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SACCHARIDES ON TERATOCARCINOMA CELL PLASMA MEMBRANES THEIR INVESTIGATION WITH RADIOACTIVELY LABELLED LECTINS

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SUMMARY

We have studied the interaction of five lectins differing in their sugar specificity, with the surface of clonal cell lines derived from transplantable murine teratocarcinoma. The results show that the differentiation from primitive embryonal carcinoma cells into parietal yolk sac cells is accompanied by changes in cell surface saccharides. These changes consist of a marked decrease in the total number of binding sites for the L-fucose-specific lectin of *Lotus tetragonolobus* and a large increase in the total number of binding sites for wax bean agglutinin. It is suggested that these differences can be used as markers in the study of this early embryonic differentiation. No agglutination of primitive embryonal carcinoma cells or of parietal yolk sac cells by low concentrations (10 μg/ml) of concanavalin A, soybean agglutinin or the fucose binding proteins was observed.

INTRODUCTION

Murine testicular teratomas are tumors of the 129 mice, defined by the proliferation of malignant primitive stem cells (known as embryonal carcinoma and resembling early embryonic cells) and of differentiated cell types deriving from these same stem cells (reviewed in ref. 1). Various cell lines have been established in vitro from transplantable teratomas. Some of them, the primitive teratocarcinoma cell lines (PTC), have retained the capacity of the original embryonal carcinoma to differentiate into most types of embryonic tissues, either in vivo or in vitro. These cell lines of embryonal carcinoma are thus a convenient alternative to early normal embryos in the study of the processes of early differentiation (reviewed in refs. 2 and 3). On the other hand, several differentiated cell lines derived from teratomas have also been established, and have been histologically defined as myoblasts [4], parietal yolk sac [5] and embryonal endoderm lines [6]. Up until now, the emphasis has been placed on the immunological similarities between cells of these primitive and differen-

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tiated lines, and cells of normal embryos. Antisera were prepared in syngeneic 129 mice against several primitive and differentiated cell types. The use of these antisera has led to the description of several cell surface antigens of restricted distribution that are also expressed on embryonic cells at certain stages of their normal development (refs. 6 and 7, and reviewed in refs. 2, 3 and 8).

In addition to these immunologically defined cell surface markers, there must exist at the surface of embryonic cells other molecules recognized as "self" by the immunized syngeneic animals, which could be used as markers in the description of the cell surface properties of both teratocarcinoma and embryonic cells. The present experiments were undertaken to examine the interaction of various lectins with the surface of three clonal cell lines derived from teratocarcinoma, in an attempt to identify such additional markers. The cell lines used in this study were F9-41 [9] a clonal line of embryonal carcinoma which has lost the ability to differentiate; PCC3, a clonal line of embryonal carcinoma capable of differentiating in vitro and in vivo [10, 11] Pys-2, a differentiated clonal line which resembles parietal yolk sac cells and thus corresponds to one of the first differentiations seen in the embryo [5].

Five purified lectins with different sugar specificities were used: concanavalin A (specific for D-glucose and D-mannose), fucose binding protein (L-fucose), soybean agglutinin (*N*-acetyl-D-galactosamine and D-galactose), wax bean agglutinin (fetuin glycopeptides) and wheat germ agglutinin ($\beta 1 \rightarrow 4$ linked oligosaccharides of *N*-acetyl-D-glucosamine, reviewed in ref. 12). The number of sites on the cell surface interacting with each of these lectins has been determined and some properties of the lectins-receptor interaction described. Agglutination of these cells by the lectins was also examined.

MATERIALS AND METHODS

Cell lines. (a) F9 was isolated from embryoid bodies of the teratocarcinoma OTT 6050 [9]. Clone F9-41 used here is a PPLO-free line reisolated from a tumor issued of F9 [13]; it is propagated as a culture of embryonal carcinoma cells; it is unable to differentiate in vivo.

