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Plant Lectins Detect Age and Region Specific Differences in Cell Surface Carbohydrates and Cell Reassociation Behavior of Embryonic Mouse Cerebellar Cells

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When plated at high cell density in a microwell culture system, freshly dissociated embryonic mouse cerebellar cells assemble into reproducible, 3-dimensional patterns. The addition of the dimeric lectin Succinyl Concanavalin A blocks reversibly the formation of the microwell pattern, suggesting that cell surface carbohydrates affect the reassociation behavior of embryonic mouse cerebellar cells.

Agglutination studies of dissociated cell populations harvested from different regions of the embryonic brain reveal that different lectins agglutinate cell populations from different embryonic brain regions. Cells from E13 cerebellum are agglutinated with Concanavalin A, wheat germ agglutinin, Ricinus communis agglutinin, mol wt 60,000, Ricinus communis agglutinin, mol wt 120,000, and Lens culinaris, but not by soybean agglutinin or a fucose-binding protein. Cells from the midbrain are agglutinated only with Concanavalin A, Ricinus communis agglutinin, mol wt 60,000 and Ricinus communis agglutinin, mol wt 120,000; those from the cerebral cortex are agglutinated only with Lens culinaris; and those from the medulla are agglutinated only with Ricinus communis agglutinin, mol wt 60,000, and Ricinus communis agglutinin, mol wt 120,000. In addition, agglutination of cerebellar cells with Concanavalin A, wheat germ agglutinin, and Ricinus communis agglutinin is diminished over the course of development from embryonic day 13 to postnatal day 7. These studies suggest regional differences in the cell surfaces of the developling brain that are further modulated during the differentiation of the tissues.

On a poly(D-lysine) treated substrate in microwell cultures, cell migration is unique to the cerebellum of the 4 brain regions studied. Surfaces treated with carbohydrate-derivatized poly(D-lysine) are currently being tested for their efficacy as substrates for differential cell migration.

Key words: plant lectins, microwell cultures, cell migration

Abbreviations: CMF – calcium- and magnesium-free-balanced salt solution; ConA – Concanavalin A; E13 – embryonic day 13; LCA – Lens culinaris hemagglutinin A; LCB – Lens culinaris hemagglutinin B; PBS – phosphate-buffered saline; RCAI – Ricinus communis agglutinin, mol wt 60,000; RCAII – Ricinus communis agglutinin, mol wt 120,000; SBA – soybean agglutinin; Succ-ConA – Succinyl Concanavalin A; WGA – wheat germ agglutinin.

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During embryonic mouse cerebellar development, immature neurons migrate from the sites where they are generated to the positions they will occupy in the mature neuronal network (1, 2). When embryonic cerebellar tissue is removed and plated as a single-cell suspension of high cell density in microwell cultures, a particular pattern of cell reassociation is evident (3). Microwell cultures will be used as a model system to monitor the reassociation behavior of suspensions of single cells from embryonic mouse cerebellum, as well as to measure in vitro cell migration.

Plant lectins are useful probes of micromolecules with exposed carbohydrates at cell surfaces (4–6). With 8 lectins the agglutination of cells harvested from various brain regions at different developmental stages was assayed to survey cell surface carbohydrates. In addition, nontoxic dimeric lecitns were used in microwell cultures to assess the influence of cell surface carbohydrates on cell reassociation behavior (7).

