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COMPARATIVE LECTIN-BINDING AND AGGLUTINATION PROPERTIES OF THE STRAIN-SPECIFIC, TA3-St, AND THE NON-STRAIN-SPECIFIC, TA3-Ha, MURINE MAMMARY CARCINOMA ASCITES SUBLINES. FURTHER STUDIES OF RECEPTORS IN EPIGLYCANIN*

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ABSTRACT

The complex carbohydrates at the cell surfaces of two TA3, murine mammary carcinoma ascites sublines (the strain-specific, TA3-St subline and the nonstrain-specific, TA3-Ha line) were compared by binding studies with ^{125}I-labelled concanavalin A (con A), *Ricinis communis* agglutinin (RCA), and eel-serum agglutinin (ESA). The TA3-Ha cell bound equal amounts of con A, 1.5-fold more RCA, and 4-fold more ESA than the TA3-St cell. Binding-inhibition studies by these lectins and two others [wheat-germ agglutinin (WGA) and potato lectin (STA)] suggest complementary binding-sites between con A and both RCA and ESA. Quantitative agglutination studies with the five lectins, and inhibition determinations by both neuraminidase-treated and untreated epiglycanin revealed that TA3-St, but not TA3-Ha, cells were agglutinated by con A, and that epiglycanin inhibited this agglutination, as well as the agglutination of rabbit erythrocytes by con A. The presence of a con A receptor on epiglycanin was also suggested by the binding of epiglycanin to con A–Sepharose, and its specific elution with methyl α-D-mannopyranoside. TA3-St cells were agglutinated at a 10–15-fold lower concentration of either STA or RCA than TA3-Ha cells, but both cells were agglutinated by the same concentration of WGA and ESA. Inhibition by epiglycanin of agglutination of TA3-St cells by either STA or ESA occurred at a concentration lower than that of TA3-Ha cells, but epiglycanin inhibited RCA agglutination of TA3-Ha cells at a concentration

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lower than that of TA3-St cells. Epiglycanin, but not asialoepiglycanin, inhibited the agglutination of TA3-Ha cells by WGA

INTRODUCTION

Two sublines of the TA3, mammary carcinoma ascites cell of the strain A mouse have been extensively investigated, because of the possible relevance of allotransplantability in experimental animals to metastasis in human cancer[1]. The characteristics of a nonstrain-specific subline of this tumor, TA3-Ha, have been compared with those of a strain-specific subline, TA3-St, by several laboratories[1-5]. We had previously demonstrated a direct correlation between the presence of a glycoprotein of high molecular weight (epiglycanin) at the cell surface and the capacity of the TA3-Ha cell, as well as other nonstrain-specific ascites sublines of the TA3 tumor, to grow progressively as ascites cells in mice of foreign strains, and to resist absorption by histocompatibility ($H\text{-}2^a$) antibody[5-8]. Some physicochemical[9,10] and immunochemical[10-12] properties of epiglycanin have been reported, and the detailed structures of four types of O-linked carbohydrate chains have been elucidated[13].

Although we demonstrated that nonstrain specificity in the TA3 ascites tumor system may be associated with the presence of epiglycanin, it is plausible that other qualitative or quantitative changes in complex carbohydrates at the cell surface may be implicated. In order to investigate the possible occurrence of biochemical differences of this type, we have undertaken a study of the relative proportion of specific receptors at the cell surfaces of the two sublines by the adsorption of ^{125}I-labelled lectins and by measuring the relative agglutinability of the two cell-lines with various lectins. Determination of the inhibitory activity of epiglycanin for these agglutination reactions suggests the presence of new carbohydrate structures in epiglycanin.

EXPERIMENTAL

Preparation of TA3 ascites cells. — The TA3-St and TA3-Ha cells of the strain A mouse were harvested, and washed three to six times with a balanced salt solution[14], without Ca^{2+} and Mg^{2+}, as previously described[8]. Neuraminidase treatment of the cells was performed as previously reported[8].

Lectins. — *Ricinus communis* agglutinin (RCA) was prepared from castor beans according to Tomita *et al.*[15]. Concanavalin A (con A) was purified from jack-bean meal (Sigma Chemical Co., St. Louis, MO 63178) according to Agrawal and Goldstein[16]. Eel-serum agglutinin (ESA) was purified as previously described[17]. Wheat-germ agglutinin (WGA) was purified by the method of Marchesi[18]. Potato lectin (STA) was prepared by the method of Marinkovich[19]. The purified lectins were iodinated with ^{125}I by use of the Chloramine T method, as previously described[20]. The specific activities of the lectins were 0.2–8.0 $\times$ 10^5 c.p.m. per mg of protein.

