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Use of lectins for probing differentiated human embryonic stem cells for carbohydrates

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The carbohydrates present on the surface of differentiated human embryonic stem cells (hESCs) are not yet well established. Here, we have employed a panel of lectins and several anti-carbohydrate antibodies to determine the carbohydrates that are present at day 12 of hESC differentiation as embryoid bodies (EBs). On the basis of staining with fluorescein-labeled lectins, we have determined the presence of both terminal and internally linked α-D-mannopyranosyl groups, poly-*N*-acetyllactosaminyl chains, both α2,3- and α2,6-linked *N*-acetylneuraminic acid (Neu5Ac), α1,6-linked L-fucosyl, and β-D-galactosyl groups, and more specifically, the T, Tn, and sialyl-Tn antigens. However, no α1,2-linked L-fucosyl, terminal nonreducing α-D-galactosyl, *N*-acetyl-β-D-glucosaminyl, nor *N*-acetyl-α-D-galactosaminyl groups were found by this approach. We also established the presence of Neu5Acα2,3/2,6-Galβ1,4 GlcNAc-terminated chains on the surfaces of 12-day-old EBs, as indicated by the great enhancement of staining by *Erythrina cristagalli* agglutinin (ECA) after treatment with neuraminidase. In each case, inhibition of binding by a haptenic sugar or treatment with neuraminidase was used to eliminate the possibility of nonspecific binding of the lectins. A comparison with undifferentiated cell staining revealed an increase in α2,3-linked Neu5Ac as well as a change to exclusively α1,6-linked L-fucose upon differentiation.

Key words: carbohydrates/differentiation/embryoid body/human embryonic stem cells/lectin

Introduction

Cell surface carbohydrates are importantly involved in cell–cell interaction and communication, cell adhesion, and many developmental processes. The question of the function of cell surface glycans during the early development of organisms is an important area for investigation as these glycans may play a role in differentiation (Haltiwanger and Lowe, 2004). Several studies have identified antigenic glycoproteins and glycolipids in the earliest development of the embryo. Two of these antigens, SSEA-3 and SSEA-4, have been identified as appearing during the four- to eight-cell stage of mouse embryos and human teratocarcinoma cell lines (Kannagi *et al*., 1982; Shevinsky *et al*., 1982). Two obvious tools with which to identify and map cell surface carbohydrates are lectins and antibodies. These probes, used with appropriate controls (blocking sugar ligands and glycosidases), can provide important information on the presence and changes during development of sugar residues on the surface of stem cells and differentiating embryoid bodies (EBs). A recent study on undifferentiated human embryonic stem cells (hESCs) used lectins to establish the presence of sugar residues on the surface of BG01 and BG02 undifferentiated hESCs (Venable *et al*., 2005). The present work employs many more lectins and carbohydrate antibodies to identify the presence of carbohydrates on the surface of differentiating EBs. Using the same methodology, we compared our results on differentiated EBs with those on undifferentiated cells.

Results

We employed 34 fluorescein-labeled lectins[1] (Goldstein and Poretz, 1986; Goldstein *et al*., 1997; Sharon and Lis, 2003) and several monoclonal anti-carbohydrate antibodies to assess carbohydrates expressed by EBs. In all cases, we established specificity of lectin and antibody binding with the use of specific carbohydrate inhibitors of binding and/or specific glycosidases, for example, neuraminidase to abolish binding by the elderberry bark lectin (*Sambucus nigra* agglutinin, SNA). Table I summarizes the lectins and antibodies used in this study along with data for the conditions employed in their use such as concentration used for staining and sugar haptens and glycosidases used to establish specificity of lectin binding.

High amounts of α-mannopyranosyl[2] groups are present as indicated by strong staining with concanavalin A (Con A) and the banana lectin (BanLec, *Musa acuminata*) (Figure 1) and moderate staining by the snowdrop bulb lectin (*Galanthus nivalis* agglutinin, GNA). All recognize terminal nonreducing α-mannosyl groups, whereas Con A and BanLec also recognize glucose and the internal branched Man_3 core trisaccharide (Man α-1,6[Man α1–3] Man) of *N*-linked glycans. The BanLec also can interact with internal α1,3-linked Man groups. In all cases, lectin binding was abolished in the presence of the specific inhibitor, methyl α-mannopyranoside.

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[1]The authors have not provided references for the carbohydrate-binding specificity of each lectin employed in this article. Rather they have cited several monographs, which provide the information on the carbohydrate-binding properties of the lectins employed in this study. When appropriate, citations that provide more recent or relative information are noted.
[2]All carbohydrates reported in this article are of the D-configuration except for fucose, which is of the L-configuration.

Table I. Data regarding the lectins and antibodies used in this study

Lectin	Binding specificity	Concentration (μg/mL)	Percentage of EBs with stain[a] (%)	Intensity (scale 1–5)[b]	Minimum agglutinating concentration (μg/mL)	Blocking agent(s)	Enhancing enzyme
α Man							
BanLec	Terminal α Man, Man α3[Man α6] Man -3-0-α-Man	10	100	4	60–75	0.4 M Me α Man	
Con A	Terminal α Man, Man α3[Man α6] Man	10	100	4	<1.5	0.4 M Me α Man	
GNA	Terminal α Man	10	34	3	8	0.4 M Me α Man	
α GalNAc							
DBA	GalNAc α1,3 GalNAc/Gal	20	0	0	12.5–25		
HPA	αGalNAc (terminal)	20	0	1	<6		
LBL	GalNAc α(1,3)[L-fuc α(1,2)]Gal, GalNAc	25	0	0	>2000		
SBA	Terminal α GalNAc and Tn antigen	10	76	4	25–50	50 mM GalNAc	
α Gal							
GS I-B_4	Terminal αGal	20	0	1	30–60		
MOA[c]	Gal α(1,3) Galβ1,4GlcNAc/Glc and terminal LacNAc	20	26	2	5	20 mM Gal α1,3 Gal	Neuraminidase
Neu5Ac							
LFA	Neu5Ac	30	100	4	8–16	0.2 M Neu5Ac, neuraminidase	
LPA + calcium	Neu5Ac	35	100	3	?	0.2 M Neu5Ac	
MAA	Neu5Ac α(2,3)Gal	10	89	4	125–500	50 mM α2,3 sialylactose	
PSL	Neu5Ac α 2,6Gal β 1,4 GlcNAc/Glc	20	100	4	<6	0.5 M Lactose, neuraminidase	
PVL[c]	Neu5Ac α2,6-Gal, GlcNAc	1	33	5	<4	0.4 M GlcNAc, neuraminidase	
SNA	Neu5Ac α2,6Gal or α2,6GalNAc	5	100	5	3–6	20 mM Human milk sialyllactose, neuraminidase	
WGA[c]	(Neu5Ac) (Gal β4GlcNAc)$_{1–3,4}$ (GlcNAc β4GlcNAc)$_{1–3,4}$	1	100	5	<0.3	250 mM LacNAc, neuraminidase	
LacNAc							
DSA	Terminal LacNAc	20	100	3	<4	40 mM Chitotriose	Neuraminidase
ECA	Terminal LacNAc	10	32	4	3–6	250 mM LacNAc	Neuraminidase
LEA	Three consecutive LacNAc residues	20	91	4	<6	40 mM Chitotriose	
MOA[c]	Gal α(1,3) Gal β1,4GlcNAc/Glc and terminal LacNAc	20	26	2	5	20 mM Gal α1,3 Gal	Neuraminidase
STL	LacNAc	10	82	4	<6	40 mM Chitotriose	
WGA[c]	(Neu5Ac) (Gal β4GlcNAc)$_{1–3,4}$ (GlcNAc β4GlcNAc)$_{1–3,4}$	1	100	5	<0.3	250 mM LacNAc, neuraminidase	
WGA-succ[c]	(Gal β4GlcNAc)$_{1–3,4}$ (GlcNAc β4GlcNAc)$_{1–3,4}$	5	100	5	NA[d]	40 mM Chitotriose	

