Production of Laminin and Fibronectin by Schwannoma Cells: Cell-Protein Interactions In Vitro and Protein Localization in Peripheral Nerve In Vivo

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ABSTRACT We studied a rat Schwannoma cell line (RN22F) to determine if it produced the basement membrane glycoproteins laminin and fibronectin, and how it interacted with these proteins in vitro. We used antisera to laminin and fibronectin for immunoprecipitation experiments and immunocytochemical localization at the electron microscope level. Polyacrylamide gels of antilaminin immunoprecipitates of conditioned medium and solubilized Schwannoma cells contained bands of reduced Mr 200,000 and 150,000. Antilaminin immunoprecipitates of conditioned medium contained nonreduced bands of 850,000 daltons and 150,000, and immunoprecipitates of solubilized cells contained nonreduced bands of 850,000, 400,000, 200,000, and 150,000 daltons. Antifibronectin immunoprecipitates of conditioned medium contained a reduced band of 220,000 daltons, and nonreduced bands of 440,000 and 220,000 daltons. Radio-labeled protein was not detected in antifibronectin immunoprecipitates of solubilized cells. By immunocytochemistry, laminin was found along the cell surface in a continuous band, whereas fibronectin was only sparsely distributed along the cell surface. In cell adhesion assays, Schwannoma cells bound preferentially to laminin-coated substrates as compared to fibronectin or noncoated substrates. A number of Schwannoma cells displayed a curved and elongated morphology on laminin substrates, as compared to a uniformly spread morphology on fibronectin, and a round, nonspread morphology on noncoated substrates. Immunofluorescent staining showed laminin in the endoneurium and perineurium and fibronectin predominantly in the perineurium of mouse sciatic nerve in vivo. The production of laminin and fibronectin by Schwann cells may be important in the development and myelination of peripheral nerves, and the proper regeneration of axons following nerve injury.

Schwann cells are the supporting structures for nerve processes in the peripheral nervous system. In myelinated nerves, the Schwann cells form the myelin sheath. Schwann cells are associated with a basement membrane. From in vivo and in vitro studies of neurons and Schwann cells, it has been found that basement membrane is produced by the Schwann cells (1–3). The nerve sheath formed by Schwann cells is important in proper regeneration of damaged axons (4). In vitro, Schwann cells do not produce a basement membrane until they have become committed to an axon or another Schwann cell (5). Nerve cells, which do not produce a basal lamina in culture, appear to exert some trophic influence on Schwann cells, stimulating the Schwann cells to produce basement membrane and collagen fibrils (3).

Basement membranes, such as produced by Schwann cells, serve several functions in the body. Basement membranes form the semipermeable membrane of the kidney glomerulus and act as supporting structures in the lens capsule of the eye and under vascular endothelium. Basement membranes can serve as a matrix or form to guide tissue regeneration (for review, see reference 6). At present, there are three known major constituents of basement membrane: collagens, specifically type IV collagen, possible some type V collagen; noncollagenous glycoproteins, among which are laminin, fibronectin, and entactin (7–10); and glycosaminoglycans, such as heparan sulfate (11). Laminin was first isolated from the mouse EHS tumor (12), and independently from a mouse embryonal carcinoma-derived cell line, where it was called GP-2 (13). Laminin is a high...
molecular weight glycoprotein of $M_r$ 850,000 or greater (14, 15). Laminin is composed of disulfide-bonded subunit chains of 400 and 200 kdaltons, with a possible stoichiometry of one 400-kdalton chain to three 200-kdalton chains (12, 15). In vivo, laminin has been found in basement membranes throughout the body, including the glomerular basement membrane, vascular endothelial basement membrane, and underlying the epithelium of the skin (7, 8). Laminin promotes the attachment of epithelial cells to type IV collagen (16), and increases the adhesion of carcinoma cells to substrates in vitro (17). Cells from regenerating liver show enhanced adhesion to laminin during the regeneration period (18). A number of cell lines grown in vitro produce laminin, including cells from endothelium, embryonal carcinomas, parietal yolk sac tumor, breast adenocarcinoma, rat liver epithelium, and rat breast epithelium. The laminin molecule can bind to glycosaminoglycans (19), particularly heparin (20).

