

TA3-Ha cells were sequentially treated three times with TPCK-trypsin, washed with BSS, and then three times with papain. These protease-treated cells, after being washed, were shown by dye exclusion to have a viability greater than 80%, and their adsorption capacity for the *V. graminea* lectin was measured again. After proteolysis, only 10% of the original lectin-adsorbing capacity remained (Table I). The release of glycoprotein-bound sialic acid and *V. graminea* receptors was monitored in the supernatant solutions following each proteolysis (Table II). In addition, the hemagglutination inhibitory activities of these supernatant solutions were measured after neuraminidase treatment. In this experiment, proteolysis of the TA3-Ha cell released 60–70% as much sialic acid as can be removed by extensive treatment of viable cells with neuraminidase (Codington *et al.*, 1974).

The total inhibitory activity in the supernatant solutions was equivalent to 5.1 mg of standard fraction C before neuraminidase treatment and 12.4 mg after removal of sialic acid. These activities are strikingly similar to the total adsorptive capacity of the intact Ha cells before and after neuraminidase treatment. The supernatant solution of the first proteolysis had the highest amount of inhibitory activity and bound sialic acid, and there was a general decrease in values for each determination with successive enzyme treatments, except that each of the three papain treatments gave a release of sialic acid slightly higher than that of the third trypsinization. In addition, the ratio of the sialic acid content to the inhibitory activity was found to be higher for each supernatant solution after papain treatment than for any supernatant solution after trypsin treatment.

In a separate experiment, not shown in Table II, Ha cells were first subjected to three sequential treatments with papain, and then three treatments with trypsin. The results were similar to those of the previous experiment: most of the sialic acid and the inhibitory activity were removed during the early protease treatments. In each experiment, the ratio of sialic acid released to inhibitory activity released was higher when papain was employed than when trypsin was used.

Strain-specific, TA3-St cells were incubated two times in succession with TPCK-trypsin or two times in succession with papain (Table II). The combined supernatant solutions obtained after incubation with trypsin and the combined supernatant solutions resulting after papain treatment contained, respectively, 25 and 34% of the total surface sialic acid removable by neuraminidase (Codington *et al.*, 1973). However, no *V. graminea* inhibitory activity could be detected in any of the proteolysis supernatant solutions, either before or even after neuraminidase treatment.

The material solubilized from the Ha cell surface by TPCK-trypsin was fractionated on columns of Bio-Gel P-4 and P-30, as previously reported (Codington *et al.*, 1972). Sequential pools from each column were assayed for *V. graminea* inhibiting activity. All the active material recov-

Table IV: *Vicia graminea* Inhibiting Activity of GPF-I Fractions, Purified by Column Chromatography, from 10^9 TA3-Ha Cells.

Enzyme Used for Cleavage	Column Gel	Fraction[c]	Activity[e]: Untreated Material	Activity[e]: Neuraminidase-Treated Material
TPCK-trypsin, then papain[a]	Bio-Gel P-100	A	1.2	3.4
TPCK-trypsin[b]	Sepharose 4B	A	0.27	2.8
	Sepharose 4B	B	1.1	1.7
	Sepharose 4B	C	1.0[d]	5.4

[a] Material from pool of six successive proteolyses (see Table II). [b] Material from pool of three successive trypsinizations (see Table II). [c] Fraction pooled as described in Codington *et al.*, 1972 and Slayter and Codington, 1973. [d] Used for standard in *Vicia graminea* inhibition assay. [e] Expressed as milligram equivalents of fraction C.

ered from the column was found in fraction 4A, the material excluded from the P-4 column (Table III), which contained GPF-1, as well as lower molecular weight material. Likewise, material containing over 95% of the activity eluted from a Bio-Gel P-30 column was found in fraction 30A, the material excluded from the column. The specific activity of the 4A fraction was less than that of the standard, fraction C, whereas the 30A fraction varied from 1.7 to 3.1 times that of the standard, fraction C.

Fractionation on a column of Bio-Gel P-4 of supernatant solutions obtained by incubation of TA3-St cells with TPCK-trypsin failed to yield any active material, as was anticipated, since the supernatant solutions themselves were inactive.

The P-30 excluded material was further fractionated on Bio-Gel P-100 (Codington *et al.*, 1972) and on Sepharose 4B (Slayter and Codington, 1973) columns. The excluded fraction from the P-100 column (GPF-I) and the three pooled fractions from the Sepharose 4B column (fractions A, B, and C) were examined for activity before and after neuraminidase treatment (Table IV). The activity relative to fraction C, used as the standard reference throughout this study, was 1.2 for the P-100 excluded material, GPF-I, and 0.27 and 1.1 for fractions A and B from the Sepharose 4B column, respectively. The activity of each of these fractions increased to varying degrees after neuraminidase treatment (Table IV).

