Laminin Is Produced by Early Rat Astrocytes in Primary Culture

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ABSTRACT The production of laminin by early rat astrocytes in primary culture was investigated by double immunofluorescence staining for laminin and the glial fibrillary acidic protein (GFAP), a defined astrocyte marker. In early cultures (3 d in vitro; 3 DIV) cytoplasmic laminin was detected in all the GFAP-positive cells which formed the major population (80%) of the nonneuronal cells present in cultures from 20–21-d embryonic, newborn, or 5-d-old rat brains. Monensin treatment (10 μM, 4 h) resulted in accumulation of laminin in the Golgi region, located using labeled wheat germ agglutinin. Laminin started gradually to disappear from the cells with the time in culture, was absent in star-shaped, apparently mature astrocytes, but remained as pericellular matrix deposits. The disappearance of cellular laminin was dependent on the age of the animal and the time in culture so that it started earlier in cultures from 5-d-old rat brains (5 DIV) and approximately following the in vivo age difference in cultures from newborn (12 DIV) and embryonic (14 DIV) rat brains.

Our results indicate that laminin is a protein of early astrocytes and also deposited by them in primary culture, thus suggesting a role for this glycoprotein in the development of the central nervous system.

The development and migration of brain cells have been extensively studied (1–3), and the timing for the appearance of the glial fibrillary acidic protein, a defined astrocyte marker (4–6) both in brain and in primary culture, has been well documented (4, 7–9).

In several studies on primary cultures the development of neurons and glial cells have been shown to closely follow the pattern of the in vivo development (8–13); tissue culture systems may thus serve as experimental models for central development and cell interactions (14, 15).

Laminin, one of the matrix glycoproteins (16), is present in all basement membranes so far investigated (16–18) and is produced by various epithelial cells in culture (16, 19). It is also produced by mouse neuroblastoma cells (20), in which its pattern of localization suggests a role for laminin as their adhesion protein.[1] In adult mouse brain, laminin has been found only in connection with blood vessel walls (21), but little is known about the expression of laminin in normal developing cells of neuroectodermal origin. Fibronectin, the other major noncollagenous matrix glycoprotein, important in normal development of several types of cells (22, 23), has been demonstrated to be present in vessel walls in developing and adult rat brain (24). In primary brain cell cultures, fibronectin is thought to be due to mesenchymal cell contamination (25).

In the present study, we used primary cultures from early rat brain to study the possible role of laminin for development of central cells of neuroectodermal origin. We demonstrate that laminin, but not fibronectin, is produced by all astrocytes in early primary cultures from embryonic, newborn, and 5-d-old rat brains and also deposited by them. These results suggest a role for laminin in the development of central nervous system and possible involvement in brain cell interactions.

MATERIALS AND METHODS

Primary Cultures: Brains of 20–21-d embryos ($n = 15$), newborn ($n = 30$), and 5-d-old ($n = 15$) albino rats (*Sprague-Dawley strain*) were used to prepare cell cultures of cerebellum, neostriatum, and substantia nigra, as previously described (26). The cells were seeded onto glass coverslips and maintained at +37°C in a humidified 5% CO_2 atmosphere, fed with Eagle's minimum essential medium supplemented with 20% fibronectin-free fetal calf serum, prepared as previously described (27), and no antibiotics. After 3 d in vitro (3 DIV) the cells had attached to the glass coverslips and could be used for experiments.

Monensin Treatment of the Cultures: The cells were cultured for 3 d, then fresh culture medium was changed and the cell layers were incubated for 4 h at +37°C in the presence of 10 μM monensin (sodium salt; Calbiochem-Behring Corp., La Jolla, CA), known to block transport of secretory proteins from the Golgi complex (28). The monensin exposure was terminated by a short rinse in 0.01 M sodium phosphate, 0.14 M sodium chloride, pH 7.4 (PBS),

[1] Liesi, P. 1983. Laminin in cultured mouse C1300 neuroblastoma cells: immunocytochemical localization by pre- and postembedding electron microscope procedures. *J. Histochem. Cytochem.* In press.

