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Direct Modification of the Glycocalyx of a Cultured Muscle Cell Line by Incorporation of Foreign Gangliosides and an Integral Membrane Glycoprotein

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As part of a program to better understand the cause-or-effect nature of the relationship between cell surface carbohydrate and cell properties and behaviour, experiments have been carried out on direct modification of the glycocalyx of cultured cells. Modification was by incorporation of gangliosides and an integral membrane glycoprotein chosen to be dissimilar to species occurring naturally in the cell line. Two methods of incorporation were investigated: simple addition of the new components to the culture medium for various times, or assembly of the components into the walls of lipid vesicles which were subsequently fused with cells. Gangliosides from beef brain and glycophorin, the major human erythrocyte sialoglycoprotein, were successfully added to the surface of myoblasts in quantities sufficient to represent a significant perturbation. Changes in cell adhesion, morphology, and viability were observed which seem to be a direct result of glycocalyx modification.

Key words: gangliosides, glycophorin, myoblasts, glycocalyx modification

The involvement of mammalian cell surface carbohydrate in a wide variety of processes related to differentiation, attachment, recognition, and growth control has been extensively documented in recent years. However, the mechanisms of this involvement are unclear. Often when altered surface carbohydrate has been correlated with altered cell behaviour one can only speculate as to whether carbohydrate changes are a primary reason for some aspect of the behaviour difference, or are simply an unrelated secondary effect. We have begun attacking this problem by attempting to modify the existing glycocalyx of cultured cells via incorporation of integral membrane components foreign to the host species. This is already known to be feasible in the case of gangliosides which may be associated with cell membranes by simple addition to the culture medium. In several cases it has been shown that this method can incorporate at least some amount of material in a form sufficiently natural to increase the sensitivity of mammalian cells to an external toxin [cholera toxin (1) or ricin (2)] directed against the glycolipid in question. However, these experiments do not prove that the new glycolipid so added mimics original host cell glycolipid,

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since the orientation and arrangement requirements for these processes are unknown. In fact, it has been suggested that exogenous gangliosides added to cell surfaces by dissolution in the surrounding medium become bound to cells in some way which is distinct from the binding of naturally inserted gangliosides (3).

The incorporation of foreign integral membrane glycoproteins "into" the membranes of cultured cells has not been studied previously. We have examined this process in a parallel study with gangliosides. Regardless of what happens to gangliosides added free in the medium, it seems unlikely on thermodynamic grounds that a transmembrane protein added under similar conditions will insert itself in a natural state; however, one can likely expect a certain amount of sticking to the cell surface. We have previously described methods whereby glycolipids and transmembrane glycoproteins such as glycophorin may be assembled into lipid bilayers while retaining their binding specificity (4–6). We have been fusing these structures with cells in culture. Potential advantages of such an approach are: a) the possibility of directly incorporating into cell membranes species which are water-insoluble or (conformationally) unstable in the absence of lipid; b) the fact that a fusion mechanism is capable of inserting integral membrane components in a "natural" state.

The L6 9/1 cell line has a simplified set of four gangliosides (7) (mainly monosialo derivatives). Beef brain gangliosides are a complex mixture (8) rich in di- and trisialo species and should provide an example of a change in surface ganglioside composition. Glycophorin is a family of small integral membrane glycoproteins of the human erythrocyte. It has a molecular weight of 31,000 [glycophorin A (9)] and carries several blood group specificities, viral binding sites, and lectin receptors (10, 11). Since it bears some 15 neuraminic acid residues per polypeptide monomer and is about 60 wt. % carbohydrate, it should represent a considerable glycoprotein change at the surface of cultured cells. In general the approach we have taken to cell surface modification has been to use brief, intense pulses of exogenous material rather than prolonged exposures to lower concentrations.

