Embryonic Neural Retinal Cell Response to Extracellular Matrix Proteins: Developmental Changes and Effects of the Cell Substratum Attachment Antibody (CSAT)

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Abstract. Cell attachment and neurite outgrowth by embryonic neural retinal cells were measured in separate quantitative assays to define differences in substrate preference and to demonstrate developmentally regulated changes in cellular response to different extracellular matrix glycoproteins. Cells attached to laminin, fibronectin, and collagen IV in a concentration-dependent fashion, though fibronectin was less effective for attachment than the other two substrates. Neurite outgrowth was much more extensive on laminin than on fibronectin or collagen IV. These results suggest that different substrates have distinct effects on neuronal differentiation.

Neural retinal cell attachment and neurite outgrowth were inhibited on all three substrates by two antibodies, cell substratum attachment antibody (CSAT) and JG22, which recognize a cell surface glycoprotein complex required for cell interactions with several extracellular matrix constituents. In addition, retinal cells grew neurites on substrates coated with the CSAT antibodies. These results suggest that cell surface molecules recognized by this antibody are directly involved in cell attachment and neurite extension.

Neural retinal cells from embryos of different ages varied in their capacity to interact with extracellular matrix substrates. Cells of all ages, embryonic day 6 (E6) to E12, attached to collagen IV and CSAT antibody substrates. In contrast, cell attachment to laminin and fibronectin diminished with increasing embryonic age. Age-dependent differences were found in the profile of proteins precipitated by the CSAT antibody, raising the possibility that modifications of these proteins are responsible for the dramatic changes in substrate preference of retinal cells between E6 and E12.

Neuronal interaction with defined tissue culture substrates has been a useful experimental paradigm leading to the elucidation of some of the molecular requirements for cell attachment and neurite outgrowth, as well as cellular events leading to neurite extension. Defined factors from both conditioned medium (Collins, 1978; Adler et al., 1981; Coughlin et al., 1981; Lander et al., 1982) and extracellular matrices (Lander et al., 1982) stimulate neurite extension by cultured neurons. The extracellular matrix protein, laminin, is active alone and appears to account for the neurite outgrowth promoting activity of conditioned medium (Lander et al., 1985; Davis et al., 1985). Substrate-attached laminin stimulates neurite outgrowth by diverse neuronal cell types from both the peripheral and central nervous systems (Baron von Evercooren et al., 1982; Manthorpe et al., 1983; Smalheiser et al., 1984; Rogers et al., 1983; Lander et al., 1983; Unsicker et al., 1985; Faivre-Bauman et al., 1984; Adler et al., 1985). Fibronectin is also an effective substrate for neurite outgrowth by peripheral neurons (Baron von Evercooren et al., 1982; Carbonetto et al., 1983; Rogers et al., 1983), but less so for central neurons (Carbonetto et al., 1983; Rogers et al., 1983). Collagens I and IV support neurite extension under some conditions (Carbonetto et al., 1983; Vladowsky et al., 1982).

Embryonic neural retina, the subject of the present study, is a useful system for studying neuronal interactions with extracellular matrices. Previous work implicates the extracellular matrix in regulating the development of retinal ganglion cells. In the goldfish and chick retinas, axons are initiated in proximity to a basement membrane, the inner limiting membrane (Easter et al., 1984; McLoon et al., 1984). Within the eye, axons grow on three substrates: existing axons, Muller glial endfeet, and the inner limiting membrane. Enzymatic removal of the inner limiting membrane during the period of axon outgrowth results in disorganized growth of axons but does not affect preexisting axons (Halffer and Deiss, 1984). Therefore, axonal growth within the eye seems to depend upon cell interactions with molecules present in the basement membrane. Laminin is present in the inner limiting membrane of developing chick (McLoon, 1984; Adler et al., 1985) and rat retina, as well as transiently in rat optic stalk (personal communication, Dr. S. McLoon, University of Minnesota, Minneapolis, MN). As laminin stimulates neurite outgrowth by neural retinal cells in vitro (Rogers et al., 1983; Adler et al., 1985) and is appropriately localized in vivo, it is likely to influence axon initiation and growth in the developing retina.