Fibronectin is a high molecular weight glycoprotein of $M_r$ 440,000 with two apparently similar disulfide-bonded chains of ~220,000. Fibronectin is found in plasma, and is a major component of basal lamina, connective tissue, and the extracellular matrix produced by fibroblasts in vitro and in vivo (for reviews, see references 21–24). Fibronectin is involved in the adhesion of various cells to collagen in vitro, including fibroblasts, Chinese hamster ovary cells, and platelets (25–27). The fibronectin molecule binds to fibrin (28), heparin (29), and staphylococci (30). In development, fibronectin may play a role in muscle morphogenesis (31), in the interaction of mesenchymal cells with collagenous matrices during endochondral bone formation (32), and in the spatial organization of cells in developing chick wing (33).

A clonal line of rat Schwann cells (RN2) has been described that synthesizes collagens in vitro (34). A subclone of this line (RN22) undergoes an apparent differentiation, as evidenced by morphological change, when treated with cyclic nucleotide analogues (35). In this study, we examined cell line RN22F to determine if the line made noncollagenous basement membrane associated proteins, and whether these cells interacted with basement membrane proteins in vitro.

**MATERIALS AND METHODS**

**Cells**

Cells used in this study were line RN22F, a clonal line of rat Schwannoma cells. The cells were a gift from Dr. John R. Sheppard (Dight Institute of Genetics, University of Minnesota). This clone was derived in Dr. Sheppard's lab from line RN2, a rat Schwannoma cell line originally established from Schwann tumors (neurilemmomas) of rats induced chemically by Dr. S. E. Sheppard's lab from line RN22, a rat Schwannoma cell line originally established in Dr. Pfeiffer (University of Connecticut). The RN22F dnnal line retains the ability of staphylococci (30). In development, fibronectin may play a role in muscle morphogenesis (31), in the interaction of mesenchymal cells with collagenous matrices during endochondral bone formation (32), and in the spatial organization of cells in developing chick wing (33).

**Fibronectin**

Fibronectin was isolated from human plasma using gelatin affinity chromatography, as previously described (36). Laminin was isolated from the mouse EHS tumor (kindly provided by Dr. George Martin [National Institutes of Health]) using a modification of the method of Timpl et al. (12). Tumor was homogenized in 3.4 M NaCl, 0.01 M phosphate buffer pH 7.4, with 50 mg/ml phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, MO) and 50 mg/ml p-hydroxymercuribenzoate (Sigma Chemical Co.), washed once with the same buffer, then extracted overnight with 0.5 M NaCl, 0.01 M phosphate, pH 7.4, and 50 mg/ml each of PMSF and p-hydroxymercuribenzoate. Type IV collagen was removed by raising the salt concentration to 1.7 M followed by stirring for 1 h at 4°C, and centrifugation (10,000 rpm, 30 min). Laminin was precipitated from the extract with 30% saturation ammonium sulfate. The precipitate was resuspended in 0.5 M NaCl, 0.01 M phosphate, pH 7.4, and dialyzed against the same buffer. Laminin was isolated from the precipitate by gel filtration chromatography on Sephacyr S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) (1.26 x 100 cm column) in the 0.5 M NaCl buffer, where it eluted just after the void volume. The laminin containing peak was dialyzed against 0.14 M NaCl, 0.01 M phosphate buffer, pH 7.4 (phosphate-buffered saline [PBS]), then chromatographed on a heparin-Sepharose affinity column (Pharmacia Fine Chemicals) eluted with PBS, and eluted with 0.5 M NaCl, 0.01 M phosphate, pH 7.4. The protein solution was boiled by evaporation while in a dialysis bag, redialyzed against 0.5 M NaCl, 0.01 M phosphate, pH 7.4, and stored at ~70°C or filter sterilized with a Millipore 0.45-µm pore sterile filter (Millipore Corp., Bedford, MA) and refrigerated.

**Antibody Production and Isolation**

We immunized New Zealand White rabbits with multiple subcutaneous injections of laminin and fibronectin. Antibodies to laminin antigen were made from rabbits immunized with enzyme-linked-immunosorbent assay (ELISA) antigen prepared from rat antiserum to entactin was a generous gift from Dr. Albert Chung (University of Pittsburgh).