Binding studies. — Binding assays were performed according to the method

previously described[20]. The reaction mixture contained 1.0–2.3 × 10^6 cells and 2–200 pmol of ^{125}I-labelled lectin in a final volume of 0.2 mL of a balanced salt solution[14] with 0.25% of bovine serum-albumin. In binding-inhibition assays, equal amounts of "cold" lectin, or various amounts of inhibitor sugars, in balanced salt solution–0.25% bovine serum-albumin (0.1 mL) were mixed with the cells just before the addition of ^{125}I-labelled lectin. Purified, blood-group substance A + H was prepared from hog gastric-mucin according to Kabat[21].

Inhibitors. — The epiglycanin utilized consisted of the fraction of highest mol. wt. obtained by gel filtration (Bio-Gel P-100) of material cleaved from viable TA3-Ha cells by incubation with a modified trypsin, as previously described[8].

Inhibition of agglutination of tumor cells. — As 10–30% of TA3-Ha and TA3-St ascites cells are normally agglutinated, and as an agglutination of 90–100% can rarely be achieved, even at high lectin-concentrations, the increase in agglutination by lectins was quantitatively measured by counting in a hemacytometer. Agglutinations were performed in a series of tubes containing cells (0.05 mL, 4 × 10^6 cells/mL) added to buffer containing the lectin (0.05 mL) at appropriate, 2-fold, serial dilutions. With all the lectins used, except con A, a balanced salt solution[14] (pH 7) devoid of Ca^{2+} and Mg^{2+} was utilized, but, in experiments with con A, the buffer contained both of these ions at mM concentration. After incubation for ~30 and ~60 min with periodic mixing, the total number of cells and the total number of agglutinated cells, as counted in the hemacytometer, were recorded.

The inhibition experiments utilized the concentration of lectin found to produce ~50% of the agglutination between the maximum and minimum agglutinations recorded. In these experiments, epiglycanin, or neuraminidase-treated epiglycanin, at various dilutions was incubated for 3 min with lectin prior to the addition of tumor cells. The percentages of agglutinated cells at 30 and 60 min were plotted against four concentrations of inhibitors. As complete inhibition of agglutination of the tumor cells was difficult to obtain, concentrations of inhibitor were recorded for an inhibition of 50%.

RESULTS

Binding of ^{125}I-labelled RCA, con A, and ESA to TA3 cells. — The results of binding ^{125}I-labelled RCA to TA3-St and TA3-Ha cells were plotted according to Steck and Wallach[22] (see Fig. 1). The values obtained for TA3-St cells before and after removal of sialic acid by neuraminidase were identical. The association constant was 7.1 μM^{-1}, and the number of binding sites was 2.0 × 10^7 per cell (see Table I). For TA3-Ha cells, treatment with neuraminidase did not modify the number of sites (3.1 × 10^7 per cell), but removal of sialic acid did increase the association constant from 4.9 to 5.7 μM^{-1}. The specificity of the RCA binding was studied by inhibition with lactose (see Fig. 2). For the TA3-St cell, neuraminidase treatment did not alter the results, and an inhibition of 50% was obtained with 0.15 μmol of lactose/mL. The concentration of lactose required for the inhibition of RCA binding

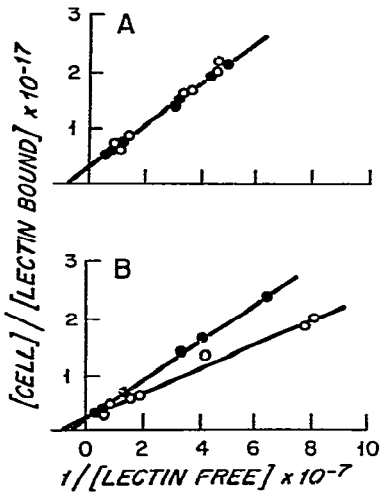

Fig. 1. Binding of ^{125}I-labelled RCA to TA3-St cells (A) and TA3-Ha cells (B). Increasing amounts of ^{125}I-labelled RCA were added to cells (●), or to cells pretreated with neuraminidase (○). After incubation for 1 h at room temperature, the proportion of RCA to the washed cells was determined by means of an autogamma counter. The data were plotted by the method of Steck and Wallach[22].