MATERIALS AND METHODS

Preparation of Single Cell Suspensions From Embryonic Mouse Brain

The embryos from litters at the 13th day of gestation were removed by laparotomy from freshly killed C57Bl/6J female mice. The cerebellum was removed by dissection (3), and transferred to 2.0 ml of CMF (NaCl, 8.00 g; KCl, 0.30 g; Glc, 2.00 g; $NaH_2PO_4 \cdot 2H_2O$, 0.5 g; KH_2PO_4, 0.25 g; 2.00 ml of 5% $NaHCO_3$; 0.5 ml of a 0.5% solution of phenol red in 480 ml distilled H_2O). The tissue was washed 3 times by sedimentation at 1 × g, and a single-cell suspension was prepared by gentle trituration with 2 or 3 fire-polished Pasteur pipets of decreasing bore size. This cell suspension was 85–95% single cells, and any remaining clumps of cells were removed by passing the cells through a Swinnex filter fitted with a double thickness of lens paper. The cells were sedimented (600 rpm in a Sorvall, model 6CC-1, Rotor HL-4 [Sorvall, Newton Conn.], 5 min, 4°C), and the pellet was resuspended in Eagle's basal medium (Hank's salts) supplemented with horse serum (10%), glucose (6 mM), glutamine (4 mM), and penicillin-streptomycin (25 units/ml each) for microwell cultures, or in PBS (NaCl, 8 g; KCl, 0.200 g; $Na_2HPO_4 \cdot 2H_2O$, 1.440 g; KH_2PO_4, 0.200 g; $CaCl_2 \cdot 2H_2O$, 0.132 g; $MgCl_2 \cdot 6H_2O$, 0.100 g per liter distilled H_2O) for agglutination assays. For culture assays, the cells were resuspended at 3–4 × 10^6 cells/ml. For agglutination assays, the cells were resuspended at 1–2 × 10^6 cells/ml. The viability of this preparation was 85–95% by Trypan blue exclusion (3).

Primary Microwell Cultures

Falcon microtest plates were pretreated with poly(D-lysine) (mol wt 150,000; 1.0 μg/ml in H_2O) for 2 h at 37°C as described (3). The plates were washed with distilled H_2O and air dried. Cells (10 μl) from the cell suspension prepared as above were introduced into the microwells with a Finnpipette fitted with a sterile tip. The viability of the cells 24 h after plating (3) was 60–80%. Incubation of the cultures was at 35.5°C with 5% CO_2 and 100% humidity.

One major advantage of microtest plates is the number of duplicate cultures possible (60 per plate). From each single-cell preparation from a litter of 6–8 embryos, 80–100 individual microwell cultures were established. The results reported are based upon cultures from 40 E13 litters (2,400–4,000 individual microwell cultures).

In 4 microwell experiments (240 microwell cultures), Succinyl-ConA (Succ-ConA) (1–500 μg/ml in Eagle's basal medium supplemented as above) was added to microwell

cultures 4 h after plating by exchanging 5 μl of the growth medium for 5 μl of the Succ-ConA solution.

Quantitation of the incidence of reaggregates, monolayer, and cables of processes were assayed on growing cultures by phase contrast on an inverted microscope.

Lectin-induced Agglutination Assays

These were performed according to Burger (8) with the single-cell preparation described above. The cells were tested for Trypan blue exclusion at the beginning and conclusion of the agglutination assays, and all results reported were from cell suspensions with 85–95% viability at both time points.

For tissues from postnatal animals, single-cell suspensions were prepared as described (3) with gentle trypsinization [crystalline 10 μg/ml, 10 min, 20°C, reaction stopped by ovomucoid (1 mg/ml), and trypsin removed by washing 3 times at 600 rpm, 5 min, 4°C]. Prior to the agglutination assay, the cells were preincubated in Eagle's basal medium supplemented with horse serum (1%) for 6 h at 35.5°C. The cells were washed 3 times with PBS to remove the serum. In some experiments, agglutination was assayed directly after the trypsinization step. All of the results reported in Tables I and II derive from 6 or more separate cell preparations. For each cell preparation, agglutination assays were performed in quadruplicate.

Materials

Microtest plates were obtained from Falcon Plastics (Brooklyn, New York) (No. 3034). Basal Medium Eagle's (Hank's salts), penicillin-streptomycin (25,000 units per ml, Lot 91035) and L-glutamine (200 mM in 0.85% NaCl) were purchased from Microbiological Associates (Bethesda, Maryland). Sera were purchased from GIBCO (horse serum, lot numbers 8350120 and 165322) and from Microbiological Associates (horse serum lot numbers 81428, 90111, 90467; fetal calf serum lot numbers 85260 and 86141; calf serum lot number 88985). Poly(D-lysine) (mol wt 150,000, type 1-B, hydrobromide, No. P-7761), methyl-α-D-mannoside, d-biotin, and ovomucoid (trypsin inhibitor, type 11-0, T9253) were

TABLE I. Region-specific Lectin-induced Agglutination of E13 Cells Harvested From Several Brain Regions*

Lectin	Hapten	Cerebellum	Midbrain	Cerebral cortex	Medulla	Liver
ConA	α-M-Man	200	200	1,000	1,000	115
WGA	D-Glc-NAc	50	500	500	500	25
RCAI	D-Gal	150	200	1,000	125	250
RCAII	D-Gal	75	125	1,000	80	175
SBA	D-Gal-NAc	1,000	1,000	1,000	1,000	1,000
Lotus	D-Fuc	1,000	1,000	1,000	1,000	1,000
LCA	D-Glc	50	250	75	1,000	250
LCB	D-Glc	100	500	75	1,000	500

*Cell suspensions were prepared as described in Materials and Methods without the use of enzymes. Agglutination was assayed according to Burger (8). Values are given as lectin required for half maximal agglutination (μg/ml). For WGA, values less than 400 represent good agglutination. For the other lectins tested, values less than 500 indicate agglutinable cells.