**Immunoprecipitation**

Confluent RN22F cultures in 35-mm tissue culture plates (Falcon 3001; Falcon Labware, Oxnard, CA) were radio-labeled by overnight incubation with 1 ml of serum free medium containing 30 µCi/ml [H]leucine (110 Ci/mmol) or [H]-amino acids (both from New England Nuclear, Boston, MA). Conditioned medium was collected, the cell layer washed with PBS with Ca ++ and Mg ++, and the cells solubilized with 1 ml per plate of 100 mM NaCl, 2 mM EDTA, 10 mM Tris, pH 7.2, 1% Nonidet P-40 (NP-40, Particle Data Services, Inc., Elmhurst, IL), 0.5% sodium deoxycholate and 1% Trisyl (Sigma Chemical Co.). The cell extract was clarified by centrifugation for 1 h at 15,000 rpm. For each sample, cells or medium from two 35-mm plates were pooled, and the solutions incubated with 20 µl of antiserum or pre-immune control serum for 30 min at 22°C. Immune complexes were adsorbed by adding 200 µl of a 10% suspension of washed Protein A-containing Staphylococcus aureus (Pansorb, Calbiochem-Behring Corp., La Jolla, CA). The bacteria/immune complexes were washed once in 1 M NaCl, 10 mM Tris, pH 7.2, 0.1% NP-40, and twice in 150 mM NaCl, 10 mM Tris, pH 7.2, 1% Triton X-100 and 1% SDS. Precipitates were dissociated under reducing or nonreducing conditions. For reducing conditions, samples were heated for 1 min, 100°C, in sample buffer containing 0.08 M Tris, pH 6.8, 3% SDS, 15% glycerol, 5% mercaptoethanol, and 0.5% bromophenol blue. For nonreducing conditions, samples were mixed at 22°C with buffer containing 1 M NaOH, 5% SDS, 15% glycerol, and 0.01% bromophenol blue. The bacteria were removed by centrifugation and supernatants applied to polyacrylamide gels consisting of a 2% stacking gel on a 7.5% resolving gel, where it eluted just after the void volume. The laminin containing peak was purified over laminin, fibronectin, and type IV collagen affinity columns. Anti-serum to entactin was a generous gift from Dr. Albert Chung (University of Pittsburgh).

**Immunocytochemistry on RN22F Cells**

To define the epitopes recognized by antibodies to laminin and fibronectin, the pre-immune rabbit serum control were used at 1:100 dilution in PBS with 1% goat serum. Secondary antiserum (goat anti-rabbit IgG, Cappel Laboratories, Cochranville, PA) was used at 1:100 dilution, and Zn(II) peroxidase anti-peroxidase (Cappel Laboratories) at 1:1,000 dilution. Diaminobenzidine reaction, postfixation, and Epon embedding were performed as described (39). Sections were viewed on a Philips EM 300 transmission electron microscope.

**Cell-binding Assay**

Plastic petri dishes (Falcon 1008, 35 mm) were coated with protein by incubating with 1 ml of protein-containing solution at a given concentration in 0.05 M sodium carbonate buffer, pH 9.6, for 3 h at 37°C. Plates were prepared fresh for each assay. To quantitate the amount of protein that specifically bound to the plates, a set of plates was made using laminin and fibronectin labeled with £H using the reductive methylation technique of Jentoft and Dearborn (40). Nonbound protein was removed by washing with PBS, and bound protein solubilized in 0.5 N NaOH, 1% SDS, added to Aqualor II (New England Nuclear) and counted in a Beckman LS230 scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). The counts bound to the plates were compared with total counts available in the protein solution in order to estimate the amount of protein bound.

**Antibodies**

Cells for the assay were trypsinized from culture flasks, washed once in DMEM with 10% horse serum, and twice in serum free DMEM. Cells were counted and adjusted to 1 x 10^5 cells/ml in serum free DMEM, added to each plate in a volume of 1 ml and inoculated without agitation for 90 min in a 37°C humidified incubator with 5% CO2, 95% air atmosphere. Nonadherent cells were removed from the plates by washing × 5 with PBS with Ca ++ and Mg ++. Bound cells were quantified by one of two methods. Nonradio-labeled cells were trypsinized from the assay plates, and the number of cells counted with a Coulter counter.

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Alternatively, cells were radio-labeled with [³H]thymidine (5 μCi/ml, 6.7 Ci/mmol) for 48 h, or [³H]amino acids (25 μCi/ml) for 24 h prior to the assay, and quantitated by solubilizing the cells in 2 ml of 0.5 N NaOH, 1% SDS, adding 15 ml of Aquasol II, and counting in a scintillation counter. Determinations for both methodologies were done in triplicate.

To determine if binding to the protein substrate was specific for the protein, affinity purified antilaminin or antifibronectin (50 to 100 μg/plate) was added with cells in the assay. To determine if new protein synthesis was required for binding to occur, cells were incubated in medium containing 10 to 25 μg/ml cycloheximide for 4 h prior to the assay and during the assay.