TABLE I

BINDING OF ^{125}I-LABELLED LECTINS TO TA3 CELLS

Lectin	*TA3 cell*			
	St		*Ha*	
	C[a]	*N*[b]	*C*	*N*
	Number of receptor sites per cell (× 10^{-6})			
RCA	20	20	31	31
Con A	1.0	1.0	1.0	1.0
ESA	3.3	3.3	14	14
	Association constant (μM^{-1})			
RCA	7.1	7.1	4.9	5.7
Con A	75	47	47	47
ESA	2.2	1.1	1.1	1.1

[a]C, control cells. [b]N, neuraminidase-treated cells.

to TA3-Ha cells was 0.1mM; after neuraminidase treatment, however, 0.2mM was required. This result is consistent with the increase in the affinity constant for TA3-Ha cells observed after neuraminidase treatment. As recorded in Table I, TA3-Ha cells were found to possess 10^7 more binding sites for RCA than TA3-St cells.

The number of binding sites to con A was found to be the same for both TA3-Ha

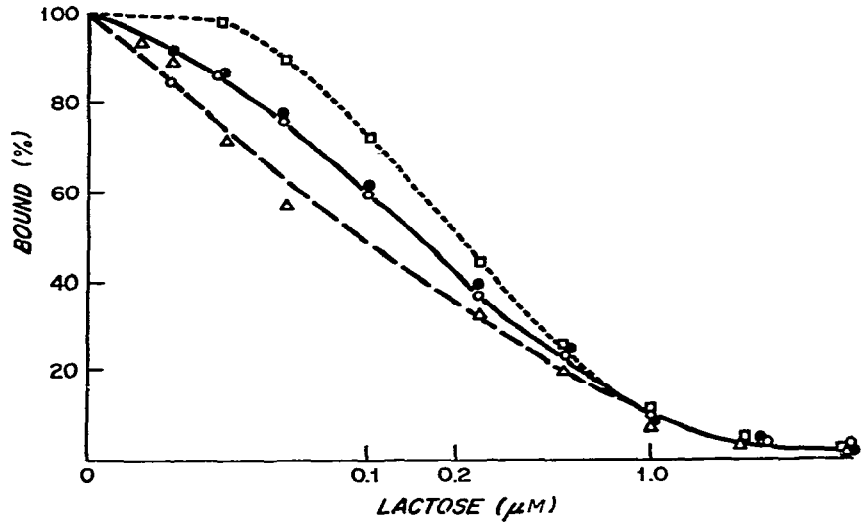

Fig. 2. Effect of lactose on binding of ^{125}I-labelled RCA to TA3-St cells (untreated, ○, and neuraminidase-treated, ●) and TA3-Ha cells (untreated, △, and neuraminidase-treated, □).

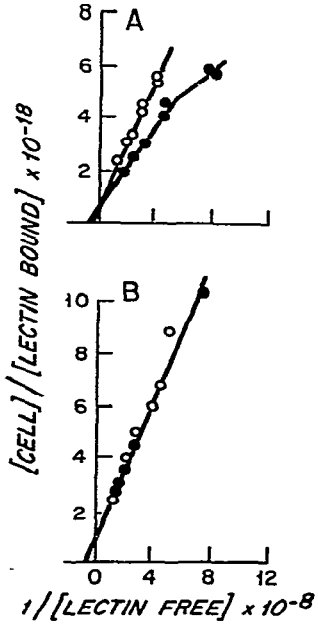

Fig. 3. Binding of ^{125}I-labelled con A to TA3-St cells (A), and TA3-Ha cells (B). A procedure similar to that described in the legend to Fig. 1 was followed.

and TA3-St cell-lines (see Table I), in agreement with a report of Friberg *et al.*[23], although the present value (1.0×10^6 receptor sites per cell) is lower than that reported[23] by them. No change was noted in the number of receptor sites after incubation of either cell line with neuraminidase (see Fig. 3 and Table I). However,