Table I. continued

Lectin	Binding specificity	Concentration (μg/mL)	Percentage of EBs with stain[a] (%)	Intensity (scale 1–5)[b]	Minimum agglutinating concentration (μg/mL)	Blocking agent(s)	Enhancing enzyme
Fucose							
AAA	α(1,2) L-fucose	25	0	0	250		
AAL	α(1,6) L-fucose	5	100	4	<0.3	0.7 M Fucose	
EEA[e]	Gal α 1,3 [L-Fuc α1,2] Gal β1,3 or 4 GlcNAc	10	49	3	<7		
Lotus	α(1,2) L-fucose (terminal)	35	0	0	<7		
UEA1	α(1,2) L-fucose	30	0	0	250–500		
βGal							
ABA	Gal β(1,3) GalNAc and β Gal	35	71	3	~25	13 mg/mL Benzyl T-antigen	Neuraminidase
ACA	Gal β(1,3) GalNAc	1	83	5	4–8	7 mg/mL Benzyl T-antigen	
IRL	β-Gal (lactose)	10	100	4	8–12	0.5 M Lactose	
PNA	Terminal βGal, Gal β(1,3)GalNAc	20	67	3	<4	0.5 M Lactose	
RCA	Terminal βGal	5	100	4	<0.3	0.5 M Lactose	
β GlcNAc							
GS II	Terminal α or β GlcNAc	25	0	1	NA[d]		
PVL[c]	GlcNAc, Neu5Ac α2,6-Gal	1	33	5	<4	0.4 M GlcNAc	
WGA[c]	(Neu5Ac) (Gal β4GlcNAc)$_{1–3,4}$ (GlcNAc β4GlcNAc)$_{1–3,4}$	1	100	5	<0.3	250 mM LacNAc, neuraminidase	
WGA-succ[c]	(Gal β4GlcNAc)$_{1–3,4}$ (GlcNAc β4GlcNAc)$_{1–3,4}$	5	100	5	NA[d]	40 mM Chitotriose	
PHA-E[e]	(Gal β 1,4 GlcNAc)$_2$ β1,2,6 Man	10	23	4	<6		
PHA-L[e]	Gal β 1,4 GlcNAc β(1,2) Man	10	19	3	125		
Tn antigens							
Antibody	Sialyl-Tn antigen	–[f,g]	100	3	–	Neuraminidase	
Antibody	Tn antigen	–[f,h]	83	4	–		
SBA	Tn antigen and terminal α GalNAc	10	76	4	25–50	50 mM GalNAc	

NA, not available.
[a]A minimum of 20 EBs were counted for this value.
[b]Intensity scale where 5 = very intense and 0 = no stain.
[c]Several of these lectins are listed in more than one location, as they have more than one binding specificity. These include MOA, PVL, WGA, and WGA-succ.
[d]Does not agglutinate red blood cells.
[e]The carbohydrate-binding specificity of PHA-E, PHA-L, and EEA is complex, and although listed as binding to 12-day-old EBs, we are unable to conduct sugar hapten inhibition of binding studies.
[f]Primary antibody was diluted 1:50.
[g]Secondary antibody was diluted 1:100.
[h]Secondary antibody was diluted 1:200.

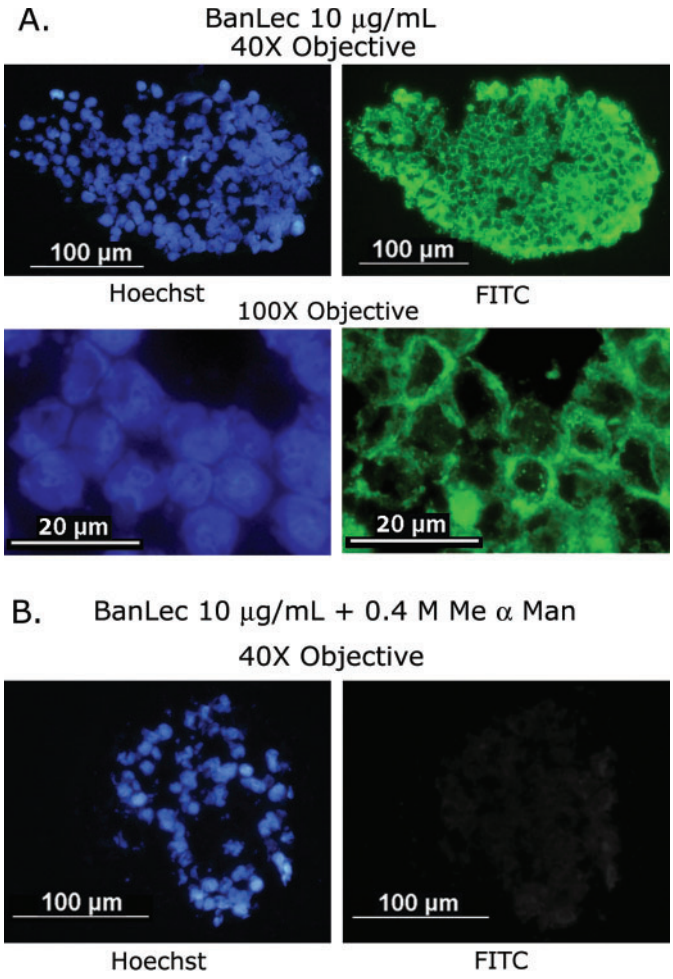

Fig. 1. Effect of methyl α-mannoside on the staining of EBs at day 12 of differentiation by 10 μg/mL fluorescein-labeled BanLec (green), double-stained with 1 × Hoechst nuclear stain (blue). (**A**) No methyl α-mannoside. (**B**) With 0.4 M methyl α-mannoside.