Cell Morphology on Protein-coated Substrates: The morphology of adherent cells was evaluated by performing adhesion as described above, using substrates coated with 10 μg of laminin or fibronectin per plate, followed by fixation of the adherent cells with 1% glutaraldehyde in PBS for 30 min. Adherent cells were classified into one of three groups: round, spread, or curved and elongated. A total of 200 cells were classified on each substrate.

Immunofluorescence on Sciatic Nerve: To evaluate the presence of laminin and fibronectin in vivo, we performed immunofluorescent localizations on sections of mouse sciatic nerve. Adult BALB/c mice were killed by cervical dislocation; and the sciatic nerves of both hind limbs removed by dissection. The nerves were fixed in cold ethanol, dehydrated, and embedded in paraffin following the procedure of Sainte-Marie (41). Several nerves were fixed in 2.5% glutaraldehyde in PBS for 2 h, dehydrated through ethanols and embedded in Epox 812 for histological staining with hemotoxlin and eosin, or Lilie’s Allochrome stain (42). Sections were deparaffinized, hydrated to PBS, and reacted with primary antiserum or pre-immune rabbit serum (1:20 dilution). The reaction was localized with fluorescein or rhodamine-conjugated goat anti rabbit IgG (Cappel Laboratories). Sections were viewed with an Olympus BH fluorescence microscope with epi-illumination using a 40× oil immersion lens. Photos were taken on Kodak Ektachrome 400 using 2 to 3-min exposures.

RESULTS

Immunoprecipitation

REDUCED GELS: Immunoprecipitates of RN22F conditioned medium using antilaminin antiserum produced major bands at 200 and 150 kdaltons when complexes were dissociated under reducing conditions (Fig. 1 b, lane 3). The 200-kdalton band co-migrated with the 200-kdalton band of reduced purified laminin from the EHS tumor (Fig. 1 a, lane R).

Antifibronectin antiserum precipitated protein of 220 kdaltons from conditioned medium (Fig. 1 b, lane 2). This band co-migrated with reduced human plasma fibronectin.

Antilaminin precipitates of the solubilized cell layer produced a major band at 200 kdaltons and a weaker band at 150 kdaltons (Fig. 1 c, lane 3). Normal rabbit serum did not precipitate any protein from conditioned medium or solubilized cells (Fig. 1 a, lanes 1 and 2). A faint extra band at 220 kdalton seen in all three lanes of Fig. 1 b is due to small amounts of entactin, a 158-kdalton protein, or the 150-kdalton band seen using antilaminin antisera, from solubilized cells or medium.

NONREDUCED GELS: A 1 M acetic acid sample buffer was used to dissociate complexes without reducing disulfide bonds. Using this sample buffer, we found that antilaminin immunoprecipitates of RN22F-conditioned medium contained bands of 850 and 150 kdaltons (Fig. 2 b, lane 3). The 850-kdalton band co-migrated with nonreduced purified laminin from the EHS tumor (Fig. 2 a, lane NR). Antifibronectin antiserum precipitated protein of 220 kdaltons from conditioned medium (Fig. 1 b, lane 2). This band co-migrated with reduced human plasma fibronectin.

Antilaminin precipitates of the solubilized cell layer produced a major band at 200 kdaltons and a weaker band at 150 kdaltons (Fig. 1 c, lane 3). Antifibronectin antiserum did not precipitate detectable amounts of protein from the solubilized cells (Fig. 1 c, lane 2). Normal rabbit serum did not precipitate any protein from conditioned medium or solubilized cells (Fig. 1 b and c, lanes 1). A faint extra band at 220 kdalton seen in all three lanes of Fig. 1 b is due to small amounts of fibronectin binding directly to the Staphylococcus without the antibody intermediate, as has been shown by other groups previously (30, 43). Antientactin antiserum did not precipitate detectable amounts of entactin, a 158-kdalton protein, or the 150-kdalton band seen using antilaminin antisera, from solubilized cells or medium.