TABLE II. Developmental-stage-specific Agglutination of Cerebellar Cells*

Lectin	Hapten	E13	PO	P7
ConA	α-M-Man	200[a]	800 (200)	1,000 (200)
WGA	D-Glc-NAc	50[a]	450 (50)	500 (50)
RCAI	D-Gal	75[a]	1,000 (150)	1,000 (150)
SBA	D-Gal-NAc	1,000[a]	1,000[a]	1,000[a]
Lotus	D-Fuc	1,000[a]	1,000[a]	1,000[a]
LCA	D-Glc	50[a]	50[a]	60[a]
LCB	D-Glc	1,000[a]	150[a]	120[a]

*Cells were harvested from animals at postnatal days zero (P0) or 7 (P7) as described (3). Agglutinations were performed as described by Burger (8). Values are given as lectin required for half maximal agglutination (μg/ml). In some experiments, cells were trypsinized (0.01%, 10 min), pelleted, resuspended, and assayed for agglutination. Values in parentheses represent half maximal agglutination after trypsinization. For WGA, values less than 400 indicate good agglutination. For the other lectins tested, values less than 500 indicate good agglutination.

[a]Agglutinability not increased by trypsinization

purchased from Sigma Chemical Company (St. Louis, Missouri). Trypsin (crystalline, TRL 36C897, 194 μ/mg) was obtained from Worthington Biochemicals.

Highly purified ConA and WGA were the generous gifts of Dr. Max M. Burger, Biozentrum, Basel, Switzerland. All other lectins were purchased from Miles and further purified by affinity chromatography (3). Succ-ConA was the gift of Dr. R. J. Mannino, Biozentrum, Basel, Switzerland and its purity was as described (7).

In vitro cell migration was measured by a modification of the method of Bürk (9). A wound was made in the culture by gently vacuuming off a lane of cells with a micropipet mounted on a micromanipulator. The growth medium was removed and replaced with fresh medium without serum. The number of cells migrating into the wound was assayed by phase contrast with an inverted microscope.

RESULTS AND DISCUSSION

The studies reported here with a microwell culture assay of cell interactions in vitro and with plant lectins as probes of the molecular architecture of embryonic and postnatal brain cells reveal differences in cell reassociation behavior and cell surface features among tissues from several brain regions at different developmental ages.

A reproducible 3-dimensional pattern of reaggregates and interconnecting structures (Fig. 1) was formed by dissociated embryonic mouse cerebellar cells in microwell cultures. Within 4 h after the addition of the single-cell suspension to the microwells, reaggregates of 2,000–10,000 cells each had formed. After 6–24 h, the reaggregates were larger and straight interconnecting structures ("cables") were observed between them. These cables were invariably populated with a large number of neurons as well as cell processes. After 24–48 h in culture, a monolayer of phase-bright, highly refractile cells with processes was observed as a skirt around individual reaggregates. No further change in the organization of the pattern was observed for periods up to 6 weeks in culture.

These results suggest that embryonic mouse cerebellar cells have, under the described conditions, a particular reassociation behavior in microwell cultures. As described elsewhere (3), the details of this behavior depend on the serum supplement and the growth