MATERIALS AND METHODS

A clone of Yaffe's L6 rat skeletal myoblast line designated L6 9/1 was used throughout this work. The cell line was routinely maintained at 37°C in monolayer cultures (in Falcon tissue culture flasks) in Dulbecco modified Eagle's medium (DME) supplemented with 10% horse serum. The incubation atmosphere was 95% air and 5% CO_2.

Egg phosphatidylcholine was from Sigma (type 3-E) and was further purified by chromatography on silicic acid. Phosphatidylserine and cholesterol were obtained from Serdary Research, London, Ontario. ^{14}C-phosphatidylcholine was from New England Nuclear. Glycophorin was isolated from human erythrocyte ghosts according to the method of Marchesi and Andrews (12). Gangliosides were extracted from bovine gray matter and purified by a modification of the method of Kanfer (13), in which a silicic acid chromatography step was used to purify the crude ganglioside fraction obtained from the initial Folch extraction. ^{3}H-labeled gangliosides and glycophorin were prepared via the sodium borotritiide procedure of Van Lenten and Ashwell (14).

Lipid mixtures were made by dissolving appropriate amounts of each in chloroform/methanol prior to drying under a stream of nitrogen and exhaustive pumping in vacuo. Dried lipids were suspended in phosphate-buffered saline (PBS) by vortexing at 22–37°C followed by sonication at 22°C (several two-minute bursts with the 3 mm microprobe of

a Branson Sonicator model W140). Sonication was followed by centrifugation for 10 min at 12,000 g to remove any traces of titanium. Vesicles containing glycophorin were prepared by sonicating (as above) glycoprotein-containing lipid bilayers generated by the method of Grant and McConnell (4).

The basic approach toward treatment of cells in culture with carbohydrate-bearing material was as follows: 25 ml flasks were seeded with 5×10^5 cells and incubated for 24 h at 37°C. Growth medium was then removed and the cells were washed once in situ with PBS prior to readdition of 1.0 ml of serum-free medium. This was supplemented with 1.0 ml of PBS containing lipid vesicles or free gangliosides or glycophorin and was followed by added horse serum where appropriate. After incubation for the desired length of time, the medium was removed and centrifuged to collect any dislodged cells. Flasks were then incubated for 10 min at 37°C with 2.0 ml of citrate saline solution in order to remove remaining cells: These were collected by centrifugation and pooled with the previous lot. The total cell pellet was washed twice by resuspension and centrifugation at 23°C in PBS (1,000 g). Cells not requiring further treatment were dissolved in 0.5 ml of 2 N NaOH. After neutralization the entire lysate was added to 10 ml scintillation fluid. Cells to be centrifuged through sucrose were suspended in 0.5 ml PBS and layered on 10 ml of 10 mM phosphate buffer, pH 7.0, containing 10% (w/w) sucrose. They were centrifuged at 2,000 rpm for 15 min in a GLC bench-top centrifuge (Sorval). The tubes were counted in 0.5 ml fractions using a Searle Mark III scintillation counter. The cell pellet plus the bottom 0.5 ml of sucrose were dissolved in 0.5 ml 13.5 N NaOH prior to neutralization and addition to 10 ml scintillation fluid.

Freeze-fracture replicas for electron microscopy of cells in culture were prepared with a Balzers BAF 301 freeze-etch unit fitted with a Pfenninger device. Cells were grown on gold grids coated with rat-tail collagen prior to being fixed, quenched in freon cooled with liquid nitrogen, and fractured in situ as described by Pfenninger and Rinderer (15).