FIGURE 2 2-10% SDS polyacrylamide gradient gel. (a) Purified EHS laminin. R, reduced. NR, nonreduced. Coomassie Blue stain. (b) Fluorograph of immunoprecipitates of conditioned medium from RN22F cultures metabolically labeled with [³H]leucine. 1 M acetic acid sample buffer. Lane 1, pre-immune rabbit serum. Lane 2, antifibronectin antiserum. Lane 3, antilaminin antiserum. (c) Fluorograph of immunoprecipitates of solubilized RN22F cell layer metabolically labeled with [³H]leucine. 1 M acetic acid sample buffer. Lanes are as in b.
Electron micrographs of extracellular immunochemical staining of RN22F Schwannoma cells. (a) Antilaminin antiserum, \(x\) 12,000. (b) Pre-immune rabbit serum, \(x\) 11,000. (c) Antifibronectin antiserum, \(x\) 12,000.

**Immunocytochemistry**

The RN22F cells were immunochemically stained using rabbit antilaminin and antifibronectin antisera in the unlabeled antibody immunoperoxidase technique. Pre-immune rabbit serum and goat serum were used as controls for nonspecific staining by the primary and secondary antisera, respectively. Laminin antisera produced staining along the periphery of the cells (Fig. 3 a). No staining was observed on or around cells of the control samples (Fig. 3 b). Staining with antifibronectin was sparse along the cell periphery, with large areas of no staining (Fig. 3 c). Some of the small amount of fibronectin staining seen by electron microscopy may be due to fibronectin from the growth medium adhering to Schwannoma cell surfaces. As the fibronectin in the growth medium was not radio-labeled, it would not be detected by immunoprecipitation with autoradiography.

**Cell-binding Assay**

The results of one cell-binding assay using the Coulter Counter for quantitation are presented in Fig. 4. This is representative of a series of five assays using both radio-labeled and nonlabeled cells. This particular assay used plates coated with 1, 10, or 20 \(\mu\)g of protein. The amount of protein binding to plates at various concentrations is given in Table I. One-tailed Student’s \(t\) test for the difference of two independent means was performed to determine if cell binding to laminin- or fibronectin-coated plates was significantly greater than binding to noncoated plates. In the above assay, binding was significantly greater to laminin at all three concentrations (1, 10, and 20 \(\mu\)g/plate) than to control plates \((P < 0.01\) in all cases). Binding was greater to fibronectin at 20 \(\mu\)g/plate than binding to control plates \((0.025 < P < 0.05)\), but not at 10 or 1 \(\mu\)g/plate \((P < 0.05)\). Binding to laminin was significantly greater than binding to fibronectin at all levels \((P < 0.01\) for 1 and 10 \(\mu\)g, 0.01 < \(P\) < 0.025 for 20 \(\mu\)g/plate, one-tailed Student’s \(t\) test). Comparison of the data from the five assays performed, using the values for the 10 \(\mu\)g/plate samples, shows that laminin significantly improved binding compared with noncoated plates \((P < 0.01\) for 4 assays, 0.01 < \(P\) < 0.05 for the fifth assay). In three of the five assays, fibronectin also provided a better substrate than control plates \((0.01 < P < 0.05)\). However, in three of the assays, laminin was very significantly better than fibronectin at promoting adhesion \((P < 0.01)\), and in no case was fibronectin significantly better than binding to laminin. The binding to laminin and fibronectin was inhibited by adding affinity purified antibodies with the cells during the incubation period, but was not blocked by treatment of the cells with cycloheximide (data not shown).
FIGURE 4 Results of cell-binding assay using nonradio-labeled cells and Coulter counter. Dashed line represents percent of cells applied that bind to the noncoated, bacteriological plastic control plate. Percent of cells bound = (number of cells bound per 10^5 cells applied) x 100%.

**Table I**

| Protein       | cpm Available* | cpm Bound  | Estimated µg protein bound† |
|---------------|----------------|------------|-----------------------------|
| Laminin       |                |            |                             |
| 1 µg          | 2.3 x 10^3     | 8.0 x 10^2 | 0.3                         |
| 10 µg         | 5.5 x 10^6     | 9.3 x 10^4 | 1.7                         |
| 100 µg        | ~5             | 5.2 x 10^6 | ~10-20†                     |
| Fibronectin   |                |            |                             |
| 1 µg          | 4.0 x 10^3     | 2.0 x 10^3 | 0.5                         |
| 10 µg         | 8.1 x 10^4     | 1.9 x 10^4 | 2.3                         |
| 100 µg        | 3.2 x 10^5     | 5.8 x 10^4 | 17.9                        |

* Counts per minute in 1 ml of protein solution of given concentration.† Protein bound estimated by: µg protein bound = (cpm bound/cpm available) x µg available.¶ Counts exceeded machine capacity.|| Value estimated using counts available for 1 and 10 µg.