(b) PCC3 was established from embryoid bodies of the same tumor [10]. As for all the PCC lines, this cell line has retained the ability to differentiate in vivo [10] and even in vitro [11].

(c) Pys-2 is a line derived from the transplantable teratocarcinoma OTT 6050 and consists of non-malignant parietal yolk sac cells. It produces in vitro large amounts of a polysaccharide material resembling the Reichert's membrane [5].

Cell cultures. Cells were grown on tissue culture dishes (Falcon, coated with gelatin in the case of F9) in Dulbecco's modified Eagle's medium supplemented with 15 % fetal calf serum (Gibco) [10]. Cells in their log-phase were removed from the plates with EDTA as previously described [10] and collected by centrifugation. The glycocalyx is removed by this EDTA treatment (Monneron, unpublished results). The cells were washed twice with phosphate-buffered saline containing 0.2 % bovine serum albumin, pH 7.2 (25 °C, $300 \times g$, 5 min). The washed pellet was resuspended in the same medium at a concentration of $2 \cdot 10^7$ cells/ml. The cells were found to be more than 85 % viable by trypan blue exclusion.

Lectins. Mixture of iso-fucose binding proteins [14], soybean agglutinin [15],

wax bean agglutinin [16] and wheat germ agglutinin [17] were prepared according to methods described in the literature. Concanavalin A was purchased from Sigma. All lectins were stored in lyophilized form at room temperature.

Radioactive labelling of lectins. Lectins were labelled enzymatically with ^{125}I. The lectin (5 mg) was dissolved in 1 ml of sodium phosphate buffer (0.05 M, pH 6.5). To the solution were added 2 μg lactoperoxidase (Sigma), 1 μg glucose oxidase (Böhringer), $1.5 \cdot 10^{-4}$ M KI and $30 \cdot 10^6$ cpm $Na^{125}I$ (C.E.N. Saclay, France). The reaction was initiated by the addition of glucose (100 μg) and allowed to proceed for 30 min at room temperature. It was stopped by the addition of sodium metabisulfite. Radioiodinated lectins were then dialysed against three changes of 1 l of phosphate-buffered saline. The radiolabelled lectins were kept frozen until used. They were centrifuged before use in a Beckman microfuge (maximum speed, 2 °C, 10 min). Radioactivity measurements were performed in an Intertechnique SL 30 scintillation spectrometer, using Triton-toluene scintillant (5.5 g 2.5-diphenyloxazole, 0.1 g 1,4-bis-(5-phenyloxazole-2)-benzene, 667 ml toluene and 333 ml Triton X-100). Specific radioactivities were: concanavalin A: $4.1 \cdot 10^6$ cpm/mg: fucose binding agglutinin: $5.5 \cdot 10^6$ cpm/mg; soybean agglutinin: $5.5 \cdot 10^5$ cpm/mg; wax bean agglutinin: $5.0 \cdot 10^6$ cpm/mg; wheat germ agglutinin: $5.3 \cdot 10^6$ cpm/mg.

Protein concentrations were determined according to Lowry et al. [18]. Homogeneity of the labelled proteins was checked by polyacrylamide gel electrophoresis at pH 8.9 [19]. In all cases, protein and radioactivity co-migrated on the gels.

Binding of lectins to the cells. The cell suspension (10^6 cells in 50 μl) was pipetted into a Beckman plastic microfuge tube and centrifuged (room temperature, 3 min, $450 \times g$). The pellet was suspended in 100 μl of lectin solution (0.1–500 μg/ml) in phosphate-buffered saline containing 0.2 % bovine serum albumin in the presence or absence of competing sugar (for fucose binding proteins, L-fucose, 10^{-2} M; soybean agglutinin, *N*-acetyl-D-galactosamine, $5 \cdot 10^{-3}$ M; wax bean agglutinin, fetal calf serum, 4 %; wheat germ agglutinin, *N*-acetyl-D-glucosamine, $5 \cdot 10^{-2}$ M and for concanavalin A, α-methyl-D-mannoside, $5 \cdot 10^{-3}$ M). The suspension was incubated for 30 min at 25 °C with occasional shaking. After incubation, the cells were washed three times in the same tube with phosphate-buffered saline containing 0.2 % bovine serum albumin. The washed pellet was resuspended in 50 μl of phosphate-buffered saline and the resulting suspension transferred quantitatively into a counting vial. The microfuge tube was washed again with the same volume of phosphate-buffered saline and this solution added to the same counting vial.