TABLE II

INHIBITION, BY LECTINS[a], OF LECTIN BINDING

TA3 cell[b]	*Radiolabelled lectin*	*Nonlabelled lectin*					*Inhibitor*			
		Con A	*ESA*	*RCA*	*STA*	*WGA*	*Me α-D-Man*	*A + H*	*Lac*
St(N)	con A	52	61	138	61	78	5		
St(C)	con A	69	99	181	96	124	8		
St(N)	ESA	140	47	18	66	30		1	
St(C)	ESA	306	65	71	103	30		3	
St(N)	RCA	101	61	19	63	62			0
St(C)	RCA	112	92	19	70	66			
Ha(N)	con A	59	65	185	85	94	3		
Ha(C)	con A	46	62	139	67	60	3		
Ha(N)	ESA	141	43	85	101	74		0	
Ha(C)	ESA	176	43	99	59	88		0	
Ha(N)	RCA	96	91	24	89	81			0
Ha(C)	RCA	101	113	31	91	59			0

[a]The extent of binding is expressed as the % of binding of the radiolabelled lectin alone. [b]Abbreviations: C, control; N, neuraminidase-treated; Me α-D-Man, methyl α-D-mannopyranoside; A + H, A + H blood-group active substance; Lac, lactose.

treatment with neuraminidase led to a decrease in the affinity constant for TA3-St cells, whereas no change was observed in the affinity constant for TA3-Ha cells after a similar treatment. Con A is known to bind to α-D-mannopyranosyl residues in carbohydrate chains linked to protein by N–glycosyl linkages[24]. As shown in Table II, the binding of con A was specifically inhibited by methyl α-D-mannopyranoside.

The binding of ^{125}I-labelled ESA to the TA3 sublines was specifically inhibited by blood-group substance A + H (see Table II). Treatment by neuraminidase decreased the affinity constant for the TA3-St cell line from 2.2 to 1.1mM, but no change was observed for the TA3-Ha cells after removal of sialic acid (see Fig. 4 and Table I). On the other hand, the number of binding sites that are unchanged by neuraminidase treatment is, in TA3-St cells (3.3×10^6 per cell), a quarter of that in TA3-Ha cells (1.3×10^7 per cell) subjected to the same treatment.

Inhibition, by lectins, of lectin binding. — Table II shows the results of the inhibition, by five unlabelled lectins, of the binding of radiolabelled lectins to the two TA3 sublines, before and after treatment with neuraminidase. In all cases, the binding of each of the three labelled lectins was inhibited by the corresponding, nonlabelled lectin. Con A enhanced the binding of ESA, and RCA enhanced the binding of con A. It is suggested that these results may be due to the binding of con A to the carbohydrate component(s) of ECA and RCA. The binding of con A to a different lectin, namely, phytohemagglutinin (PHA), was previously reported[23].

Agglutination of cells by lectins. — The agglutinabilities of the TA3-Ha and

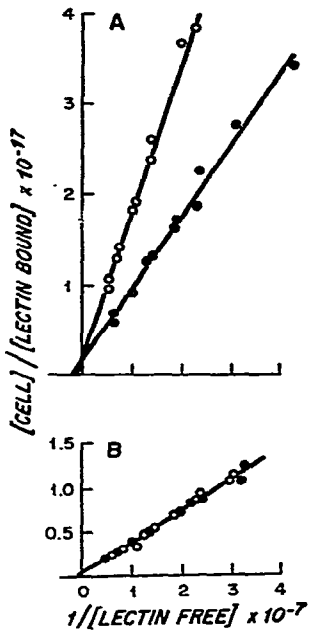

Fig. 4. Binding of ^{125}I-labelled ESA to TA3-St cells (A), and TA3-Ha cells (B). A procedure similar to that described in the legend to Fig. 1 was followed.

TABLE III

AGGLUTINATION OF TA3 ASCITES CELL-LINES BY LECTINS, AND INHIBITION OF AGGLUTINATION BY EPIGLYCANIN

Lectins	*TA3 cells (2×10^6 cells/mL)*	*Lectin agglutination (μg/mL)*[a]	*Epiglycanin inhibition (μg/mL)*[b]	
			Control	*Neuraminidase-treated*
Con A	St	4	100 ±20	
Con A	Ha	400		
Con A	RBC[c]	4	6 ±4	
RCA	St	2	110 ±20	4 ±1
RCA	Ha	30	5 ±2	5 ±2
ESA	St	600	200 ±50	150 ±50
ESA	Ha	600	>200	>200
WGA	St	8	>50	>50
WGA	Ha	8	15 ±5	>50
STA	St	8	35 ±10	35 ±10
STA	Ha	80	>150	>150

[a]Concentration required for agglutination of 50% of the cells. [b]Concentration required for inhibition, at 60 min, of the agglutination of 50% of the cells. [c]Rabbit erythrocytes at a concentration of 3×10^7 cells/mL.