**Cell Morphology on Protein-coated Substrates**

During the cell binding assay, we noticed that many cells plated on laminin adopted a different, somewhat crescent or curved, morphology as compared with cells plated on fibronectin or noncoated plastic. The typical cell morphology on noncoated plastic was round and nonspread (Fig. 5 a). These cells were bound to the plastic, i.e., they were not removed by the buffer wash, but they did not spread, a result similar to that seen for many cell types on plastic that is not treated for tissue culture. On fibronectin coated plates, a number of cells spread on the substrate, as seen in Fig. 5 b. However, on laminin, many cells adopted a curved morphology, as shown in Fig. 5 c. Curved cells were only very rarely seen on the other substrates. The results of the classification of 200 cells by morphology on each of the three substrates (noncoated, laminin 10 µg/plate, fibronectin 10 µg/plate) are given in Table II. The data were analyzed as a 3 x 3 contingency table (44, 45). In Table II, the first value listed in each cross-category is the number of cells observed in that category (OBS). The second value is the expected number of cells (EXP) in that category based on a model of independence—in other words, the number of cells one would expect to be in a given category if cell morphology were independent of the substrate the cells were plated on. (The expected value is calculated as EXP = (row total) x (column total)/N, where N is the total sample size.) The third value in each category (DIFF) is the difference between the observed number of cells and the expected number of cells in the category (OBS - EXP). These differences were used in the calculation of the χ^2 statistic (χ^2 = Σ((OBS - EXP)^2/EXP) with 4 degrees of freedom). The calculated χ^2 statistic was 350 (P < 0.005). The observed number of cells in each category was significantly different from the expected number of cells if substrate and cell morphology were independent of each other. Examining the differences between the observed and expected values gives some indication of where the greatest differences occur. In the first row of Table II (no coating), it is seen that there are two times more cells in the round category than expected, and fewer cells than expected in the spread and curved categories. For the laminin row, the differences are reversed; there are many fewer cells in the round category than expected, and more cells in the curved and spread categories. In the fibronectin row, observed values for round cells are near expected values, but there are more cells in the spread category and fewer cells in the curved category than expected. To summarize the data: The majority of the cells binding to the noncoated bacterial plastic do not spread on the substrate. When the surface is coated with fibronectin, the cells are able to spread better than on a noncoated substrate, and do so in a uniform "fried egg" shape rather than a curved shape. When laminin is used as the substrate, virtually none of the cells stay in the round nonspread morphology; two thirds of the cells take on a uniformly spread morphology, while the other third take on the curved, crescent-shaped morphology.

**Sciatic Nerve Staining**

Immunofluorescent staining of mouse sciatic nerve was performed to determine if laminin and fibronectin are present in peripheral nerve in vivo, as the results with the Schwannoma cell line might indicate. Sections were stained with rabbit antilaminin or antiﬁbronectin, with preimmune rabbit serum as the specificity control. Fig. 6 a is a micrograph of a 1-µm resin section of sciatic nerve stained with Lillie's Allochrome Stain. This stain has a periodic acid-Schiffs reagent step which stains basement membranes/glycosaminoglycans pink, and a step which stains collagen blue. In these sections the endoneurium (E) stained pinkish purple, indicating the location of basement membrane, and the perineurium (P) stained deeper purple, indicating a stronger presence of collagens. Fig. 6 b is a 6 µm paraffin section of sciatic nerve fixed in cold ethanol and stained with Lillie's Allochrome. Although the histology is not as well preserved in the cold ethanol-fixed tissue, the structures are recognizable and stain identically to the glutaraldehyde-fixed tissues. Immunofluorescent staining was performed on sections adjacent to that shown in Fig. 6 b. Fig. 6 c shows a control section treated with preimmune rabbit serum. Endoneurium (E) and perineurium (P) are indicated. There is little background staining. Fig. 6 d is a phase-contrast micrograph of the section shown in Fig. 6 c. (Different objectives had to be used for the phase and fluorescence pictures, so the field sizes are not identical.) Rabbit antilaminin stained in a very distinct band in the endoneurium around nerve fibers and in the perineurium (Fig. 6 e). In contrast to laminin, there is
FIGURE 5 Cell morphology of adherent RN22F Schwannoma cells on protein-coated substrates. Phase contrast. (a) Round cells; noncoated plastic substrate. (b) Spread cells; fibronectin-coated substrate. (c) Curved and elongated cells; laminin-coated substrate. × 400.