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followed by fixation of the cell layers with 3.5% paraformaldehyde in PBS for 15 min at room temperature. Then the cell layers were rinsed in PBS overnight at +4°C and permeabilized with cold methanol (−20°C) before they were stained for laminin. Rabbit antibodies against laminin were those used previously and their specificity was confirmed (20, 21).[1] They were used at a dilution of 1:100 and applied for 1 h at room temperature. The cell layers were then rinsed in PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit immunoglobulins (Wellcome Research Laboratories, Beckenham, U.K.) diluted 1:20 for 30 min at room temperature. After a rinse in PBS, the Golgi complexes of the cells were demonstrated. For that, the cell layers were incubated with tetramethylrhodamine-isothiocyanate (TRITC)-conjugated wheat germ agglutinin (WGA) (100 μg/ml; Vector Laboratories, Inc., Burlingame, CA) that has been shown to bind to the Golgi region (29). The cell layers were embedded in PBS-glycerol (1:1) and viewed with a Leitz Dialux EB 20 microscope with epi-illumination. The light source was a HBO 200 high pressure mercury lamp. The filterblocks K2 (excitation 470–490) nm; cut-off 515 nm) and N (excitation 530–560 nm; cut-off 580 nm) for FITC and TRITC-fluorescence, respectively, were used.

Double-Immunofluorescence Staining for GFAP and Laminin Using Primary Antisera from the Same Species: The cell layers were fixed with 3.5% paraformaldehyde and permeabilized with cold (−20°C) methanol as described above and were then rehydrated through alcohols (30) before the first antibody step. This consisted of rabbit antilaminin antibodies diluted 1:100, applied for 1 h at room temperature. The cell layers were then rinsed in PBS and incubated with FITC-conjugated sheep anti-rabbit immunoglobulins. After a wash in PBS the same cell layers were incubated with rabbit anti-GFAP antibodies diluted 1:100 for 1 h at room temperature. The specificity of this antiserum has been confirmed previously (4, 5, 31). The cell layers were rinsed in PBS after which TRITC-conjugated goat anti-rabbit immunoglobulins (Nordic Immunological Laboratories, Tilburg, The Netherlands) diluted 1:20 were applied.

In control experiments either antilaminin or anti-GFAP were substituted with PBS or antibodies preabsorbed with 50 μg/ml of the corresponding antigen purified as described (31; mouse laminin was a kind gift from Dr. R. Timpl, Max-Planck Institut für Biochemie, W. Germany). All the cell layers were mounted in PBS-glycerol (1:1).

Double-Immunofluorescence Staining for GFAP and Laminin Using Primary Antisera from Different Species: Laminin staining was performed as described above. Then the cell layers were incubated with mouse anti-GFAP antibodies diluted 1:100 for 1 h at room temperature. Production and use of this antiserum has been described elsewhere (9). Then the cell layers were rinsed in PBS and incubated with TRITC-conjugated sheep anti-mouse immunoglobulins (N. L. Cappel Laboratories Inc., Cochranville, PA) diluted 1:40. After a final rinse in PBS they were mounted in PBS-glycerol (1:1).

Immunofluorescence Staining for Fibronectin and Neurofilaments: The cell layers were treated as done for double-staining till the first antibody step. This now consisted of either rabbit antifibronectin antibodies (preparation and specificity described in reference 32) diluted 1:200 or rabbit anti-chicken neurofilament antibodies (33, 34) diluted 1:100, applied for 1 h at room temperature. After a rinse in PBS the cell layers were incubated with FITC-conjugated anti-rabbit immunoglobulins rinsed again and mounted in PBS-glycerol (1:1).