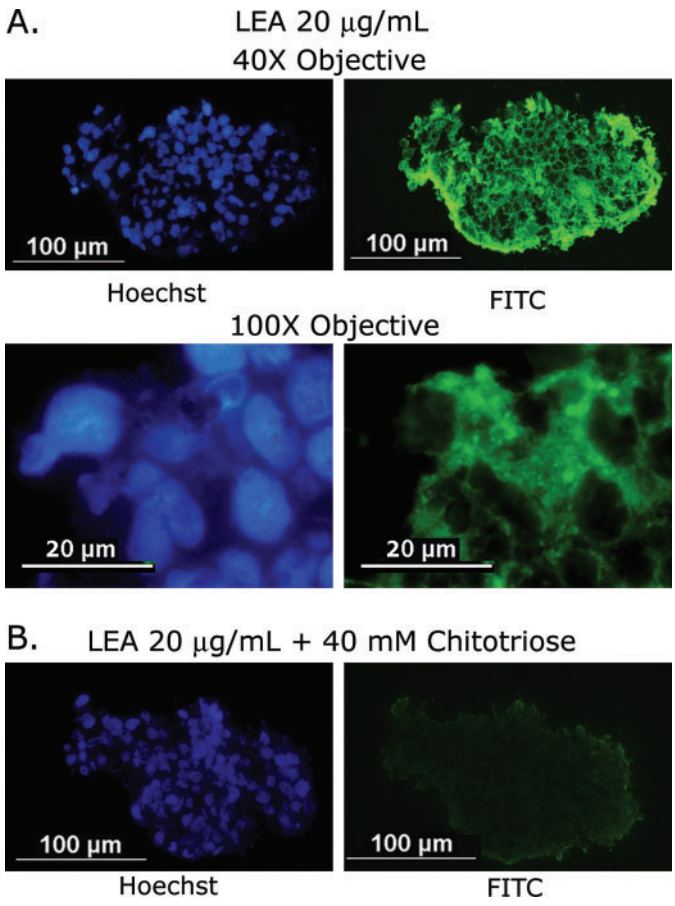

Fig. 2. Effect of *N*,*N*′,*N*″-triacetylchitotriose on the staining of EBs at day 12 of differentiation by 20 μg/mL fluorescein-labeled LEA (green), double-stained with 1 × Hoechst nuclear stain (blue). (**A**) No chitotriose. (**B**) With 40 mM chitotriose.

The presence of poly-*N*-acetyllactosaminyl chains (LacNAc) is clearly demonstrated by the intense staining by fluorescein-labeled *Erythrina cristagalli* agglutinin (ECA), wheat germ agglutinin (WGA, *Triticum vulgaris*), and succinylated WGA (WGA-succ), tomato lectin (*Lycopersicon esculentum* agglutinin, LEA) (Figure 2), potato lectin (*Solanum tuberosum* lectin, STL), and jimson weed lectin (*Datura stramonium* agglutinin, DSA), all of which strongly recognize this carbohydrate epitope. Inhibition of staining using LacNAc or *N*,*N*′,*N*″-triacetylchitotriose established specificity of binding by these lectins. It should be noted that although several of these lectins (e.g., WGA, LEA, and STL) are most often described as GlcNAc-dextrin-binding lectins, they also react with LacNAc and its homologs (Kawashima *et al.*, 1990). Consistent with the presence of terminal *N*-acetyllactosaminyl residues is the staining by *Ricinus communis* agglutinin (RCA) and *Ischnoderma resinosum* lectin (IRL) (Figure 3), both terminal β-galactosyl-recognizing lectins. Staining with ECA was strongly enhanced following treatment with neuraminidase, indicating the presence of Neu5Ac α2,3/2,6-Gal β1,4GlcNAc-terminated chains (Figure 4).

Twelve-day differentiated hESCs exhibit both the Tn (GalNAc α-*O*-Ser/Thr) (Figure 5) and sialyl-Tn (Neu5Ac α-2,6-GalNAc α-*O*-Ser/Thr) (Figure 6) antigens. This is indicated by positive staining with soybean agglutinin (SBA, *Glycine max*) and anti-Tn antibody and by SNA and anti-sialyl-Tn antibody, respectively. Prior treatment of hESCs with neuraminidase abolished the reaction with anti-sialyl-Tn antibody. The presence of the T-disaccharide (Gal β1,3 GalNAc) was shown by staining with the peanut lectin (PNA, *Arachis hypogaea*), amaranthin (*Amaranthus caudatus* agglutinin, ACA), and the *Agaricus bisporus* agglutinin (ABA) mushroom lectin.

The presence of sialic acid is clearly indicated by staining by the following lectins: slug (*Limax flavus* agglutinin, LFA), horseshoe crab (*Limulus polyphemus* agglutinin, LPA), *Maackia amurensis* agglutinin (MAA) (specific for α2,3-linked Neu5Ac), SNA (specific for Neu5Acα2,6Gal/GalNAc) (Figure 7), and *Polyporus squamosus* lectin (PSL), which recognizes sialic acid linked α2,6 to *N*-acetyllactosamine, a trisaccharide epitope that occurs only on *N*-linked chains of glycoproteins. The presence of trisialyl *N*-glycans was indicated by strong staining by the *Psathyrella velutina* mushroom lectin (PVL) (Ueda *et al.*, 2002). Neu5Ac abolished labeling by the slug and horseshoe crab lectins; α2,3-sialyllactosamine

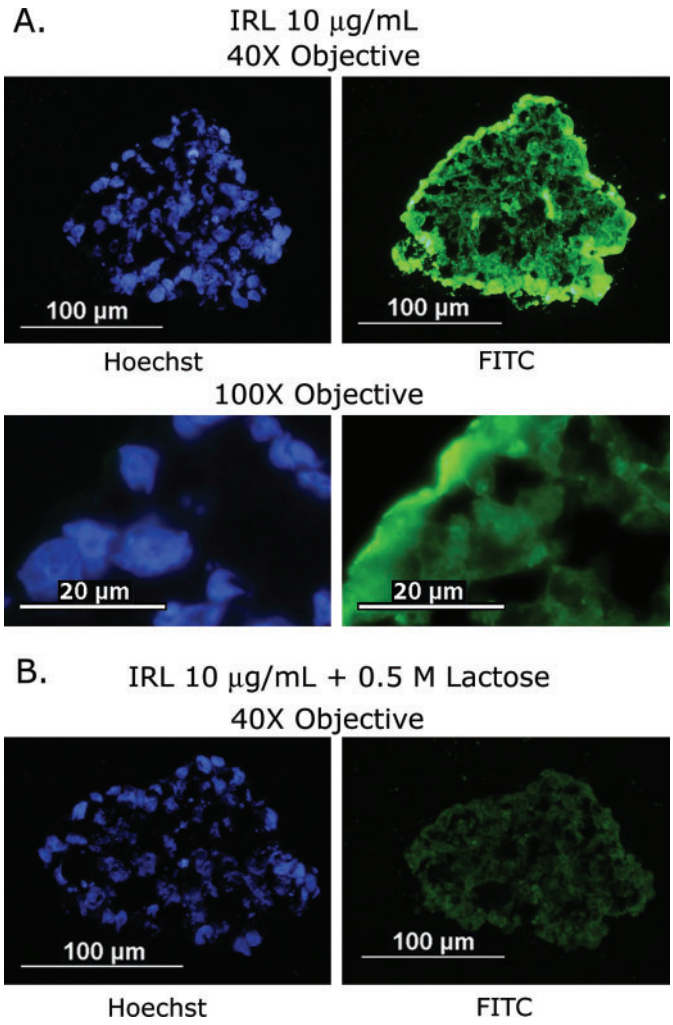

Fig. 3. Effect of lactose on the staining of EBs at day 12 of differentiation by 10 μg/mL fluorescein-labeled IRL (green), double-stained with 1 × Hoechst nuclear stain (blue). (**A**) No lactose. (**B**) With 0.5 M lactose.