RESULTS AND DISCUSSION

Glycocalyx Modification by Addition of Free Gangliosides or Glycophorin

When free gangliosides or the membrane glycoprotein, glycophorin, are added to myoblasts in culture, a certain fraction of the added material becomes associated with the cells. The extent of this phenomenon may be measured by using tritiated analogues prepared as described in the Methods section (Fig. 1 and Table I). It is important to note from Table I that the fraction which actually becomes "cell-associated" is to some extent a function of how well the cells are washed to remove unbound glycolipid or glycoprotein. Undoubtedly there is a large contribution from material which simply coats the cells. The strength of association of the foreign carbohydrate-bearing components with myoblast membranes ranges from very weak (removable by repeated gentle washing with PBS) to very strong (not removed even by centrifugation through a sucrose step gradient at 23°C). Although the efficiency of association is low, the absolute amounts of material involved are relatively large and should represent a substantial perturbation on the glycocalyx. Figure 2 shows that maximum efficiency of binding of both free gangliosides and free glycophorin occurs at reduced serum concentrations. A similar effect of serum on the uptake of free G_{M1} by human fibroblasts has been reported by Fishman et al (16). The time course for binding appears in Figure 3.

Glycocalyx Modification by Fusion With Lipid Bilayer Structures

The phenomenon of vesicle fusion with mammalian membranes was first investigated by H. M. McConnell and co-workers, who showed that some portion of the foreign lipid that becomes cell-associated when lipid vesicles are mixed with cell membranes becomes

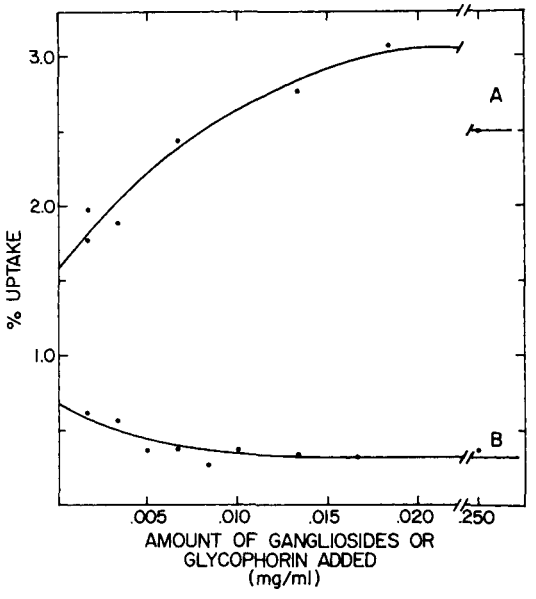

Fig. 1. Binding efficiency of (A) ^{3}H-gangliosides and (B) ^{3}H-glycophorin to L6 rat myoblasts in culture. In each of a number of flasks containing normal growth medium 5×10^{5} cells were plated. After 24 h the medium was replaced with serum-free medium containing the foreign carbohydrate-bearing material and cells were incubated for 2 h. Assay was for counts associated with the cells following removal with citrate-saline and several washings in PBS. The initial rise in efficiency of ganglioside uptake presumably results from approach to the critical micelle concentration.

TABLE I. Association of Foreign Carbohydrate-Bearing Material With Cells in Culture*

Sample added to 10^{6} cells	Final concentration (μg/ml)	% Associated cells after PBS wash	% Associated with cells after 10% sucrose wash
GA[a]	200	0.71	0.20
GL[b]	200	0.55	0.32
5:9:1[c]	1,000 (lipid)	0.23	0.11
5:9:1 + GA	1,000 (lipid) 200 (GA)	0.39	0.17
5:9:1 + GL	1,000 (lipid) 200 (GL)	0.30	0.05

*Material was added free in solution or as bilayer components of lipid vesicles.
Values reported are the average of four measurements which varied over a range of some 20% of the value quoted.
[a]GA = beef brain ganglioside.
[b]GL = glycophorin.
[c]5:9:1 indicates the weight ratio of the lipid mixture, cholesterol/egg phosphatidylcholine/phosphatidylserine.