The amount of specifically bound lectin is the difference between the amount bound in the absence and presence of the competing sugar.

Data processing. Binding data were analyzed according to Scatchard [20]. The amount of free lectin was measured on aliquots of the supernatant following removal of cells by centrifugation. Total lectin was estimated by counting an aliquot of the cell suspension before centrifugation and washing. The maximum number of bound lectin molecules was extrapolated from the Scatchard's plot. The molecular weights used for calculation were: fucose binding proteins, 120 000; soybean agglutinin, 120 000; wax bean agglutinin, 110 000; wheat germ agglutinin, 36 000; concanavalin A, 110 000. The mean surface area of the cells was estimated from their mean diameter, measured with a micrometric ocular at 400 × magnification. It was, respectively, 530 μm^2 for F9 cells, 315 μm^2 for PCC3 cells and 855 μm^2 for Pys-2 cells.

RESULTS AND DISCUSSION

In a series of preliminary experiments, the time course of binding of the lectins to F9 and to Pys-2 cells was examined. At 25 °C, about 85–95 % of the maximal binding for a given lectin, at concentrations ranging from 1 to 100 μg/ml, was attained after 1 min. Binding was nearly complete after 15 min. However, all subsequent experiments were performed using 30-min incubations, conditions which allow

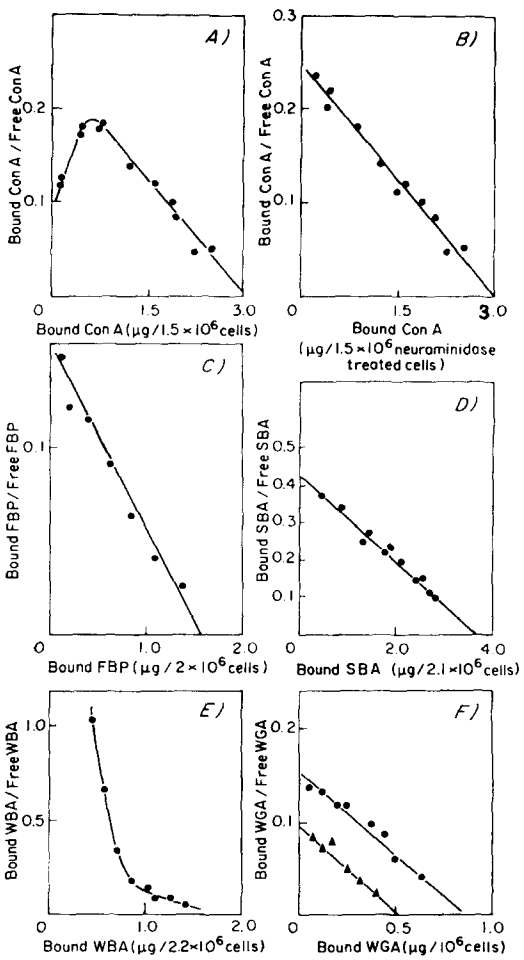

Fig. 1. Scatchard's plots of binding of ^{125}I-labelled lectins to F9-41 cells. The binding of concanavalin A, fucose binding proteins, soybean agglutinin, wax bean agglutinin and wheat germ agglutinin to F9-41 cells was performed as described in Materials and Methods, except in B: the washed F9 cells were resuspended in phosphate-buffered saline-bovine serum albumin, pH 6.5, at a density of $5 \cdot 10^7$–$7 \cdot 10^7$ cells/ml. To 800 μl of cell suspension were added 70 μl of neuraminidase (Influenza virus neuraminidase, 5.77 I.U./ml, Calbiochem). After 30 min at 37 °C, the cells were washed twice by centrifugation, suspended in phosphate-buffered saline-bovine serum albumin, pH 7.0, counted and used in binding experiments. In F, —▲— corresponds to the amount of wheat germ agglutinin which is not removed by the proper competing sugars.