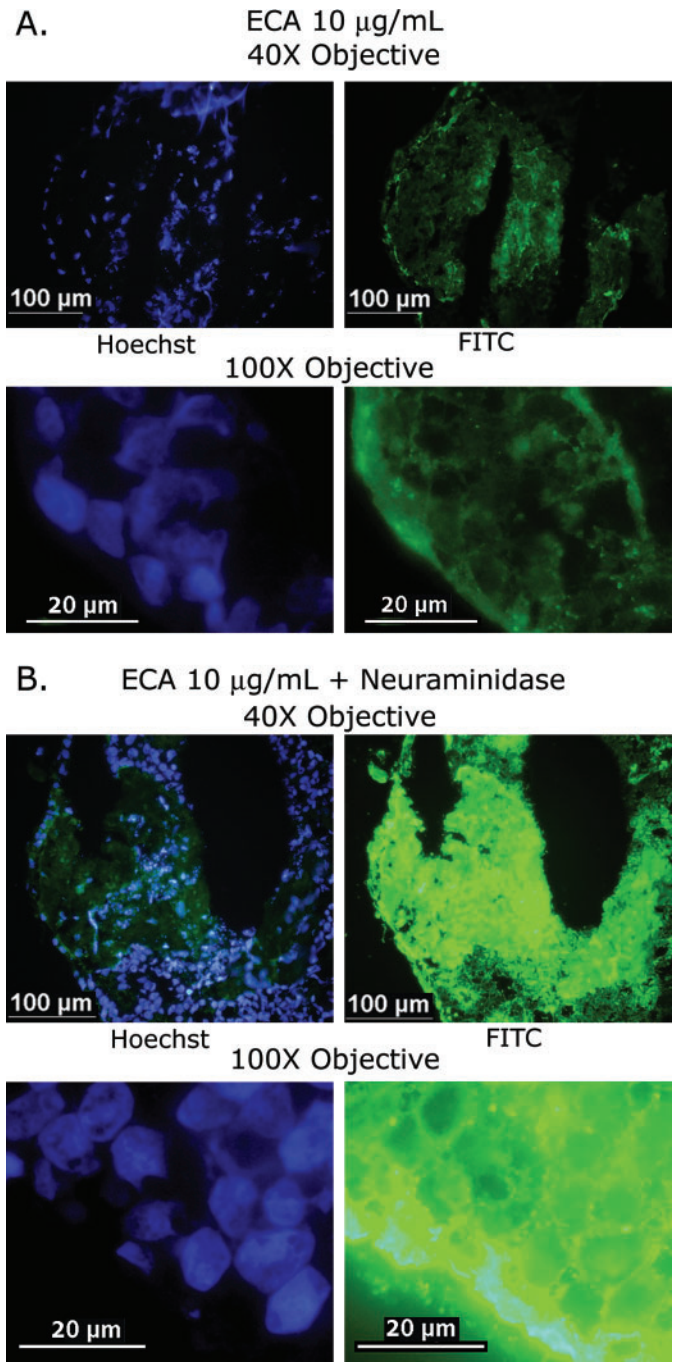

Fig. 4. Effect of neuraminidase treatment on the staining of EBs at day 12 of differentiation by 10 μg/mL fluorescein-labeled ECA, double-stained with 1 × Hoechst nuclear stain (blue). (**A**) No neuraminidase treatment. (**B**) With neuraminidase treatment before lectin staining.

blocked MAA; and the treatment with neuraminidase negated staining by SNA, LFA, PVL, and PSL. Staining by WGA, which also recognizes sialic acid bound to LacNAc, is diminished by treating cells with neuraminidase.

Of many fucosyl-binding lectins assayed for binding to hESCs, only the orange peel fungal lectin (*Aleuria aurantia* lectin, AAL) stained the cells (Figure 8). This lectin is known to recognize α1,6-linked fucosyl (see footnote 2) groups as occur on the *N*,*N*′-diacetylchitobiosyl residues in *N*-linked glycoproteins (Fukumori *et al.*, 1990). The lotus lectin (*Lotus tetragonolobus* lectin, LTL) and UEA1 (*Ulex europaeus*), which bind principally to α1,2-linked L-fucosyl groups, did not stain the cells nor did the eel serum fucosyl-binding lectin (*Anguilla anguilla* agglutinin, AAA).

Lectins reacting with nonreducing terminal α-GalNAc on oligosaccharides, including the lima bean lectin (LBL) (*Phaseolus lunatus* agglutinin, PLA), *Dolichos biflorus* agglutinin (DBA), and the snail, *Helix pomatia* agglutinin (HPA) failed to bind. The soybean lectin, also an α-GalNAc-binding lectin, gave a positive result; it alone, of all the GalNAc-binding lectins tested, most readily recognizes a single GalNAc residue *O*-linked to Ser/Thr. Terminal nonreducing β-GlcNAc groups also appear to be absent from 12-day-old EBs, from the observation that the *Griffonia simplicifolia* II (GS II) lectin did not bind. We did not detect significant amounts of terminal α-galactosyl groups by the *Griffonia simplicifolia* I B_4 (GS I-B_4) isolectin; however, slight binding by *Marasmius oreades* agglutinin (MOA), which is highly specific for the Gal α1,3Gal β1,4GlcNAc/Glc epitope, was apparent. LacNAc reacts very weakly