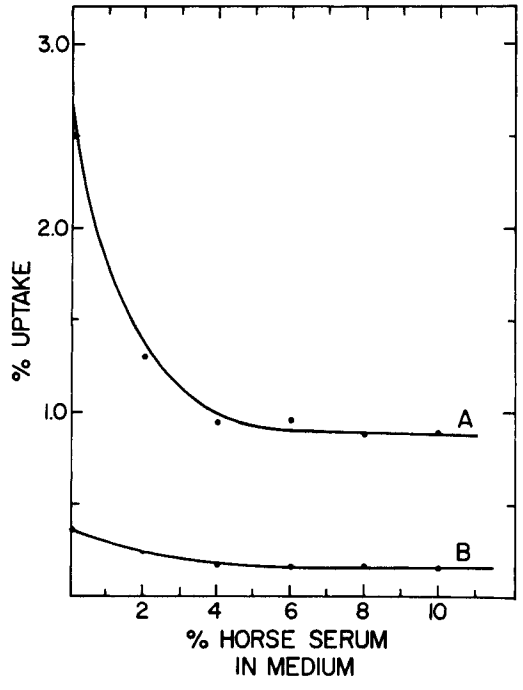

Fig. 2. Serum dependence of the binding of (A) ^{3}H-gangliosides and (B) ^{3}H-glycophorin to L6 rat myoblasts in culture. The conditions for the experiment were as described in Fig. 1 with gangliosides and glycophorin added at 0.25 mg/ml and incubated for 2 h. Each point is an average of two determinations.

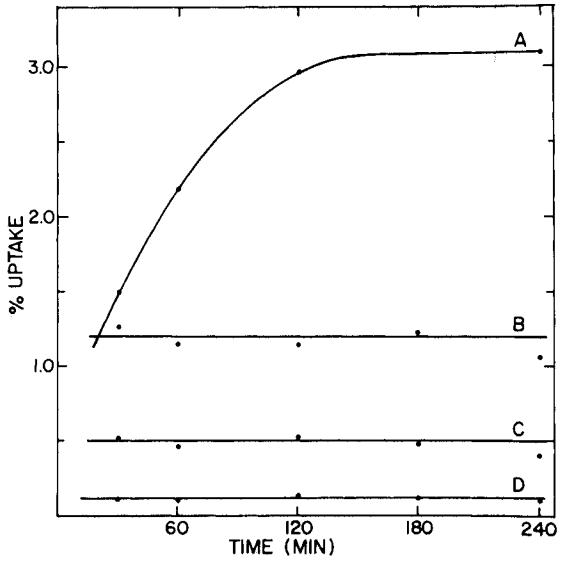

Fig. 3. Time course for binding of ^{3}H-gangliosides in the absence (A) and presence (B) of 10% serum, and of ^{3}H-glycophorin in the absence (C) and presence (D) of 10% serum. Final concentration of ganglioside or glycophorin was 0.25 mg/ml.

part of the cell membrane and diffuses laterally into it (17). These same workers reported that some lipid simply "stuck" to the membrane and could only be washed away by rather vigorous treatments such as sucrose-density gradients. Fusion of lipid vesicles with viable cells was first reported by one of us in H.M. McConnell's laboratory (18) and has since been actively pursued using mammalian cell lines by a variety of workers (19–22). In the majority of cases the motivation for these experiments seems to have been use of the fusion event to transfer vesicle aqueous contents (a membrane-impermeable substance) to the cell cytoplasm. Very likely in such experiments, apart from the fraction which has fused with cells, appreciable quantities of lipid are also endocytosed or simply stuck to the surface.