comparison with the antibody binding experiments performed on the same cells.

The association constants (K_a) and the total number of molecules bound were derived from the Scatchard's plots (see typical examples in Fig. 1). The values obtained for F9, PCC3 and Pys-2 are listed in Tables I, II and III, and summarized in Table IV. Examination of the results shows that the binding constants vary from $0.5 \cdot 10^6$ to $17 \cdot 10^6$, and are within the range commonly found with different lectins for many other cell types (reviewed in ref. 21). No consistent pattern can be seen for these values. In several cases, the labelled lectins could not be entirely removed from the cell surface by high concentrations of the competing monosaccharide. This binding is a specific one, since it obeys saturation kinetics; it is likely reflecting heterogeneities in

TABLE I

BINDING OF LECTINS TO F9-41 CELLS

Lectin tested	K_a ($\cdot 10^{-6}$)	Maximum number of molecules bound per cell		Cooperativity at low lectin concentration	Agglutinability (at 10 μg/ml of lectin)
		Removable by competing sugars	Not removable by competing sugars		
Concanavalin A	5	$1.1 \cdot 10^7$	n.d.	+	0
Fucose binding proteins	0.8	$7.8 \cdot 10^6$	n.d.	0	0
Soybean agglutinin	12	$9.5 \cdot 10^6$	n.d.	0	+/−
Wax bean agglutinin	(a)	$5.5 \cdot 10^6$	n.d.	?	+++
Wheat germ agglutinin	2.6	$8.7 \cdot 10^6$	$5.5 \cdot 10^6$	0	++++

(a) Not measurable due to the heterogeneity of the population of binding sites.
n.d., not detectable.

TABLE II

BINDING OF LECTINS TO PCC3 CELLS

Lectin tested	K_a ($\cdot 10^{-6}$)	Maximum number of molecules bound per cell		Cooperativity at low lectin concentration	Agglutinability (at 10 μg/ml of lectin)
		Removable by competing sugars	Not removable by competing sugars		
Concanavalin A	17	$7.7 \cdot 10^6$	n.d.	0	0
Fucose binding proteins	(a)	$2.2 \cdot 10^6$	$1 \cdot 10^6$	0	0
Soybean agglutinin	(a)	$2 \cdot 10^6$	n.d.	0	+/−
Wax bean agglutinin	11	$2.1 \cdot 10^7$	−	+	++++
Wheat germ agglutinin	(a)	$6 \cdot 10^6$	$4.8 \cdot 10^6$	+	++++

(a) Not measurable due to the heterogeneity of the population of binding sites.
n.d., not detectable.

TABLE III

BINDING OF LECTINS TO Pys-2CELLS

Lectin tested	K_a ($\cdot 10^{-6}$)	Maximum number of molecules bound per cell		Cooperativity at low lectin concentration	Agglutinability (at 10 μg/ml of lectin)
		Removable by competing sugars	Not removable by competing sugars		
Concanavalin A	4.1	$4 \cdot 10^7$	n.d.	0	0
Fucose binding proteins	-	0	$3 \cdot 10^{5}$[b]	-	0
Soybean agglutinin	8.5	$4.1 \cdot 10^7$	n.d.	+	+/-
Wax bean agglutinin	(a)	$7.5 \cdot 10^7$	-	0	+++
Wheat germ agglutinin	1.6	$2.4 \cdot 10^7$	$0.9 \cdot 10^7$	+	+/-

(a) Not measurable due to the heterogeneity of the population of binding sites
(b) Presumably not specific background; increases linearly with lectin concentrations.
n.d., not detectable.