TA3-St ascites cells by five lectins are shown in Table III. The observation of Friberg[1] on the difference in susceptibility of the two sublines to agglutination by con A was confirmed. Of the TA3-St cells, 50% were agglutinated at a concentration of only 4 μg of lectin/mL, the same concentration that resulted in the agglutination of rabbit erythrocytes. The TA3-Ha cell, however, showed no agglutination at 100 times the concentration. With two other lectins, STA and RCA, the TA3-St cell also exhibited far more agglutinability (at a 10- and 15-fold lower lectin concentration, respectively) than the TA3-Ha cell. However, the two cell lines exhibited an equal capacity to agglutinate with either WGA or ESA, only 8 μg of WGA/mL being required.

Inhibition, by epiglycanin, of the agglutination of cells by lectins. — Epiglycanin or asialoepiglycanin was effective in inhibiting the agglutination of one, or both, of the TA3 sublines by each of the lectins studied (see Table III). The effectiveness of epiglycanin in inhibiting the agglutination both of TA3-St cells and rabbit erythrocytes by con A was surprising, as the presence of an N–glycosyl-linked, carbohydrate chain in epiglycanin had not been demonstrated, but the presence of such a chain had been suggested by the detection of ~0.3% of D-mannose in all epiglycanin samples studied[8]. Although this D-mannose content suggests the presence of only ~1 D-mannose per 100 D-galactose residues, this content was nevertheless considered significant. The presence of an N–glycosyl-linked, carbohydrate chain was further demonstrated by adsorbing, on a column of con A–Sepharose 4B, a sample of protease-cleaved epiglycanin similar to that used in the inhibition experiments (see Table III), and washing the column with water. After incubation with neuraminidase, fractions were monitored for their content of free sialic acid[25]. The results show that ~60% of the bound sialic acid placed on the column was eluted with water. Subsequent elution with methyl α-D-mannopyranoside gave fractions that were also monitored for sialic acid but, in this instance, each fraction was exhaustively dialyzed against water to remove all mannoside prior to testing for sialic acid. The results suggest that ~40% of the epiglycanin was specifically bound to the column. The carbohydrate and amino acid compositions of the two fractions appear to be similar[26].

Undegraded epiglycanin was a potent inhibitor of agglutination by WGA of the TA3-Ha cell, but not of the TA3-St cell, whereas asialoepiglycanin was not an inhibitor of either cell.

Neuraminidase-treated or -untreated epiglycanin had no effect on the agglutination of TA3-Ha cells by STA, whereas the TA3-St cells (which are more readily agglutinated by this lectin) were inhibited equally well by epiglycanin and asialoepiglycanin.

Both cell-lines were agglutinated by ESA, but only the agglutination of the TA3-St cells was inhibited by epiglycanin. This inhibition was weak, and 150 μg of asialoepiglycanin/mL was required before an inhibition of 50% was observed.

DISCUSSION

No correlation appears to exist between the number of receptor sites for each

lectin at the surfaces of either the TA3-Ha or TA3-St cell (see Table I) and the relative agglutinabilities (see Table III) of the cells by the five lectins studied. Indeed, both cell-lines were found to possess large numbers of receptor sites for the three labelled lectins, namely, con A, RCA, and ESA (see Table I). The complexity of the agglutination phenomenon, and some of the factors that may influence agglutination, have been discussed by others[27].

The failure of the TA3-Ha cells to be agglutinated by con A, even at high concentrations of the lectin (see Table III), and their failure to be agglutinated, except at 10–15-fold higher concentrations than required, for TA3-St cells, by the two other lectins, RCA and STA, may be explained by two unusual, topographical features. Electron microscopy has demonstrated that the TA3-Ha cell possesses a great number of regularly spaced, long, thin microvilli at its surface, in contrast to the irregularly spaced bridges and folds present at the surface of the TA3-St cell[28]. Perhaps more important is the presence at the surface of the TA3-Ha cell, but not at the surface of the TA3-St cell, of $\sim 4 \times 10^6$ large, endogenous glycoprotein (epiglycanin) molecules in extended conformation[9,10]. The similar agglutinabilities of the two cell-lines by the two other lectins investigated, namely, ESA and WGA, serve, however, as a reminder of the complexities of the mammalian cell-surface.