RESULTS

Rat Brain Cell Cultures

Primary cultures were established from three specific brain regions (cerebellum, neostriatum, substantia nigra) of embryonic (20–21 d of gestation), newborn, and 5-d-old rats. After 3 DIV, regardless of the age of the animal used to initiate cultures, nonneuronal cells formed monolayers that were attached to glass coverslips, and neuronlike cells could be seen attached to this monolayer. Neurons were identified by their characteristic morphology and, in some cultures, by using neurofilament antibodies and astrocytes using GFAP as marker

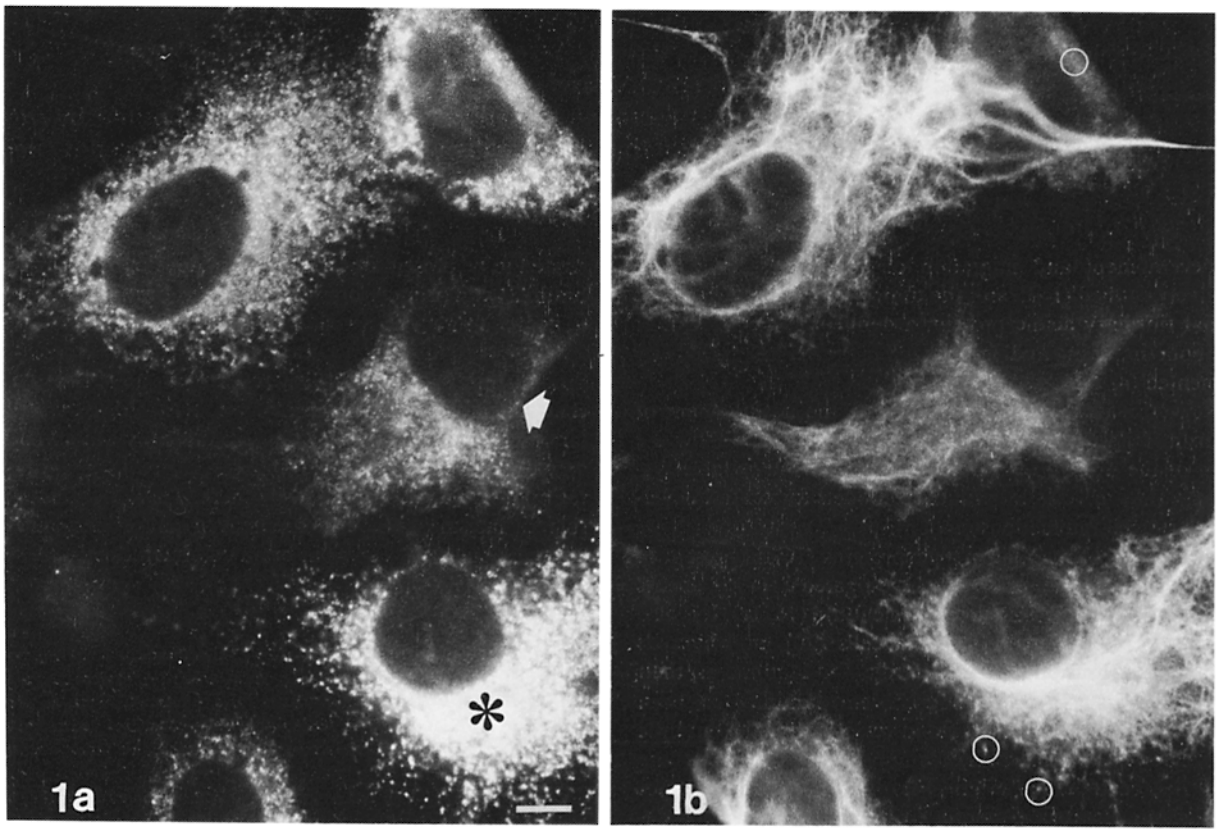

FIGURE 1 Double-immunofluorescence staining of a primary culture from newborn rat brain (cerebellum). 3 DIV. (*a*) Laminin staining using rabbit antiserum shows that the nonneuronal cells have moderate (arrow) to intense (asterisk) immunoreactivity. FITC-fluorescence. (*b*) The same nonneuronal cells are astrocytes as revealed by immunofluorescence staining for GFAP by using rabbit antiserum. TRITC-fluorescence. Note that some of the laminin is also stained (open circles) by the TRITC-conjugated anti-rabbit IgG. Bar, 10 μm. × 800.