Recently, progress has been made in identifying neuronal
surface molecules that may mediate retinal cell interactions with basement membranes. The monoclonal antibody, T61, disorganizes and inhibits axonal growth in retinal explants (Halfter and Deiss, 1986). Two other monoclonal antibodies, cell substratum attachment antibody (CSAT)\(^{1}\) and JG22, which compete with each other, perturb cell–matrix interactions in a variety of cell types (Neff et al., 1982; Horwitz et al., 1985; Decker et al., 1984; Bozymcko and Horwitz, 1986; Chen et al., 1985b; Greve and Gottlieb, 1982). The glycoproteins recognized by these antibodies include a fibronectin receptor (Akiyama et al., 1986; Horwitz et al., 1985) and have some properties of a laminin receptor (Horwitz et al., 1985). The cDNA-derived protein sequences of the subunits of the fibronectin receptor indicate that they contain hydrophobic transmembrane segments (Tamkum et al., 1986; Argraves et al., 1986). The CSAT proteins colocalize and interact with microfilament-associated proteins (Damsky et al., 1985; Chen et al., 1985a, 1985b; Greve and Gottlieb, 1982). Therefore, these proteins have properties appropriate for mediating interactions between the matrix and cytoskeleton. In our experiments, the CSAT and JG22 antibodies were used to determine whether neural retinal cells also possess molecules that mediate neural responses to purified extracellular matrix molecules.

**Materials and Methods**

**Materials**

Linbro 96-well flat bottom tissue culture plates were purchased from Flow Laboratories, McLean, VA. ENPHANCE was purchased from New England Nuclear, Boston, MA. Na\(^{2+}\) and L-\(^{35}\)S]methionine were obtained from Amersham, Arlington Heights, IL. Iodogen was from Pierce Chemical Co., Rockford, IL. Protein A Sepharose CL-4B and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Fertile White Leghorn chicks were from Feather Hill Farm, Petaluma, CA, and mice were from Simonson Laboratories, Gilroy, CA. Mouse Immunoglobulin (IgG) was purchased from Cappell Laboratories, Malvern, PA. Affinity purified polyclonal antibodies to murine collagen IV were obtained from Dr. H. N. P. Horwitz, University of Pennsylvania, Philadelphia, PA, and Dr. D. Gottlieb, Washington University, St. Louis, MO. Rabbit anti-neural cell adhesion molecule (anti-N-CAM) antibodies and IgG were prepared by Dr. J. Bixby (Bixby and Reichardt, 1987).

**Protein Purification and Antibody Preparation**

Murine collagen IV and laminin were purified from the Engelbreth-Holm-Swarm sarcoma by published procedures (Timpl et al., 1982). Human cell fibronectin, a gift from Dr. S. Fisher, University of California, San Francisco, was prepared as described (Chou-Rong Zhu et al., 1994). Proteins were radioiodinated by the iodogen method (Fraker and Speck, 1978). Hybridoma cells secreting CSAT and JG22 antibodies were gifts from Dr. A. F. Horwitz, University of Pennsylvania, Philadelphia, PA, and Dr. D. Gottlieb, Washington University, St. Louis, MO, respectively, and were grown as described (Neff et al., 1982). To prepare large amounts of antibodies, hybridoma cells were injected into pristane-primed 8-wk-old female Balb/c mice. After ascites fluid was collected, the CSAT antibodies were purified by protein A-Sepharose CL-4B chromatography (Neff et al., 1982). Rabbit anti-neural cell adhesion molecule (anti-N-CAM) antibodies and IgG were prepared by Dr. J. Bixby (Bixby and Reichardt, 1987).

**Neurite Outgrowth and Attachment Assays**

Both the neurite outgrowth and attachment assays had four major steps; substrate preparation, neural retinal cell preparation, plating and incubation of neuronal retinal cells, and analysis for eitherattachment or neurite outgrowth. Lower cell densities and longer incubation times were used on the plates to be analyzed for neurite outgrowth. Otherwise, the two assays used identical protocols.

To prepare substrates, sterile 96-well plates were coated with 100 \(\mu\)l per well of laminin, collagen IV, or antibody diluted in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS; 200 mg/liter KCl, 200 mg/liter K\(_2\)SO\(_4\), 8.0 \(\mu\)liter NaCl, and 2.16 \(\mu\)liter Na\(_2\)HPO\(_4\)-7H\(_2\)O. pH 7.36-7.45) to the concentrations indicated in the results. Fibronectin was applied in 100 \(\mu\)l per well of sterile 0.1 M cyclohexylaminopropane sulfonic acid buffer, pH 9.0. Plates were incubated with proteins overnight at room temperature. After rinsing three to five times with sterile CMF-PBS, the wells were blocked by incubation with 1% BSA for at least 2 h at room temperature. Plates were again rinsed three to five times with sterile CMF-PBS and 100 \(\mu\)l of culture medium was added to each well. At this time antibodies were added to the wells and plates were stored in the incubator, at 37°C and 5% CO\(_2\), until the cells were ready, approx 1 h.