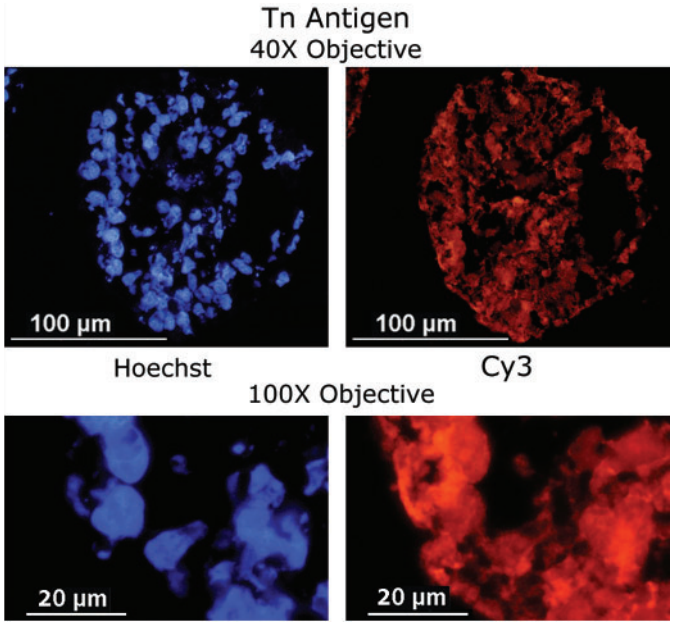

Fig. 5. Staining of EBs at day 12 of differentiation for the Tn antigen with monoclonal mouse anti-Tn (primary antibody) and donkey anti-mouse immunoglobulin (Ig)-Cy3 (secondary antibody, red), double-stained with 1 × Hoechst nuclear stain (blue).

with MOA, and, given the presence of extensive LacNAc termini as shown by the binding of ECA, LEA, WGA, STL, and DSA, this is a possible explanation for its weak binding.

Discussion

This study provides an in-depth description of the carbohydrates on the surface of 12-day differentiated EBs as revealed by the use of carbohydrate-recognizing proteins: lectins and anti-carbohydrate antibodies. Our results indicate the presence of (1) extensive α-mannosyl-terminated chains as shown by moderate staining by GNA and intense staining by Con A and the BanLec, the last two of which also recognize the branched Man_3 trisaccharide proximal to Asp-linkage in *N*-linked glycoproteins; (2) poly *N*-acetyllactosaminyl residues indicated by staining with ECA, WGA, and the tomato, potato, and jimson weed lectins. Staining by RCA and IRL confirms the presence of terminal β-galactosyl groups; (3) sialylated glycoconjugates as revealed by staining by LFA, LPA, SNA, PVL, and PSL, all of which recognize α2,6-linked Neu5Ac, and by MAA, which binds α2,3-linked sialic acid; (4) α1,6-linked fucose as shown by the binding of the orange peel fungal lectin, AAL. This fucosyl group is probably on the *N*,*N*′-diacetylchitobiosyl residue linked to an Asn residue of glycoprotein; (5) Tn and sialyl-Tn antigens as revealed by staining by SBA and antibodies against these carbohydrate antigens; and (6) The T-(Thomsen-Friedenreich) antigen (Gal α1,3GalNAc-*O*-Ser/Thr) as revealed by staining by PNA, ACA, and ABA. On the contrary, lack of lectin staining suggests the absence of the following carbohydrate residues: terminal α-galactosyl, *N*-acetylglucosaminyl, and *N*-acetylgalactosaminyl groups as well as the probable lack of α1,2-linked fucosyl groups.

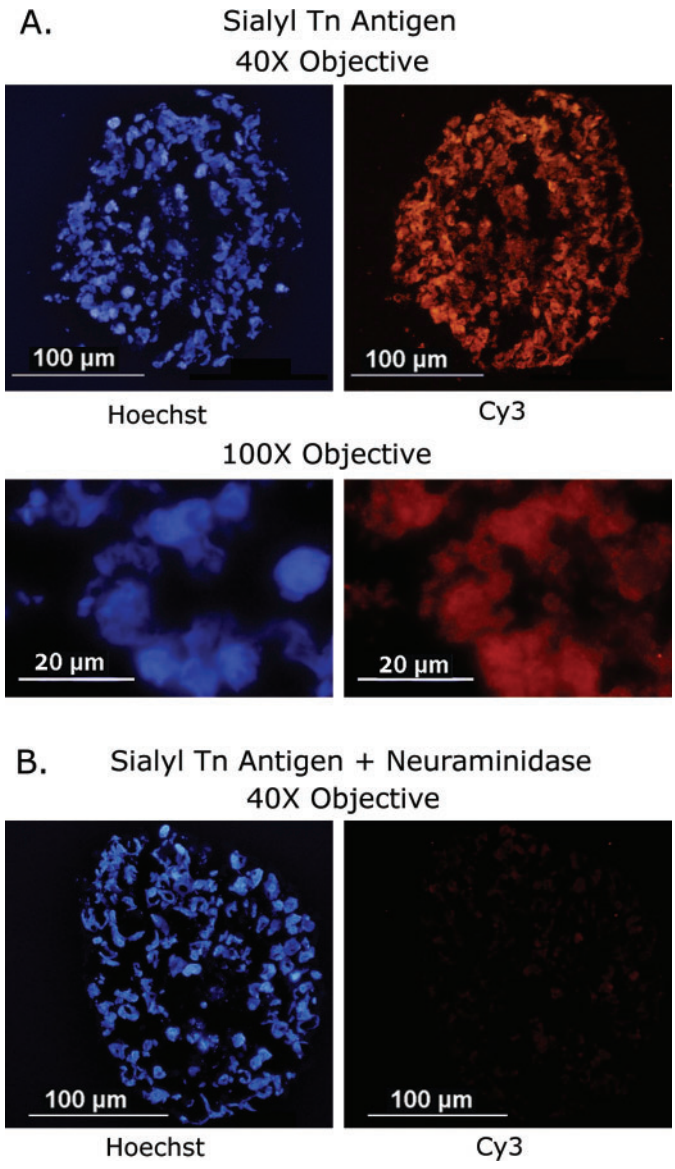

Fig. 6. Effect of neuraminidase on the staining of EBs at day 12 of differentiation with monoclonal mouse anti-sialyl-Tn (primary antibody) and donkey anti-mouse immunoglobulin (Ig)-Cy3 (secondary antibody, red), double-stained with 1 × Hoechst nuclear stain (blue). (**A**) No neuraminidase treatment. (**B**) With neuraminidase treatment before antibody staining.

Many of the lectins stain only the cell surface and/or cytoplasm of the ES cells. This is demonstrated by BanLec (Figure 1), LEA (Figure 2), and IRL (Figure 3). Other lectins appear to stain all parts of the cells—nucleus, cytoplasm, and cell surface—and entire EBs, for example, SNA (Figure 7) and AAL (Figure 8). Still other lectins stain entire cells, but only those located at the edges or in sporadic locations of the EBs. ECA (Figure 4) is an example of a lectin that normally stains only edges and sporadic locations within an EB. Following neuraminidase treatment of the cells, this lectin appears to stain all cell surfaces and cytoplasm but not nuclei. No correlation is evident between specificity and location of stain because different lectins