| Coating          | Morphology | Row total |
|------------------|------------|-----------|
|                  | Round | Spread | Curved |     |
| No coating       | OBS    | 171    | 28     | 1   | 200 |
|                  | EXP    | 85.6   | 93.6   | 20.6|     |
|                  | DIFF   | +85.4  | −65.6  | −19.6|    |
| Laminin, 10 µg   | OBS    | 5      | 134    | 61  | 200 |
|                  | EXP    | 85.6   | 93.6   | 20.6|     |
|                  | DIFF   | −80.6  | +40.4  | +40.4|    |
| Fibronectin, 10 µg| OBS   | 81     | 119    | 0   | 200 |
|                  | EXP    | 85.6   | 93.6   | 20.6|     |
|                  | DIFF   | −4.6   | +25.4  | −20.6|    |
| Column total     |        | 257    | 281    | 62  | 600 |

Cells (1 x 10⁵/plate) were incubated on protein-coated 35-mm bacteriological plastic plates for 90 min. Nonbound cells were washed away, and bound cells fixed with glutaraldehyde. A total of 200 cells from each plate were classified by morphology. OBS, observed number of cells in category. EXP, expected number of cells in category (see calculating formula in text). DIFF, difference between the observed and expected number of cells (OBS − EXP).

little staining of the endoneurium with antifibronectin, but the perineurium stains very strongly (Fig. 6f). The antifibronectin staining that is seen in the endoneurium is very diffuse as compared with the very distinct bands seen with antilaminin antiserum. The location of laminin and fibronectin in vivo appear to correlate with the behavior of the Schwannoma cells on laminin and fibronectin coated substrates. The Schwannoma cells bind better to laminin in vitro, and in vivo laminin is close to Schwann cells, suggesting its production by, or association with, these cells.

DISCUSSION

Our study shows that a rat Schwannoma cell line, RN22F, produces both laminin and fibronectin as detected by immunoprecipitation. The laminin appears in immunoprecipitates of solubilized cell layer and conditioned medium. In immune precipitates using antilaminin a 150-kdalton band is precipitated along with a 200-kdalton reduced laminin band. Under nonreducing conditions the 150-kdalton band can be separated from the 850-kdalton nonreduced laminin. Antifibronectin antiserum precipitates a band of 220 kdaltons (reduced) from conditioned medium. Under nonreducing conditions there are bands of 440 and 220-kdalton. The 440-kdalton band co-migrates with human plasma fibronectin run on gels in the acetic acid sample buffer, and the 220-kdalton band co-migrates with reduced human plasma fibronectin. Fibronectin was not detected in immunoprecipitates of the cell layers suggesting that it is largely shed from the cells.

Immunocytochemical localization of laminin and fibronectin at the electron microscope level indicates that laminin is distributed along the surface of the Schwannoma cells in culture. Fibronectin is sparsely distributed along the cell surface. As indicated by immunoprecipitation, almost all of the fibronectin produced by the Schwannoma cells is released into the culture medium.

The cell binding assays show that the Schwannoma cells bind better to laminin than to fibronectin or noncoated bacteriological plastic. Classification of cells by their morphology on the different substrates indicated that the cells will spread on a fibronectin substrate, but more will spread on laminin, and a large number of the cells undergo a change in morphology to a curved-crescent shape on laminin. The change in morphology was similar to a morphological change observed when RN22 cells were treated with cyclic nucleotide analogs (35). Finally, by immunofluorescence, it was found that laminin and fibronectin are present in sciatic nerve. The laminin was localized in the endoneurium and perineurium, while the fibronectin was found mainly in the perineurium.