Retinae were dissected from embryonic chickens in CMF-PBS containing 6% glucose and 100 U/ml penicillin and streptomycin. The retinal cells were dissociated by trypsinization in 0.03% trypsin for 10 min in CMF-PBS followed by trituration. Dissociated cells were collected by centrifugation and resuspended for preplating in F12 nutrient mixture with insulin (5 \(\mu\)g/ml), selenium (30 \(\mu\)M), iron-saturated owtoswerr (25 \(\mu\)g/ml), and 100 U/ml penicillin and streptomycin (Bottenstein et al., 1980). Cells were preplanted on tissue culture plates for 45 min at 37°C in a 5% CO\(_2\) atmosphere to remove nonneuronal cells. At the end of the preplating step, the supernatants containing neuronal cells were harvested and the cell number was adjusted for cell attachment and neurite outgrowth experiments. For attachment assays, the neuronal retinal cells were suspended at 5×10\(^4\) to 10\(^5\) cells/ml. For the neurite outgrowth assay, cells were used at 10\(^4\) cells/ml. Lower initial plating densities were used for the neurite outgrowth assay, since it was difficult to assess neurite number in wells with more than about a thousand cells. For both assays, 100 \(\mu\)l of cells per well were added to the previously prepared plates and these were centrifuged in a TJ-6 centrifuge (Beckman Instruments, Inc., Palo Alto, CA) at 48 g for 5 min. This centrifugation step greatly increased the reproducibility of the attachment assay since it removed the variability inherent in settling of cells onto the culture plate surface.

Attachment assay plates were analyzed after incubation at 37°C in a 5% CO\(_2\) atmosphere for 1.5 h. Unattached cells were removed by the brisk addition of warm F12 nutrient mixture to each well, followed by gentle vacuum suction with a narrow bore pipet. The cells were fixed overnight at 4°C in 2% glutaraldehyde in F12 nutrient mixture and stained for 2.4 h the following morning by the addition of trypsin blue to a final concentration of 0.08% (Koda et al., 1986). After thorough rinsing, the A\(_{560}\) of individual wells was measured on a microtiter plate reader (Flow Laboratories). In early experiments we determined that cell number was proportional to A\(_{560}\). Attachment assays, the CSAT and JG22 antibodies were used to determine whether neural retinal cells also possess molecules that mediate neural responses to purified extracellular matrix molecules.

**Immunoprecipitation with CSAT Antibody**

Chick embryo fibroblasts prepared as previously described by Rein and Rudin (1968) and retinal cells prepared as in the previous section were metabolically labeled by incubation overnight at 37°C in a 5% CO\(_2\) atmosphere with 60 \(\mu\)Ci/ml \([\text{\textsuperscript{35}}\text{S}]\)methionine in methionine-deficient F12 medium. After labeling, the fibroblasts and neural retinal cells were incubated for 10 min at 37°C in CMF-PBS with 5 mM EDTA and then removed from the dish by gentle pipetting. Cells were resuspended in 0.5 ml Hepes-buffered F12 nutrient mixture, and 40 \(\mu\)g of antibody (CSAT or mouse immunoglobulin-A) was added to the wells. After 1 h at 4°C, 0.5 ml of 10% (NH\(_4\)\(_2\))\(_2\)SO\(_4\) was added and the cells were centrifuged at 15,000×g for 10 min. The cell pellets were washed three times in 50 mM Tris, pH 7.5, containing 150 mM Na\(_2\)SO\(_4\), 5 mM EDTA, and 0.5% BSA. The pellets were dissolved in 0.5 ml 0.1 M Tris-HCl, pH 7.5, containing 20% glycerol and 0.5 mM phenylmethylsulfonyl fluoride, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Abbreviations used in this paper:**

- CMF-PBS, calcium- and magnesium-free phosphate-buffered saline
- CSAT, cell substrate attachment
- MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
- \(A_{560}\) (experimental well) - \(A_{560}\) (BSA-coated well) × 100
- \(A_{560}\) (poly-D-lysine-coated well) - \(A_{560}\) (BSA-coated well)