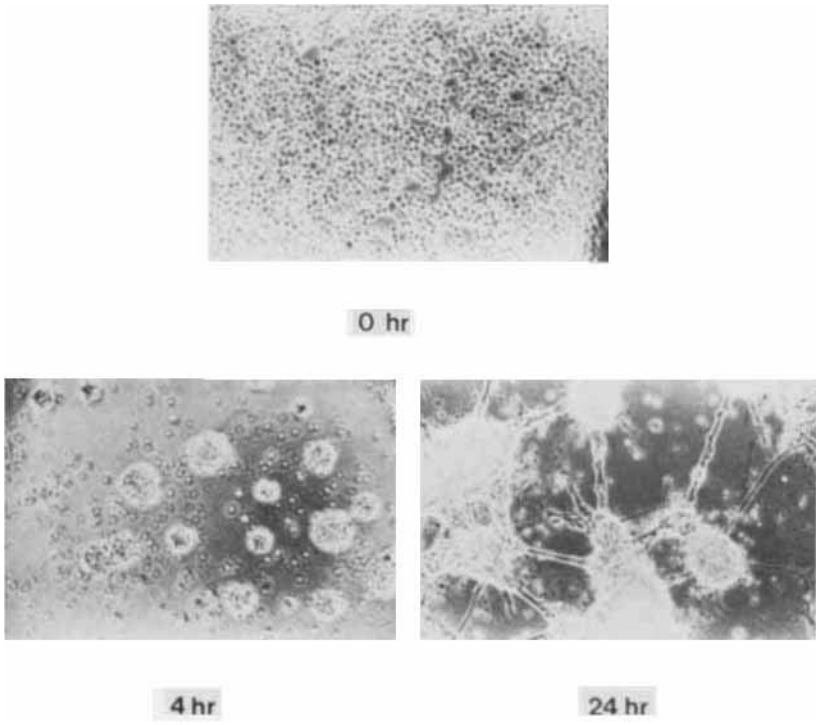

Fig. 1. The course of pattern formation by E13 cerebellar cells in microwell culture. Cerebellum was removed by dissection, washed twice in CMF, and triturated into a single-cell suspension with a series of fire-polished Pasteur pipets of decreasing bore. The cells were pelleted and resuspended at 3×10^6 cells/ml in Eagle's medium supplemented with glucose (6 mM), penicillin-streptomycin (25 units/ml), and horse serum (10%). The substratum was pretreated with poly(D-lysine). The pattern of reaggregates (R) and interconnecting cables (C) was formed after 24 h in culture. For details, see Ref. 3. × 150.

substratum, as well as the brain region and developmental stage of the tissue from which the cells were harvested. The question as to which cell types or interactions in the microwell cultures are the primary organizers of the pattern remains to be clarified. Available electron microscopic and electrophysiologic evidence (Fischbach and Hatten, unpublished) do, however, indicate that the E13 cerebellar cell population is predominantly neuronal.

The reassociation events required to form the described microwell pattern were influenced strongly by addition of the nontoxic, dimeric lectin Succ-Con A. In a growth medium supplemented with 6 mM glucose, low concentrations of Succ-ConA inhibited cable extension in the cultures by a mechanism that was reversed within 4 h by the removal of Succ-ConA and was blocked by preincubation of the cultures with methyl-α-D-mannoside (Fig. 2). The dose dependence of this effect is given in Fig. 3. These observations suggest that cable extension in microwell cultures relates to cell surface carbohydrates.

The binding of Succ-ConA could interrupt cable extension by several general mechanisms. Since Succ-ConA is added 4 h after plating, it is unlikely that it interferes with cell attachment to the substratum or the initial aggregation of the cells. However, Succ-ConA could influence cell interactions (or movements) within the reaggregates subsequent to the initial aggregation that signals cable extension. Alternatively, the binding of