The 150-kdalton band, seen in immunoprecipitates with antilaminin appears to be a protein chain that is not covalently linked into the laminin complex. Because of the 150,000 mol wt of this protein, it was thought that the protein might be entactin, a 158,000 laminin-associated sulfated protein described by Carlin et al. (9). However, antiserum to entactin did not precipitate this 150-kdalton band in immunoprecipitation experiments. In contrast to laminin from the EHS tumor, the laminin produced by the RN22F cells does not appear to have a 400,000 subunit under reducing conditions, although the total molecular weight of the nonreduced complex is similar to the weight of nonreduced EHS laminin. Preliminary work is beginning to indicate that the 150-kdalton band may be a degradation product or subunit gene product that contributes to the 400-kdalton chain, with degradation of the 400-kdalton chain occurring at a fast enough rate that the 400-kdalton product cannot be detected by our method. This is based on three sets of observations: (a) The RN2 rat Schwannoma line, a cell line related to the RN22F line used here, produces 400-
Figure 6 Immunofluorescent staining of mouse sciatic nerve. (E) Endoneurium. (P) Perineurium. (a) Resin section (1 μm) of mouse sciatic nerve fixed with glutaraldehyde and stained with Lillie’s Allochrome stain. × 1,200. (b) Paraffin section (6 μm) of mouse sciatic nerve fixed with cold ethanol and stained with Lillie’s Allochrome stain. × 1,200. (c) Immunocytochemical control, stained with pre-immune rabbit serum. Paraffin section of cold ethanol-fixed sciatic nerve. × 1,200. (d) Phase-contrast micrograph of the section shown in c. × 900. (e) Immunofluorescent staining with antilaminin antiserum. Paraffin section of cold ethanol-fixed sciatic nerve. × 1,200. (f) Immunofluorescent staining with antifibronectin antiserum. Paraffin section of cold ethanol-fixed sciatic nerve. × 1,200.
and 200-kdalton chains, but no 150-kdalton chain using the same antisera as used in this study (data not shown). (b) Western blot analysis using polyclonal rabbit antialamin antisera indicates that our antisera reacts with a faint 150-kdalton band as well as the major 400- and 200-kdalton bands in laminin preparations (data not shown). Immunoprecipitation experiments using this polyclonal antisera, and a monoclonal rat anti-mouse laminin antibody preparation precipitate similar patterns of bands from crude laminin preparations, indicating that the 150-kdalton bands may be part of a laminin chain. (c) The 150-kdalton band does not seem to be one of the other major basement membrane components in that it is not precipitated by anti-entactin antisera, and it is precipitated by an antialamin antisera that has been affinity purified over columns of type IV collagen, fibronectin, and basement membrane proteoglycan to remove contaminating antibodies. This is all circumstantial evidence; further work, including peptide mapping and amino acid analysis, will be needed to positively identify the 150-kdalton band produced by the RN22F cells.

The production of laminin and fibronectin by the RN22F cell line is consistent with studies by other groups. Kurkinen and Altalo (46) have shown that a related Schwannoma cell line, RN2, produces fibronectin and also a procollagen of unknown type. The localization of laminin in the endoneurium and fibronectin in the perineurium generally agrees with the distribution of collagens in the peripheral nervous system. Shellswell et al. (47) found types IV and V collagen in the basement membrane portion of the endoneurium, and types I and III collagen in the perineurium and extra-basement membrane regions of the endoneurium. Laminin is often associated with type IV collagen in basement membranes, while fibronectin is often found in the presence of types I and III collagen.

There are two specific times when the production of laminin and fibronectin by Schwann cells may be important in the peripheral nervous system: during development, basement membranes may be important in guiding developing axons to their final location and in promoting myelination; and following injury to nerves, the basement membrane proteins produced by the Schwann cells may be important in stimulating and guiding the sprouting neurites so they can properly regenerate and reestablish the disrupted nerve pathway.

There is evidence accumulating to support a role for basement membrane proteins in nerve development. Nonneuronal cells in culture make a "substrate conditioning factor" that appears to guide elongating nerve fibers in vitro (48). Akers et al. (49) found that pre-treatment of growth substrate with fibronectin promoted retinal neurite outgrowth (49). Manthorpe et al. (50) reported that conditioned medium from RN22F Schwannoma cultures contains a factor that promotes neurite outgrowth. Fibronectin and collagen appear to play a role in neural crest migration in early embryos (51, 52).

Schwann cells and their products may be vital in the proper regeneration of injured nerves. From early studies it has been known that nerves in the peripheral nervous system had the capacity to regenerate, but central nervous system neurons rarely, if ever, regenerated (4). Recently, Aguayo et al. (53) reported that an autologous graft of sciatic nerve between lower cervical or upper thoracic spinal cord and medulla oblongata allowed spinal and brain stem axons to extend further than they usually grow in intact animals (without grafts). The change of the neurological environment from central nervous system to that of the peripheral nervous system—with the Schwann cells and basement membrane of the sciatic nerve—allowed spinal and brain stem axons to extend further than cervical or upper thoracic spinal cord and medulla oblongata allowed spinal and brain stem axons to extend further than they usually grew in intact animals (without grafts).
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