Epiglycanin, the preponderant glycoprotein of the TA3-Ha cell-surface, was found to inhibit agglutinations by the five lectins studied. This finding suggests that this glycoprotein may contribute receptors for these lectins at the surface of the cell. The surprising observation that epiglycanin inhibited the agglutination by con A both of TA3-St cells and rabbit erythrocytes gives evidence for the presence of a receptor for con A in epiglycanin. No conclusive evidence for the presence of an asparagine-linked chain having α-D-mannopyranose-containing receptors[24] had been demonstrated earlier. Further evidence for the occurrence of at least one, and possibly two, asparagine-linked chains in epiglycanin was the observation that a large fraction of epiglycanin, which was cleaved from the cell surface by a modified trypsin[8], was specifically bound to a con A–Sepharose 4B column. As both cell-lines were found to possess similar numbers of con A receptors (see Table I), such receptors, if present, must occur in the TA3-St cell in glycoproteins unrelated to epiglycanin.

Because epiglycanin was reported[10] to be a potent inhibitor of hemagglutination by RCA, the finding that this glycoprotein can inhibit the agglutination of both cell-lines by RCA was expected. After reductive cleavage by alkali–borohydride reduction, isolation of the individual, O–glycosyl-linked chain-types in epiglycanin suggested that $\sim 5\%$ of the chains may contain a β-D-Gal(1→4)-β-D-GlcNAc terminal (nonreducing) group, which is a probable receptor[29] for RCA, and an additional 3% of the chains contain this sequence in penultimate position to a terminal (nonreducing) NeuAc group. The strong inhibition of agglutination of the TA3-St cell by epiglycanin, particularly after neuraminidase treatment, suggests the presence of similar linkages at the cell surface of that cell line.

It has been reported[27] that internal 2-acetamido-2-deoxy-D-glucose or di-*N*-acetylchitobiose residues may serve as receptors for both STA and WGA. The

present results (see Table III), however, show markedly different patterns of inhibition by epiglycanin for the two lectins. No inhibition of WGA agglutination of TA3-St cells was observed with epiglycanin, either before or after removal of NeuAc. With the TA3-Ha cell, however, epiglycanin bearing terminal NeuAc groups, but not asialoepiglycanin, was an effective inhibitor of agglutination by WGA. Contrariwise, removal of sialic acid did not change the inhibitory activity of epiglycanin to STA-induced agglutination of either TA3-St or TA3-Ha cells. These results are consistent with the recent report of Bhavanandan and Katlic[30] on the role of sialic acid as a receptor in agglutination by WGA.

The possibility that carbohydrate chains in epiglycanin may contain fucose residues is suggested by the results with ESA. Both cell-lines were agglutinated with the same concentration of ESA (see Table III), although the TA3-Ha line was shown to possess 4 times as many receptor sites for this lectin as the TA3-St cell. Furthermore, binding of ESA to each cell was inhibited by A + H blood-group substance (see Table II). As the receptor for ESA exhibits H blood-group specificity, and presumably contains one fucose residue, it was interesting to consider the possibility that epiglycanin does possess H blood-group specificity, and that the greater number of sites in the TA3-Ha cell is due to epiglycanin. Although no inhibitory activity was detected against ESA-promoted agglutination of TA3-Ha cells, weak, but significant, activity was exhibited by both epiglycanin and asialoepiglycanin in the inhibition of agglutination of TA3-St cells (see Table III). No fucose has been detected in epiglycanin, and yet, H activity would require only one fucose residue per receptor site. The presence of as many as two fucose residues in a single molecule of epiglycanin of mol. wt. ~500,000 would constitute only 0.06% of the weight of the glycoprotein, a proportion not usually detectable by g.l.c.[31]. Two such receptors per epiglycanin molecule could account for 8×10^6 receptors per TA3-Ha cell, a value consistent with the approximately 11×10^6 more receptors found on the TA3-Ha than on the TA3-St cell-surface (see Table I).

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