TABLE IV

SUMMARIZED CHARACTERISTICS OF LECTINS BINDING TO F9, PCC3 AND Pys-2 CELLS

Lectins	K_a ($\cdot 10^{-6}$)			Number of molecules bound/μm^2 ($\cdot 10^2$)		
	F9-41	PCC3	Pys-2	F9-41	PCC3	Pys-2
Concanavalin A	5	17	4.1	21	25	47
Fucose binding proteins	0.8	(a)	-	14	7.4 (T = 10)	0
Soybean agglutinin	12	(a)	8.5	17	6.4	48
Wax bean agglutinin	(a)	11	(a)	10	67	88
Wheat germ agglutinin	2.6	(a)	1.6	16 (T = 26)	19 (T = 35)	28 (T = 38)

(a) Not measurable due to the heterogeneity of the population of binding sites.
T, specifically + non-specifically bound molecules.

the structure of lectin binding sites. However, examination of the number of lectin binding sites per unit of surface area (which takes into account the marked differences in cell size) revealed several important points: (a) the total number of wheat germ agglutinin and concanavalin A binding sites for the three cell lines is very similar (ranging from 21 000 to 47 000 molecules/μm^2); (b) the number of soybean agglutinin binding sites is markedly lower on the PCC3 cells than on the other cell lines; (c) PCC3 and Pys-2 cells have a similar number of wax bean agglutinin binding sites on F9 cells; (d) fucose binding proteins binding sites are absent on Pys-2 cells.

The last finding is of particular interest, since it indicates that differentiation of early embryonic cells, of which F9 is a model, into early endodermal derivatives, such

as Pys-2, is accompanied by large changes of cell surface carbohydrates. The absence of binding of fucose binding proteins to Pys-2 cells strongly suggests that in these cells the surface saccharides are devoid of terminal L-fucose residues. This absence may be due to several causes, such as a loss of a fucosyltransferase, loss of the proper receptor for the transferase or increased fucosidase activity. Another possibility is the loss of a complete glycoprotein. Whatever the cause of this change is, it may be one of the events accompanying the differentiation into this specific cell type and thus might be used as a marker in the study of early differentiation.

In some cases (concanavalin A with F9 cells; wheat germ agglutinin and wax bean agglutinin with PCC3 cells, etc.) clear evidence for cooperativity of binding at low lectin concentrations has been obtained. For the binding of concanavalin A to F9 cells this effect was abolished by treatment of the cells with neuraminidase, although the total number of concanavalin A molecules bound was not affected by such treatment. Cooperativity has been noted previously in the binding of soybean agglutinin to erythrocytes [22] and of concanavalin A to rat lymphocytes [23], but the biological meaning of this phenomenon is still not clear.

We have also studied the patterns of agglutination of the teratocarcinoma cells by lectins (Tables I, II, III). A low concentration was used (10 μg/ml), since many, if not all, biological effects of the lectins are expressed at low occupancy of sites [21–23]. It can be seen that early embryonic cells, although highly tumorigenic are not agglutinated by low concentrations of concanavalin A, soybean agglutinin and fucose binding proteins; by contrast, they are strongly and specifically agglutinated by wax bean agglutinin and wheat germ agglutinin. Pys-2 cells behave in a very similar way, except that it is poorly agglutinated by wheat germ agglutinin. Thus, the PTC cells and their derivatives do not behave like "typical" tumor cells. This is noteworthy, especially in view of the fact that the highly malignant embryonal carcinoma cells possess the unusual property to be able to become somewhat "normalized", in a quasi normal differentiation process [24, 25], a property which makes them very similar to normal embryonic cells.

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