As we have described elsewhere (4, 5, 26), it is possible to incorporate beef brain gangliosides and glycophorin into lipid bilayers in such a way that they retain their binding functions. We have fused such structures with myoblasts in culture in order to incorporate new material into the cell membranes. Once again the success of the procedure may be followed with radiolabeled material: tritiated gangliosides and glycophorin or ^{14}C-phosphatidylcholine. Figure 4 demonstrates that, as with addition of free glycophorin and gangliosides, the efficiency of incorporation is low. The data in Table I indicate the range

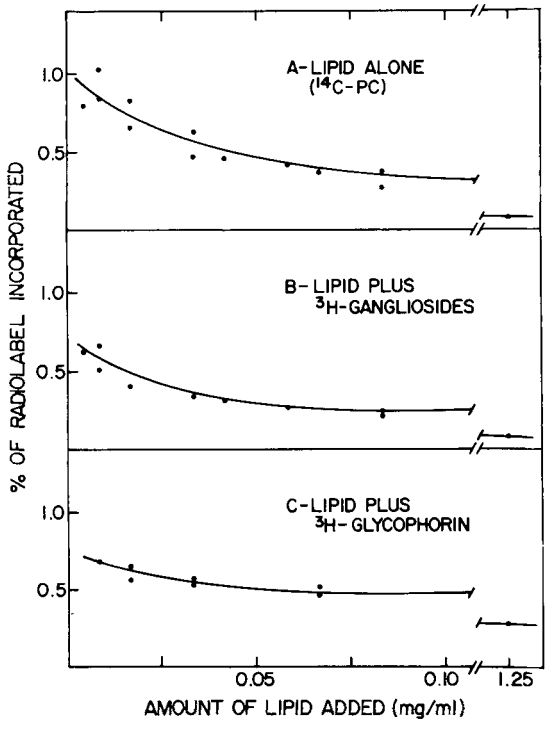

Fig. 4. Uptake by myoblasts in culture of gangliosides and the integral glycoprotein, glycophorin, via fusion with lipid bilayer structures. For curve A the cells were treated with lipid alone (5:9:1 weight ratio of cholesterol: egg phosphatidylcholine: phosphatidylserine) containing ^{14}C-phosphatidylcholine as a marker. For curve B the procedure was the same except that 20 wt. % ganglioside was included in the lipid mixture and ^{3}H-ganglioside was the marker. Curve C was generated using lipid vesicles as in A but containing 20 wt. % glycophorin in the bilayer. The experimental approach was similar to that employed in Fig. 1. Time of incubation, 2 h.

in degree of association of lipid with the cell membrane. Figure 5 illustrates the effect of serum on incorporation of lipid vesicles, and Fig 6 gives the time course for uptake.

In Fig. 7 freeze-fracture electron microscopy has been employed to monitor the in situ appearance of cells following a brief, intense pulse with unsonicated lipid structures containing bilayer-incorporated glycophorin. There is a distinct patchiness and "dilution" of the protein-related intramembranous particles presumably brought about by the massive injection of new lipid. Clearly an appreciable amount of new membrane material can be rapidly incorporated in this fashion.

Effects of Glycocalyx Modification/Perturbation

Gross effects such as reduced viability and changes in cell morphology become evident within 20 h of addition of gangliosides or glycophorin even in the presence of serum. Additions in the range of 100 μg/ml often lead to almost total cell death. The ability of exogenously added free ganglioside to reduce cell viability has been reported recently for 3T3 cells (23). Typical morphology effects we noted were a tendency to form long spindle-shaped processes (caused by both gangliosides and glycophorin) and dramatic shrinking of the cell body (caused by glycophorin). As would be expected from the uptake curves, effects were most marked when cells had been treated in the absence of serum. Glycophorin and gangliosides are both some 60% carbohydrate by weight – yet on a weight-for-weight basis glycophorin induced more dramatic effects on morphology and viability.