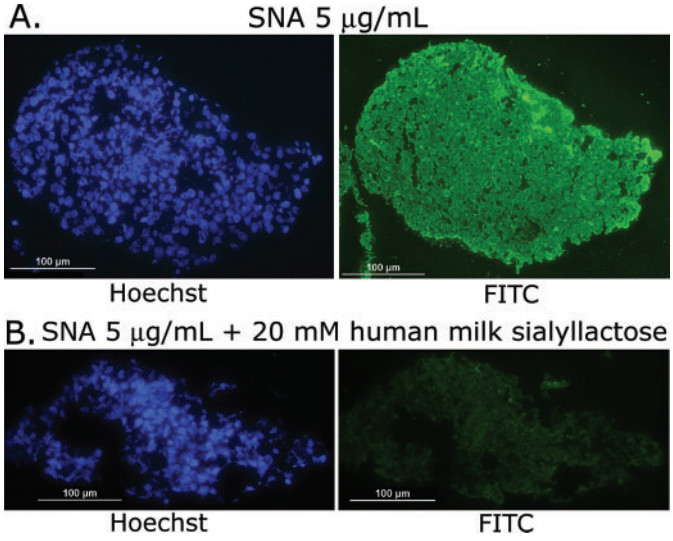

Fig. 7. Effect of human milk sialyllactose on the staining of EBs at day 12 of differentiation by 5 µg/mL fluorescein-labeled SNA, double-stained with 1 × Hoechst nuclear stain (blue). (**A**) No human milk. (**B**) With 20 mM human milk sialyllactose.

having the same general specificity display different binding locations.

To compare 12-day differentiated EBs with undifferentiated hESCs, we used similar fluorescence microscopy techniques on fixed hESCs rather than relying solely on previous data from cell-sorting techniques (Venable *et al.*, 2005) (Table II). It is interesting to note, however, that the data achieved by the two methods were very similar. Using our own undifferentiated cell data, we were able to draw the following conclusions: MAA bound 89% of 12-day EBs, whereas only 19% of the undifferentiated cells were labeled by this lectin. This binding by MAA is also consistent with the presence in human EBs of the stage-specific embryonic antigens SSEA-3 and SSEA-4, the globogangliosides occurring in human teratocarcinoma cells (Kannagi *et al.*, 1982). AAL bound to 100% of both undifferentiated and 12-day differentiated cells, indicating the presence of α1,2- and/or α1,6-linked fucose in both cell types. We employed UEA1 to determine which type of fucose was present in each type of cells. UEA1 stained 15% of the undifferentiated cells but none of the 12-day differentiated cells, indicating loss of virtually all α1,2-linked fucose during the early stage of differentiation. To our knowledge, this is the first time that such a shift has been identified. We believe that these changes in undifferentiated hESCs during the 12-day differentiation as EBs may be of importance.

We also identified for the first time the presence of the Tn and sialyl-Tn antigens in 12-day-old EBs. As positive controls, we observed positive staining of the sialyl-Tn antibody to human cervix, testis, and duodenum tissues and for the Tn antibody to the cervix and duodenum tissues (Yonezawa *et al.*, 1992). The presence of the T-disaccharide was also shown by the binding of ABA and PNA, as well as the very strong staining by ACA, all of which recognize this carbohydrate epitope (Springer, 1984).

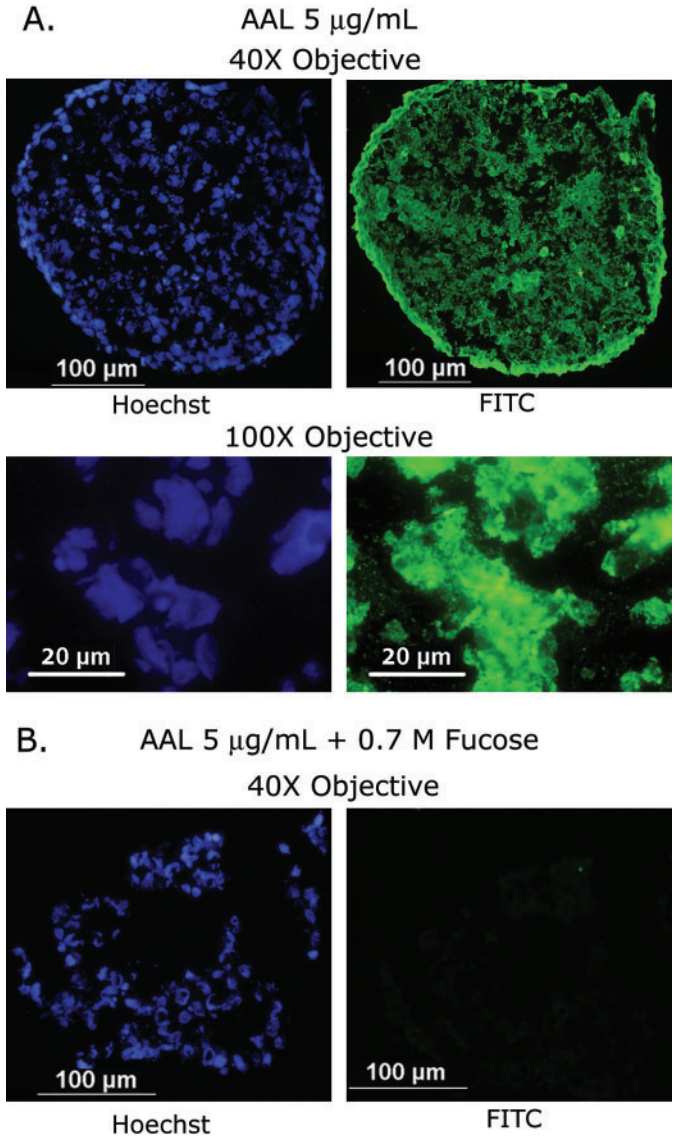

Fig. 8. Effect of fucose on the staining of EBs at day 12 of differentiation by 5 µg/mL fluorescein-labeled AAL, double-stained with 1 × Hoechst nuclear stain (blue). (**A**) No fucose. (**B**) With 0.7 M fucose.

Materials and methods

Materials

The lectins BanLec, Con A, DSA, GNA, IRL, LBL, LFA, MAA, MOA, PSL, and SNA were prepared from their natural sources and labeled with fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO) in this laboratory. The following lectins were purchased from EY laboratories (San Mateo, CA) as FITC-conjugated: AAA, ABA, GS II, HPA, LPA, PHA-E (*Phaseolus vulgaris* erythroagglutinin), PHA-L, (*Phaseolus vulgaris* leucoagglutinin), PNA, and UEA1. The lectin AAL was purchased from Vector laboratories (Burlingame, CA) and was FITC-labeled following the procedure described below. The following lectins were purchased from Vector laboratories as FITC-labeled: ACA, DBA, ECA, EEA (*Euonymus europaeus* agglutinin), GS I-B_4, LEA,

Table II. A comparison of lectin binding to undifferentiated and differentiated hESCs