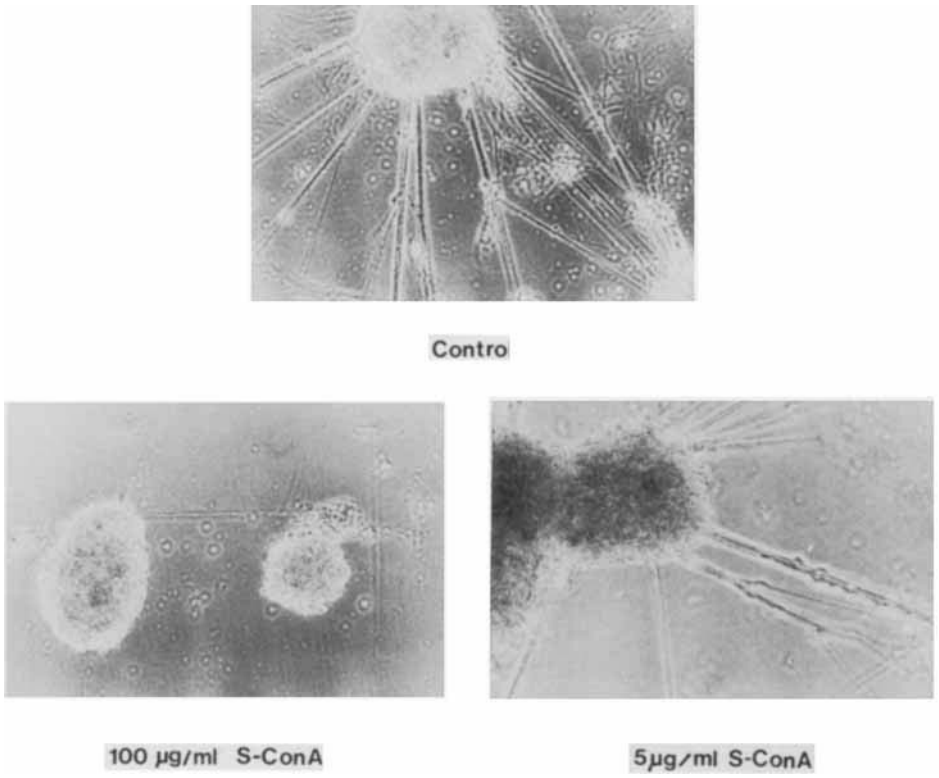

Fig. 2. Effects of Succ-ConA on pattern formation by E13 cerebellar cells in microwell culture. Succ-ConA was added at the indicated dose 4 h after plating. × 250.

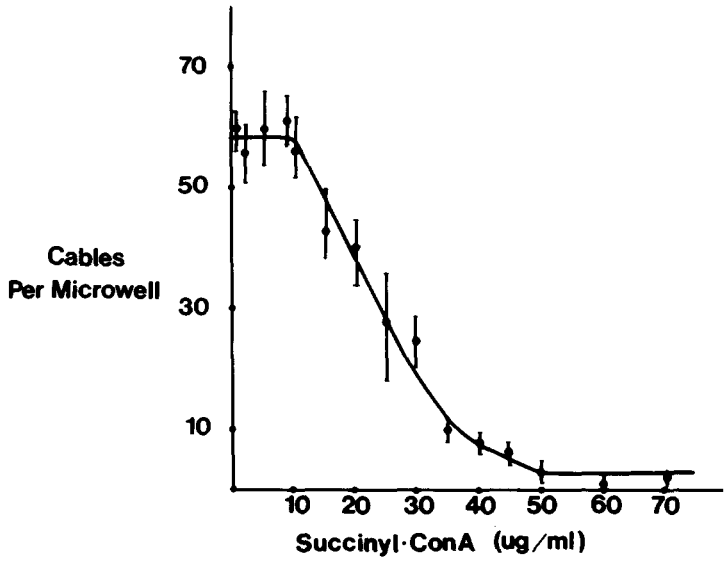

Fig. 3. Effect of Succ-ConA on cable formation in E13 cerebellar cultures. Succ-ConA was added at the indicated concentration 4 h after plating. Counts of the number of cables per microwell were made by phase contrast with an inverted microscope 96 h after plating.

Succ-ConA to the surface of fiber producing cells could inhibit the lengthening of cables per se. At any rate, these results suggests that conditions for cable extension relate, at least in part, to the cell surface.

Eight lectins were used to survey cell surface carbohydrate-containing macromolecules with cells harvested from several brain regions at different developmental stages.

Lectin-induced agglutination assays with single cell suspensions from various brain regions at several developmental stages revealed differences in agglutinating activity. E13 cells from the cerebellum, midbrain, and liver, but not cerebral cortex or medulla, were agglutinated with ConA (Table I). The reaction was temperature-dependent as previously reported for non-neuronal cells (10) and was inhibited by preincubation of the cells with methyl-α-D-mannoside. E13 cells from medulla and cerebral cortex were rendered agglutinable with ConA by gentle trypsinization (10 μg/ml, 10 min, 37°C).

E13 cells from cerebellum and liver, but not other brain regions, were agglutinated by WGA. The reaction was temperature-independent as reported for nonneuronal cells (10), and was inhibited by N-acetyl-D-glucosamine. E13 cells from midbrain, medulla, and cerebral cortex were rendered agglutinable with WGA, however, by gentle trypsin treatment (10 μg/ml, 10 min, 37°C). Cells from cerebellum, midbrain, medulla, and liver, but not cerebral cortex, were agglutinated by both Ricinus communis lectins I and II (RCAI and RCAII), a reaction that was inhibited by preincubation of the cells with D-galactose. Nonagglutinable E13 cells from cerebral cortex were rendered agglutinable with RCAI or RCAII by gentle trypsin treatment (10 μg/ml, 10 min, 37°C).