Probably more interesting than these gross effects will be the subtle effects which must occur at shorter times and/or lower concentrations of material. Within an hour of addition of free glycophorin in serum-free medium, many cells show reduced adhesion and

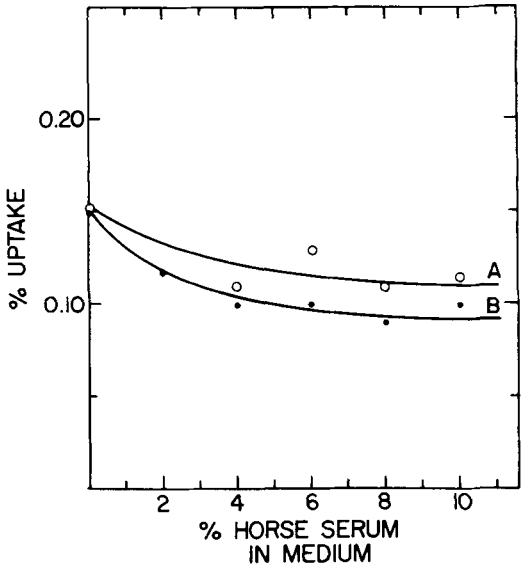

Fig. 5. Serum dependence of the "fusion" process. Vesicles of the 5:9:1 lipid mixture (weight ratio of cholesterol: egg phosphatidylcholine: phosphatidylserine) were added in PBS to cells under conditions comparable to those in the other figures (described in Fig. 1). Final lipid concentration 1.25 mg/ml. Curve A, lipid alone. Curve B, lipid containing 20 wt. % ganglioside. Each point is an average of two determinations.

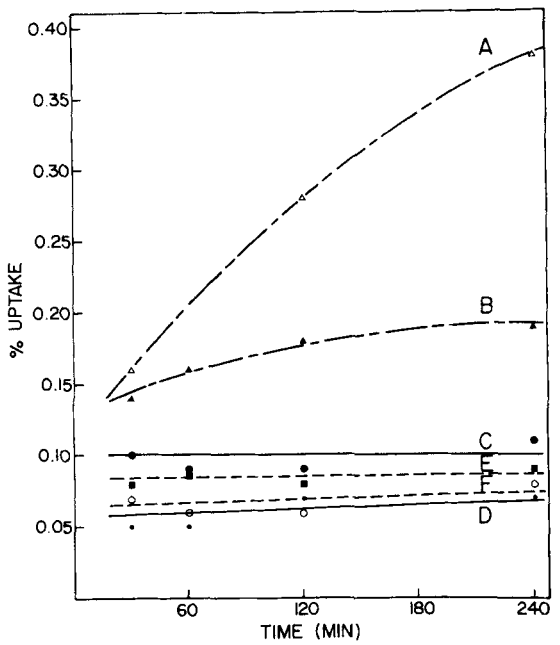

Fig. 6. Early time course for the "vesicle fusion"-mediated incorporation of gangliosides and the integral glycoprotein, glycophorin, into myoblasts in culture. Lipid mixture of the same basic 5:9:1 composition as in Figs. 4, 5, and 7 was added in PBS to produce a final concentration of 1.25 mg/ml. Curves C,D: lipid alone labeled with ^{14}C-phosphatidylcholine; curves E,F: lipid plus 20 wt. % gangliosides labeled with ^{3}H-gangliosides. curves A,B: lipid plus 20 wt. % glycophorin labeled with ^{3}H-glycophorin. The upper curve of each pair (A, C, E) are in the absence of serum, while the lower curves (B, D, F) are in the presence of 10% serum.