Lectin	Concentration	Lectin specificity	Undifferentiated result (%) (day 0)[a]	Differentiated result (%) (day 12)[b]
α Man				
Con A	10	Terminal α-Man, Man α3[Man α6] Man	99[c]	100
GNA	10	Terminal Man α(1,3)Man	25[d]	34
α GalNAc				
DBA	10, 20	GalNAc α1,3 GalNAc/Gal	0[c]	0
Neu5Ac				
MAA	10	Neu5Ac α(2,3)Gal	19[d]	89
SNA	5	Neu5Ac α2,6Gal or α2,6GalNAc	80[d]	100
WGA[e]	1	(Neu5Ac) (Gal β4GlcNAc)$_{1-3,4}$ (GlcNAc β4GlcNAc)$_{1-3,4}$	100[d]	100
LacNAc				
LEA	20	LacNAc	99[c]	91
WGA[e]	1	(Neu5Ac) (Gal β4GlcNAc)$_{1-3,4}$ (GlcNAc β4GlcNAc)$_{1-3,4}$	100[d]	100
Fucose				
AAL	5	α(1,6) L-fucose	100[d]	100
Lotus	35	α(1,2) L-fucose (terminal)	0[c]	0
UEA1	30	α(1,2) L-fucose	15[d]	0
βGal				
ACA	1	Terminal βGal, Gal β(1,3)GalNAc	100[d]	83
PNA	20	Terminal βGal, Gal β(1,3)GalNAc	81[d]	67
RCA	5	Terminal βGal	100[d]	100
β GlcNAc				
WGA[e]	1	(Neu5Ac) (Gal β4GlcNAc)$_{1-3,4}$ (GlcNAc β4GlcNAc)$_{1-3,4}$	100[d]	100
PHA-E[f]	10	(Gal β 1,4 GlcNAc)$_2$ β1,2,6, Man	55[c]	23
PHA-L[f]	10	Gal β(1,4)GlcNAc β(1,2) Man	15[c]	19

[a]Day 0 represents undifferentiated hESCs.
[b]Day 12 represents the hESCs differentiated for 12 days as EBs.
[c]Venable *et al*. (2005), cells were counted by flow cytometry.
[d]A minimum of 20 undifferentiated ES cell colonies were counted for this value.
[e]Several of these lectins are listed in more than one location, as they have more than one binding specificity. These include MOA, PVL, WGA, and WGA-succ.
[f]The carbohydrate-binding specificity of PHA-E, PHA-L, and EEA is complex, and although listed as binding to 12-day-old EBs, we are unable to conduct sugar hapten inhibition of binding studies.

LTL, RCA1, SBA (*Glycine max*), STL, WGA, and WGA-succ. One lectin, PVL, was purchased from WAKO Chemicals (Richmond, VA) and FITC-labeled in this laboratory. Before use, all lectins were checked by spectrophotometry for FITC-labeling, and, if possible, an agglutination assay was done to assure specific binding ability. The only lectin that did not demonstrate agglutinating activity, LBL, is thought to have been split into its monomers during FITC labeling. It is presumed to retain its specific binding ability, although it no longer agglutinates red blood cells (RBCs). The following haptenic sugars were purchased from Sigma: Neu5Ac (used to block LFA and LPA), α2,3 sialyllactose (used to block MAA), lactose (used to block PSL, IRL, PNA, and RCA), fucose (used to block AAL), and the benzyl T-antigen (used to block ABA and ACA). Human milk sialyllactose (80% α2,6-linked and 20% α2,3-linked), used to block SNA, was also purchased at Sigma. The *N*-acetylgalactopyrannoside used to block SBA and the *N*-acetylglucopyrannoside used to block PVL were purchased from Pfanstiehl Laboratories (Waukegan, IL). The Gal α1,3 Gal used to block MOA was purchased from V-labs, Inc., (Covington, LA). The remaining sugars used in this study, methyl α-mannoside (used to block BanLec, Con A, and GNA), *N*,*N*′,*N*″-triacetylchitotriose (used to block DSA, LEA, STL, and WGA-succ), and LacNAc (used to block WGA and ECA), were all available in this laboratory from previous studies. The anti-Tn antigen, sialylated mouse mAb antibody (clone B35.2—reacts only with NeuAcα2,6GalNAc-Ser/Thr), was purchased from Cal Bio Chem (La Jolla, CA). The monoclonal mouse antibody to the Tn antigen (clone B1.1) was purchased from Biomeda (Foster City, CA), and the secondary antibody, Cy3-conjugated donkey anti-mouse, was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). *Clostridium perfringens* neuraminidase was purchased from Sigma as a lyophilized powder. Coverslips used in undifferentiated cell culture were 18 mm in diameter and between 0.13 and 0.17 mm thick. The coverslips were purchased from Fisher (Marshalltown, IA). The Triton X-100 was purchased from Research Products International (Elk Grove Village, IL).

Fluorescein labeling of lectins

One to four milligrams of the desired lectin was placed into a dark vial and dissolved in 1 mL of phosphate-buffered saline (PBS, 2 mM sodium phosphate monobasic, 8 mM sodium phosphate dibasic, 0.15 M sodium chloride, 0.1 mM $CaCl_2$, and 6 mM sodium azide) containing 100 mM sodium carbonate at pH 9.5. Lectin-specific sugars were added to the solution to protect the active sites, mixed briefly, and allowed to stand for at least 10 min to ensure complete binding to the active site. To this solution was added ~1 mg FITC. The vial was rotated in the dark for 1–2 h at room temperature depending on the lectin being labeled, after which the solution was transferred to a dialysis bag. Dialysis into PBS proceeded in the dark until all unbound FITC and carbohydrate were removed. The solution was stored in an amber vial at 4°C until use.

Undifferentiated hESC culture

BG01 hESCs were cultured *in vitro* on a mouse embryonic fibroblast (MEF) feeder layer in hESC medium (78.7% Dulbecco's modified Eagle's medium [DMEM; Invitrogen, Carlsbad, CA], 19.7% knockout serum replacement [KOSR; Invitrogen], 1.0% nonessential amino acids [NEAA; Invitrogen], 0.2% basic fibroblast growth factor [bFGF; Invitrogen], 0.1 mM β-mercaptoethanol [Sigma], and 2 mM L-glutamine [Invitrogen]). Two confluent 60-mm hESC dishes were used for each 12-well plate of undifferentiated cells. A round 18-mm diameter coverslip 0.13–0.17 mm thick (Fisher) was placed into each well, and undifferentiated hESCs were plated on top of these.

Lectin staining of undifferentiated hESCs

Between 2 and 4 days after plating, the hESCs were fixed in a solution of 2% paraformaldehyde and 0.1% Triton X-100 in PBS (2 mM sodium phosphate monobasic, 8 mM sodium phosphate dibasic, 0.15 M sodium chloride, 0.1 mM $CaCl_2$, and 6 mM sodium azide). The fixed cells were then rinsed three times in PBS-containing 0.1% Triton X-100 for 5 min each. The cells were placed in a dark chamber, and a solution of 1% bovine serum albumin (BSA, Sigma), 0.1% Triton X-100 in PBS, was applied for 20 min, followed by treatment for 20 min with a solution in PBS-containing lectin at the specified concentration and 0.1% Triton X-100. The cells were rinsed three times for 5 min each with a solution of 0.1% Triton X-100 in PBS and stained with a solution of 1 × Hoechst 23187 (Sigma) and 0.1% Triton X-100 in PBS for 5 min. Following rinsing the cells once with PBS, the coverslips were removed from the wells, rinsed once by dipping into PBS, and applied directly to a slide containing 7–10 μL of Prolong Gold (Molecular Probes, Eugene, OR). After standing in the dark chamber overnight, the slides were stored in the dark at –20°C until they were viewed on a fluorescence microscope.