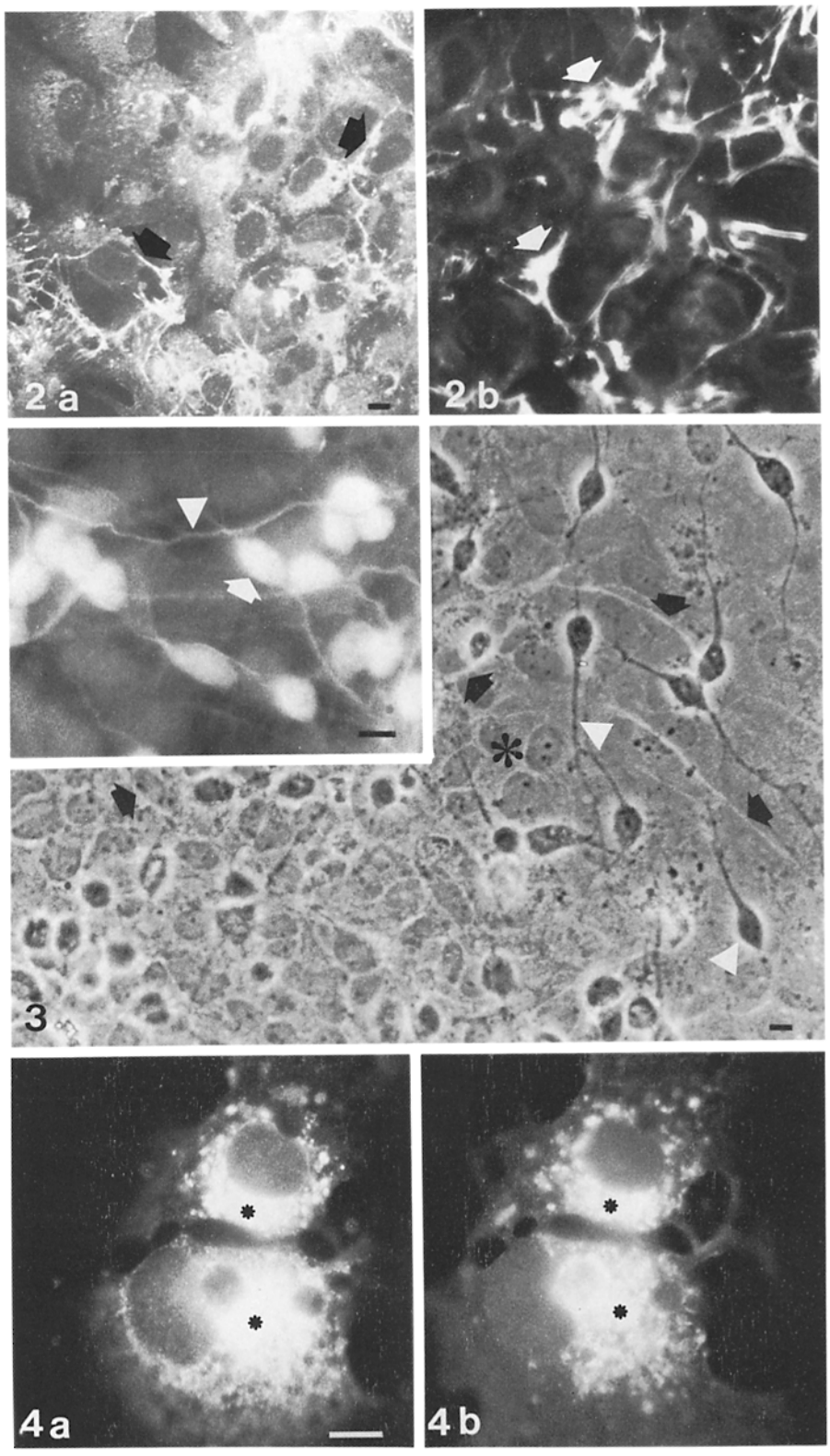

FIGURE 2-4 Laminin immunofluorescence in primary cultures from newborn rat brain (cerebellum). (*a*) 7 DIV. All the nonneuronal cells are weakly to moderately positive for laminin at the central parts of monolayer areas. A laminin matrix is visible (arrows). (*b*) 10 DIV. Cytoplasmic laminin immunoreactivity has almost completely disappeared. Weak laminin immunoreactivity can be seen in some of the monolayer cells. Laminin matrix is prominent (arrows). Bar, 10 μm. × 300 Fig. 3: A combined phase-contrast and laminin immunofluorescence micrograph of a primary culture from newborn rat brain (substantia nigra). 15 DIV. Laminin-positive fibers (arrows) on top of monolayer cells (asterisk) are frequently in connection with neuron-like cells and their fibers (arrowheads). × 300. The inset shows neuronlike cells stained with neurofilament antibodies. Both nerve cell bodies (arrow) and fibers (arrowhead) contain the specific immunofluorescence. × 500. Bar, 10 μm. Fig. 4: Double-immunofluorescence staining of a monensin-treated primary culture from newborn rat brain (cerebellum). 3 DIV. (*a*) Laminin-immunoreactivity is intense and is now confined to the perinuclear region (asterisk). FITC-fluorescence. (*b*) The Golgi complexes of the same cells (arrowheads) were demonstrated by TRITC-conjugated wheat germ agglutinin (asterisk) and a clear correspondence with laminin staining was found. TRITC-fluorescence. Bar, 10 μm. × 800.

(see below). As the nonneuronal cells had spread and formed monolayers, neurons were seen attached above. Cultures established from cerebellum contained small bipolar and tripolar neurons with thin processes and large neurons morphologically resembling Purkinje cells in situ (35, 36). With prolonged time in culture, the ratio of nonneuronal/neuronal cells appeared to increase.

Laminin in Primary Cultures

After 3 DIV, as soon as the cells had attached to glass coverslips, all the nonneuronal cells in cultures were strongly to moderately positive for laminin. Laminin had a cytoplasmic granular distribution and could be demonstrated by double immunofluorescence in the same cells as GFAP, an interme-

diate filament protein with fibrillary staining pattern. This was even possible with two primary rabbit antisera (Fig. 1) used in the following sequence: antilaminin, anti-rabbit-IgG-FITC, anti-GFAP, and anti-rabbit-IgG-TRITC. FITC now indicated only laminin and TRITC some laminin, but also GFAP in its typical fibrillar distribution. The presence of laminin and GFAP in the same cells was also verified using rabbit antilaminin and mouse anti-GFAP antibodies in double staining experiments, and specificity of each staining reaction was controlled using blocked antibodies (figures not shown).