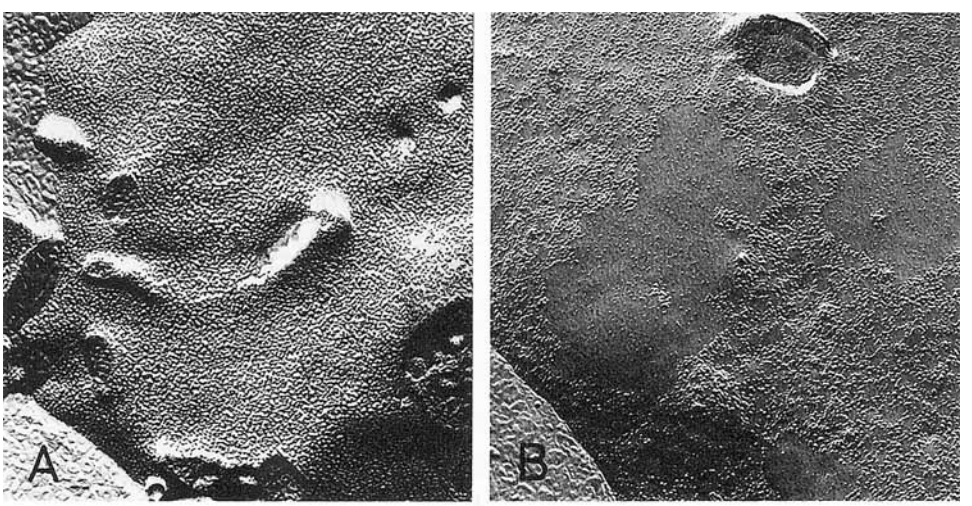

Fig. 7. Freeze-fracture electron micrographs of L6 myoblasts in culture. In each case a portion of the convex facture face of the upper surface of one cell is shown. Cells were not removed from the growth surface or physically disturbed in any way either before or after fixation. 7A) a normal cell grown to subconfluence; 7B) a similar cell following 2 h incubation with unsonicated vesicles containing glycophorin. The treated cell shows a characteristically low density of protein-related intramembranous particles and large, smooth areas. Magnification: 11, 000 X.

begin to lift off. If the floating cells are retrieved, washed, and replated in glycophorin-free, serum-containing medium they reattach and grow. This seems not to be a nonspecific effect, since on a weight-for-weight basis much more ganglioside becomes cell-associated (Fig. 1), while having a much less dramatic effect. We have not examined the dependence of this phenomenon on cell cycle or cell type, but induction of selective detachment could be a useful tool.

The effects of added gangliosides or glycophorin seem most likely to be mediated at the plasma membrane. Within the time periods involved the vast bulk of the gangliosides and glycophorin added to cell cultures will be metabolically unavailable to the cells. Pinocytosis of membrane-bound material can be expected to occur and to lead to small metabolic pools of the glycolipid and glycoprotein sugars. It may be instructive that the uptake curves for free glycophorin (which has the most dramatic effects on the cells) show little evidence of the time-dependent uptake to be expected of active endocytosis (Fig. 1). Exogenous sialidases have been shown to exist [eg, in the case of transformed, but not normal, hamster fibroblasts (24)]. However, the quantity of free sialic acid made available in this way is unlikely to exceed a few μg/ml at most, and we have observed that free N-acetyl neuraminic acid at 5 μg/ml has no effect on L6 cells over a period of 24 h. The uptake of free gangliosides in the absence of serum is quite strikingly time-dependent. However thin layer chromatographic analysis of L6 cells incubated with tritiated gangliosides for 24 h shows the bulk of the label to cochromatograph with the original gangliosides.

There seem to be at least two reasonable sources of the changes seen, both of which could result from simple addition to the glycocalyx of an unnatural (or excessive) glycolipid or glycoprotein. The first is altered adhesiveness (increased or decreased). The second is perturbation of the membrane surface architecture (eg, changes in receptor orientation, arrangement, or exposure). We suggest that both of these could be operational regardless of whether the foreign ganglioside or glycoprotein is inserted into the membrane in a "natural" configuration. That is, the gangliosides need not have their ceramide portion inserted into the bilayer, and glycophorin need not be transmembrane for these effects to occur. We have previously reported evidence for ganglioside headgroup cooperative interactions (25, 26). We have extended these studies to obtain data which strongly suggest that non-specific carbohydrate interaction among glycolipids and glycoproteins is an important phenomenon at mammalian cell surfaces (Sharom and Grant, unpublished). Hence a physical "tangling" of the oligosaccharide portion of glycolipids or glycoproteins could provide a sufficient driving force both for association of exogenous carbohydrate-bearing components with myoblasts and for some of the effects produced by this association.

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