EB formation from hESCs

BG01 hESCs were cultured *in vitro* on an MEF feeder layer before differentiation. Two confluent 60-mm hESC dishes were used for each dish of EBs prepared. The cells that had already differentiated were removed from the dishes, leaving only pluripotent hESCs in hESC medium. To generate EBs, undifferentiated hESCs were removed from the MEF feeder, then suspended in ~10 mL hESC medium, and centrifuged at 78 × *g* for 5 min. The cells were resuspended in EB medium made of 78.8% DMEM, 19.7% KOSR, 1.0% NEAA, 0.1 mM β-mercaptoethanol, and 2 mM L-glutamine and placed in a dish to form EBs. The EB medium was replaced every other day for 12 days.

Cryostat embedding and sectioning

Cryomolds (Electron Microscopy Sciences, Hatfield, PA) were filled with optimum cooling temperature (OCT) medium (TissueTek; Electron Microscopy Sciences). The 12-day EBs were placed in a 15-mL conical vial, centrifuged briefly, and the supernatant was removed. The EBs were then rinsed with 5-mL room temperature 1 × PBS (Invitrogen) and placed in a 1.5-mL tube. The tube was centrifuged briefly and the PBS removed. The pelleted EBs were removed using a glass pipet, transferred to the mold containing the OCT medium, and then frozen in a 2-methylbutane dry ice slurry. Samples were stored at –80°C until sectioning. Six-micrometer sections were cut using a Microm cryostat and collected on positively charged slides (Fisher), which were stored at –80°C until staining.

Lectin staining of differentiated cryostat sections

After thawing, samples were outlined using a pap pen (Electron Microscopy Sciences). They were rinsed once with PBS for 5 min, blocked in a solution of 1% BSA in PBS for 20 min followed by lectin solution in PBS for 20 min. The samples were rinsed three times in PBS for 5 min each and fixed in a 2% solution of paraformaldehyde in PBS for 15 min. After fixation, the samples were rinsed three times in PBS for 5 min each. A 1 × Hoechst 23187 solution (Sigma) in PBS was applied as a nuclear stain for 5 min. The slides were further rinsed one time with PBS for 5 min. Slides were coverslipped with 5–10 μL Prolong Gold (Molecular Probes) and stored at –20°C until they were viewed on an Olympus BX51 fluorescent microscope. Unless otherwise specified, all procedures were completed in low light at room temperature in a moist chamber. Control slides were treated with PBS or lectin solution incubated with the blocking carbohydrate for at least 20 min in place of the lectin solution. In the case of LPA, HEPES buffer (0.15 M sodium chloride, 0.02 M HEPES, 0.01 M calcium chloride, and 0.04% sodium azide) was used to prepare all reagents in place of PBS because LPA requires a high concentration of calcium to bind.

Antibody staining

After thawing, frozen sections were outlined with a pap pen. They were rinsed once with PBS for 5 min before applying a blocking buffer of 1% donkey serum (Jackson ImmunoResearch Laboratories) and 0.5% Triton X-100 in PBS for 1 h. After blocking, the primary antibodies were diluted in blocking buffer and applied to the slides overnight at 4°C. The slides were then rinsed three times in PBS for 5 min. The secondary antibodies were diluted in the blocking buffer and applied to the samples for 1 h at room temperature, then rinsed three times with PBS. As necessary, the

lectin procedure was followed from this point on, beginning with BSA treatment of the slide wells. For controls, blocking buffer was used in place of primary antibodies. Unless otherwise specified, all procedures were completed in diminished light at room temperature in a moist chamber.

Neuraminidase treatment

Lyophilized neuraminidase was reconstituted to a concentration of 5-units/mL in 0.1 M sodium acetate buffer at pH 5.0 containing 0.15 M sodium chloride. This solution was diluted 5-fold in the same buffer and applied to the well. The slide was placed in a moist chamber and incubated at 37°C for 1 h, after which it was brought to room temperature and rinsed three times in PBS for 5 min each. The slide was then stained by antibody or lectin following the procedure described above.

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Conflict of interest statement

None declared.

Abbreviations

AAA, *Anguilla anguilla* agglutinin, eel serum; AAL, *Aleuria aurantia* lectin, orange peel fungus; ABA, *Agaricus bisporus* agglutinin; ACA or amaranthin, *Amaranthus caudatus* agglutinin; BanLec, banana lectin, *Musa acuminata*; BSA, bovine serum albumin; Con A, concanavalin A; DBA, *Dolichos biflorus* agglutinin; DSA, *Datura stramonium* agglutinin, jimson weed; EB, embryoid body; ECA, *Erythrina cristagalli* agglutinin; EEA, *Euonymus europaeus* agglutinin; FITC, fluorescein isothiocyanate; GNA, *Galanthus nivalis* agglutinin, snowdrop bulb lectin; GS I-B_4, *Griffonia simplicifolia* I B_4; GS II, *Griffonia simplicifolia* II; hESC, human embryonic stem cell; HPA, *Helix pomatia* agglutinin, edible snail; IRL, *Ischnoderma resinosum* lectin; LacNAc, *N*-acetyllactosamine; LBL, lima bean lectin; LEA, *Lycopersicon esculentum* agglutinin, tomato lectin; LFA, *Limax flavus* agglutinin, slug; LPA, *Limulus polyphemus* agglutinin, horseshoe crab lectin; LTL, *Lotus tetragonolobus* lectin; MAA, *Maackia amurensis* agglutinin; MEF, mouse embryonic fibroblast; MOA, *Marasmius oreades* agglutinin; Neu5Ac, *N*-acetylneuraminic acid; PBS, phosphate buffered saline; PHA-E, *Phaseolus vulgaris* erythroagglutinin; PHA-L, *Phaseolus vulgaris* leucoagglutinin; PLA, *Phaseolus lunatus* agglutinin; PNA, peanut agglutinin, *Arachis hypogaea*; PSL, *Polyporus squamosus* lectin; PVL, *Psathyrella velutina* lectin; RCA, *Ricinus communis* agglutinin; SBA, *Glycine max,* soybean agglutinin; SNA, *Sambucus nigra* agglutinin, elderberry bark lectin; SSEA, stage-specific embryonic antigen; STL, *Solanum tuberosum* lectin, potato lectin; UEA1, *Ulex europaeus* agglutinin; WGA, wheat germ agglutinin, *Triticum vulgaris*; WGA-succ, succinylated WGA.

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