E13 cells from cerebellum, midbrain, and cerebral cortex were agglutinated with Lens culinaris A (LCA) lectin, a reaction that was inhibited by D-glucose. E13 cells from cerebellum and cerebral cortex, but not midbrain, medulla, or liver, were agglutinated by Lens culinaris B (LCB) lectin, a reaction inhibited by D-glucose. Trypsinization of cells that did not agglutinate with LCA or LCB lectins did not alter these results.

None of the cells tested were agglutinated with either the soybean agglutinin (SBA) or a fucose-binding protein from lotus seeds. Trypsinization (10 μg/ml, 10 min, 37°C) of the cells did not alter these results.

In addition to regional differences in the lectin-induced agglutination of developing neurons, developmental stage-specific alterations in lectin-induced agglutination were observed (Table II). The concentration of lectin required for the half maximal agglutination of E13 cerebellar cells was increased markedly for cerebellar cells harvested after embryonic day 16. As shown in Table II, postnatal cerebellar cells had little agglutinating activity with any of the 7 lectins tested except LCA. Unlike the other lectins tested in these studies, LCA agglutinates most nontransformed rather than transformed cell lines. (4, 5). Postnatal cerebellar cells were agglutinable with ConA, RCA, or WGA following gentle trypsinization (10 μg/ml, 10 min, 37°C).

The agglutination of embryonic neuronal populations appears to occur by a mechanism similar to that reported for nonneuronal cells, since the same hapten inhibition and temperature dependence is observed for both cell types (10, 12, 13). The differences reported here for lectin-induced agglutination probably do not reflect simple differences in intercellular adhesion, since recent studies (14, 15) have demonstrated that lectin-induced agglutination and spontaneous aggregation occur by separate mechanisms.

Agglutination with plant lectins is a complex process that can reflect, in addition to the carbohydrate composition of the receptor(s), more general cell surface features such as receptor availability, mobility, valence, and anchorage to a cytoskeletal network of submembranous elements (4–6). Although these results cannot be interpreted specifically as differences in carbohydrate-containing surface macromolecules among several brain

TABLE III. In Vitro Cell Migration of E13 Embryonic Cerebellar Cells*

Tissue origin	Migration (%)	Time (h)
Cerebellum	100	2
Midbrain	0	24
Cerebral cortex	0	24
Medulla	50	24

*In vitro cell migration was measured by a modification of the method of Bürk (9). A wound was made in the culture by gently vacuuming off a lane of cells with a micropipet mounted on a micromanipulator. The growth medium was removed, and replaced with fresh medium without serum. The number of cells migrating into the wound was assayed by phase contrast with an inverted microscope. Values are given as percent of wound area occupied by cells at the given time.

regions at different stages of development, they do suggest region-specific alterations in the cell surfaces of developing neurons.

Changes in lectin-induced agglutination over the course of chick retinal development have been suggested previously (11). However, one critical difference between those early studies and the present results is that these embryonic brain cells were not treated with proteases or with EGTA prior to the agglutination assays.

Studies with fluorescein-conjugated lectins are currently in progress to determine the amount of lectin bound by different cell types in particular brain regions. Preliminary evidence (Hatten, Lekić, and Schachner, unpublished) indicates that the E13 cerebellar population is heterogeneous with respect to lectin binding. It is possible that kinetic studies with lectins and carbohydrate haptens will detail further differences in carbohydrate-containing macromolecules during cerebellar development.

Cell migration is a major force in the modeling of the cerebellar neuronal network. We are therefore in the process of developing a model system to assess in vitro embryonic cerebellar cell migration in microwell cultures, and to ask whether migration events relate to the presence of particular carbohydrates in the substratum.

On a poly(D-lysine)-treated substratum, in vitro cells of the embryonic cerebellum migrated much more than cells of the other regions tested (Table III). In addition, considerable movement of whole reaggregates was observed. We are currently examining whether culture substrate treated with poly(D-lysine) derivatized with one or another of the cell surface carbohydrate moieties that had been detected in the agglutination assays will have different efficacies for cell migration.

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