The above staining procedures showed that astrocytes, after 3 DIV, comprised ~80% of all the nonneuronal cells and were positive for laminin regardless of the age of the animal used to initiate the cultures. The GFAP-negative nonneuronal cells (~20% of the nonneuronal cell population) were morphologically flat and large and some of them also were positive for fibronectin. These fibronectin-positive cells were not studied further. At this stage (3 DIV), weak laminin matrix was also present and deposited on the top of non-neuronal cells. The cytoplasmic laminin immunoreactivity in astrocytes gradually disappeared depending on the age of the animal and time in culture. In cultures started from 5-d-old rat brains, the central parts of monolayer areas were negative for laminin immunoreactivity by 5 DIV, and only at the edge of these areas were there astrocytes positive for laminin. The same was true in cultures from newborn rat brain by 12 DIV (Fig. 2) and in cultures from embryonic rat brain by 14 DIV. By 30 DIV, all the cultures were virtually devoid of laminin immunoreactive astrocytes. In contrast, the intensity of GFAP immunofluorescence increased with time in culture. No change in GFAP staining pattern was seen between the different brain regions in culture. In older cultures a few GFAP-positive, star-shaped astrocytes with thin, long extensions could be found—these cells were negative for laminin. As the cultures grew older, the laminin matrix became more extensive and was occasionally present between nonneuronal cells and more often, although not consistently, in connection with neuronlike cells and their processes (Fig. 3). The neuronlike cells never exhibited immunofluorescence for laminin.

Monensin Treatment of the Cultures

In untreated cultures some laminin immunoreactivity, when intense enough, could be located to the Golgi region, but more consistently the Golgi area showed the weakest immunoreactivity. Monensin is known to block the transport of secreted glycoproteins from the Golgi apparatus (28). Monensin caused clear vacuolization of all nonneuronal cells and accumulation of laminin within the Golgi region (Fig. 4), which also indicated that the laminin-positive cells synthesized laminin themselves. Double-staining with antilaminin and anti-GFAP confirmed that, in all the astrocytes, laminin was accumulated within the Golgi regions.

DISCUSSION

The present results demonstrate the presence of laminin, a major noncollagenous matrix glycoprotein (16), in early astrocytes cultured from developing rat brain. The monensin experiments indicate that laminin is produced by these cells and is processed, as in mouse neuroblastoma cells,[1] through the Golgi pathway. In older cultures laminin was also strongly deposited in pericellular matrix form, not under the cells as in epithelial cell cultures (37), but on top of the astrocyte monolayer commonly between them and neuronlike cells. All neuronlike cells in the primary cultures were negative for laminin.

Our results also showed that laminin disappeared from astrocytes with the time in culture depending on the initial age of the animal following closely the in vivo age difference of the brain tissue used to initiate cultures. Several studies on in vitro development of central tissues have reported a correspondence with the normal brain development in vivo (8–13). Thus the observation that laminin is detected in adult mouse brain only in association with capillary structures (21) may not be in contradiction with our work here. Previously, developmental changes were observed for laminin in differentiation of tubular epithelial cells from the mouse nephrogenic mesenchyme (38).

In the primary cultures, only few fibronectin-positive cells were present and, in agreement with previous reports (25, 39, 40), generally lacked GFAP. Cultured human cell strains with some astrocytelike properties and cultured human astrocytoma cels (41, 42) have been shown to produce fibronectin, but it is not clear how closely these cells relate to those present in primary brain cell cultures.

Little is known about the extracellular components of the central nervous system. Synaptic cleft proteins have been partially purified (43, 44) and there is also evidence for the presence of cell-to-cell contacts mediated by extracellular material that has not been identified (45, 46). The fact that, simultaneously and with the disappearance of intracellular laminin from astrocytes, extensive laminin matrix is deposited on the top of the cells and between them and neuronlike cells suggests that laminin might play a role in their interactions.

Support for a role for laminin in cell-to-cell interactions is given by the distribution of laminin in cultures of mouse neuroblastoma cells.[1] Moreover, besides diffusible factors (47, 49), contacts with artificial substrates (50), matrix proteins (51), or nonneuronal cells (52, 53) have been shown to be important for neuronal survival.

In conclusion, our results demonstrate that laminin, but not fibronectin, is produced and deposited by all astrocytes present in early cultures from the developing rat brain. We also describe the time-dependent disappearance of laminin from these cells in culture to follow the age difference of the animals used to initiate the cultures. These observations raise the possibility that laminin may have a guiding and supporting role in developing brain where the cells mature and migrate and neuronal pathways are formed.

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