Discussion

The results of this study provide strong evidence that the receptor for the *Vicia graminea* lectin on the TA3-Ha adenocarcinoma ascites cell is present predominantly or exclusively on the glycoprotein macromolecule (GPF-I) that is at the surface of the Ha, but not the St, subline. (a) The viable Ha cell has over 100–400 times the number of lectin-binding sites as does the intact St cell. Only from the Ha, and not from the St, subline, was it possible to remove from its surface GPF-I material (Codington *et al.*, 1973). (b) Mild proteolysis of the Ha cell removes simultaneously the surface GPF-I molecules and the *V. graminea* receptors; after successive proteolytic treatments, less than 10% of the original lectin-binding capacity can be detected on the surface of the Ha cell. (c) The lectin receptor is recovered from the supernatant solutions of the Ha cell after proteolysis in an amount approximately equivalent to that present on the cell surface before proteolysis. No lectin inhibitor was found in the proteolysate of the St cell. (d) During fractionation through a series of gel columns, the inhibitory activity from the Ha cells was eluted in those fractions containing the GPF-I material. (e) It was previously shown (Cooper *et al.*, 1974) that there is *in vivo* release of *V. graminea* receptors associated with a high-molecular-weight glycoprotein from TA3-Ha cells, but not from TA3-St cells, into the ascites fluid and serum of tumor-bearing syngeneic hosts.

Although no GPF-I material was found in the supernatant solutions after mild incubation of TA3-St cells with proteolytic enzymes (Table II), we did detect small, yet significant, amounts of lectin adsorption of these cells (Table I). This adsorption appears to be specific, since it was tenfold greater than that by the strain A erythrocyte or spleen cell (Table I). The question arises, therefore, as to whether these lectin receptors are part of GPF-I molecules, which may be too low in concentration to be detected in either the supernatant solutions (Table II) or column fractions (Table III). The data reveal, however, that most of these receptors cannot be part of molecules similar to GPF-I, since proteolysis of the TA3-St cell would have released from 10^9 cells (Table II) a minimum of 5 μg of fraction C equivalent material or the equivalent of 50 μg of neuraminidase-treated glycoprotein, amounts of activity that would have been readily detected in the supernatant solutions (Table II) or column fractions (Table III).

The activities of the purified fractions (Table IV) show a marked heterogeneity in GPF-I material, from 0.3 mg-equiv for fraction A, Sepharose 4B column (Slayter and Codington, 1973), to 1.8 mg-equiv for fraction A (Bio-Gel P-30 column). Since all fractions appear to possess identical amino acid compositions (Codington *et al.*, 1974), activity differences are probably due to variations of the carbohydrate moiety. All fractions contain the same four major carbohydrate components, galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and *N*-acetylneuraminic acid, but differ in their relative proportions (Codington *et al.*, 1974). A marked heterogeneity of the GPF-I material is also shown by the various degrees of masking, by sialic acid residues, of the inhibitory sites. Removal of sialic acid residues by neuraminidase (Table IV) gave an increase of inhibition, ranging from a 1.5- to a 10-fold increase. The chemical data had suggested two major chain types (Jeanloz and Codington, 1974), one being a disaccharide chain with *V. graminea* inhibitory activity (Uhlenbruch and Dahr, 1971). This chain probably occurs in far greater proportion in fraction C than in fraction A, which is consistent with the higher inhibitory activity of fraction C.

To our knowledge, this study represents the first instance in which the number of cell surface receptor sites in a neoplastic cell has been related specifically and quantitatively to glycoprotein material obtained as purified fractions after removal from the cell surface. On the basis of the inhibition and adsorption results described in this paper, it appears probable that more than 5 mg of mucin-type glycoprotein material per 10^9 cells, probably in the form of long, rod-like molecules (Slayter *et al.*, 1973), extend outward from the TA3-Ha cell membrane. The average apparent molecular weight of this material, as isolated from the ascites fluid

and serum of syngeneic mice bearing the ascites tumor (Cooper *et al.*, 1974), is estimated to be significantly greater than that of the largest size material isolated after proteolysis (fraction A, Sepharose 4B column), which was shown to have a molecular weight of 460,000 by sedimentation equilibrium (Slayter *et al.*, 1973). Based upon an estimated average molecular weight of about 5×10^5, 5 mg/10^9 cells of this glycoprotein would represent approximately 5×10^6 molecules/cell.

The quantitation of the large glycoprotein at the surface of the TA3-Ha cell by cell adsorption and hemagglutination inhibition experiments facilitates studies of a possible immunological role of this material in the serum of tumor-bearing hosts, particularly since the methods employed are sufficiently sensitive to detect the glycoprotein in amounts as low as 5 ng/ml. Another mode of action of the glycoprotein in enhancing the virilence of the TA3-Ha cell was recently suggested (Codington *et al.*, 1973; Sanford *et al.*, 1973). It was suggested that the presence of GPF-I molecules on the Ha surface is related to the ability of the Ha subline to kill allogeneic recipients, that is, to its loss of strain specificity. The proposed masking effect by this glycoprotein of surface histocompatibility antigens may be due to the physical presence of the high concentration of long, rod-like molecules (Codington *et al.*, 1973), and may not be specifically related to the presence of active *V. graminea* inhibitory carbohydrate structures. Although the *V. graminea* receptor sites may not be related to the biological function of the glycoprotein, they are highly useful as markers for further study of the biosynthesis, turnover, and membrane distribution of GPF-I molecules of the TA3-Ha cell. Furthermore, they will facilitate the screening of other TA3 sublines and of other lines of tumor cells which may have similar membrane glycoproteins.

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