Plates containing cells to be analyzed for neurite outgrowth were grown for 16–24 h at 37°C in a 5% CO\(_2\) atmosphere. Cells were fixed with 2% glutaraldehyde as described above and examined in the microscope. Neurite outgrowth was quantified by determining the percentage of cells bearing neurites greater than two cell diameters in length (Lander et al., 1982). The viability of cells cultured for longer periods was measured by the cell survival assay of Manthorpe et al. (1986). Briefly, 10 \(\mu\)l of MTT solution was added to each culture 1 h before fixation. Viable cells take up and convert MTT to a visible blue formazan product.
lin, MIg) was added. After 1 h of incubation at room temperature, cells were extracted in extraction buffer (50 mM Tris, 150 mM NaCl, 1 mM CaCl₂, 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, pH 7.4) for 30 min at 4°C with occasional vortexing. The extracts were spun at 10,000 g for 1 min to pellet insoluble material. Supernatants were cleared twice by incubation with Sepharose 4B for 1 h at 4°C followed by centrifugation for 1 min (134 g in a Beckman TJ6). Immune complexes were adsorbed by mixing with protein A-Sepharose CL-4B (75 µl of packed beads per tube). After 1 h, Sepharose beads were sedimented at 134 g (Beckman TJ6 for 10 s) and washed four times with 10 ml extraction buffer. Pellets were resuspended in 100 µl electrophoresis sample buffer and boiled for SDS-PAGE analysis (Laemmli, 1970). 7.5% gels were run under reducing (with beta-mercaptoethanol) or nonreducing conditions, treated with ENHANCE, dried, and exposed to Kodak X Omat R film.

Results

E6 Neural Retinal Cells Attach to Extracellular Matrix Glycoprotein Substrates

Neural retinal cell attachment to different constituents of the extracellular matrix was measured using a quantitative attachment assay (see Materials and Methods). Results in Fig. 1 show that E6 chick neural retinal cells attached to laminin, fibronectin, and collagen IV in a concentration-dependent fashion. At optimal concentrations of collagen IV and laminin, attachment was as efficient as to poly-D-lysine. At the highest concentrations of fibronectin tested, attachment was ~70% as efficient as to poly-D-lysine. Plates coated with 1-2 µg/ml of collagen IV and 5-10 µg/ml of laminin yielded maximal attachment. Half-maximal attachment was seen with ~0.5 µg/ml collagen IV and ~2.5 µg/ml laminin. Fibronectin-coated substrates did not support attachment of 100% of the cells plated, even at coating concentrations as high as 300 µg/ml; half maximal attachment required ~40 µg/ml of fibronectin.

![Figure 1](image1.png)

**Figure 1.** Embryonic neural retinal cells attach to tissue culture substrates coated with purified extracellular matrix molecules. A 1.5-h attachment assay was used to measure the attachment of E6 chick neural retinal cells to tissue culture substrates coated with purified laminin, fibronectin, or collagen IV as described in Materials and Methods. Wells coated with 1 mg/ml poly-D-lysine served as a positive control; attachment to poly-D-lysine was taken as 100% and was used in the calculation of percent cells attached for the other substrates. Each point is the average of at least three separate determinations. The figure shows percent of cells attached vs. coating protein concentration for collagen IV (open circles), laminin (solid squares), and fibronectin (solid triangles). The error bars show standard error of the mean.

To determine whether observed differences in cell attachment were attributable to variations in protein coating efficiency, known amounts of protein were labeled with ¹²⁵I and used to measure the efficiency of protein attachment to the plastic substrate (Fig. 2). Collagen IV was adsorbed about twofold more efficiently than fibronectin, which in turn was somewhat more efficiently adsorbed than laminin. Data from Figs. 1 and 2 show that maximal cell attachment was seen with ~7 ng of laminin and ~12 ng of collagen IV adsorbed to an area of ~0.28 cm² (bottom of well).

The specificity of neural retinal cell interactions with laminin and collagen IV-coated substrates was demonstrated using affinity purified anti-collagen IV antibody and anti-laminin antisera (Fig. 3). Cells were plated on collagen IV or laminin in the presence or absence of antibodies to laminin, collagen IV, or nonimmune serum. Results in Fig. 3 show that affinity purified anti-collagen IV antibodies inhibited cell attachment to collagen IV substrates but not to laminin substrates. Conversely, anti-laminin antibodies inhibited neural retinal cell attachment to laminin but not collagen IV substrates. These results provide strong evidence that attachment to laminin and collagen IV involve specific and distinct interactions.

Embryonic Neural Retinal Cells Extend Neurites in Culture when Plated on Purified Extracellular Matrix Proteins

When neural retinal cells were incubated for longer periods on extracellular matrix protein substrates, some cells extended neurites (Fig. 4). On laminin, E6 embryonic neural retinal cells attached quickly and assumed a characteristic polygonal shape (Fig. 4A). These cells developed neurites as early as a few hours after plating, and gave rise to exten-
Figure 3. Embryonic neural retinal cells attach to laminin and collagen IV in a specific fashion. Day 6 embryonic neural retinal cells were assayed for attachment to protein-coated tissue culture wells as described in Materials and Methods. Cells were plated onto wells that had been coated with 2 µg/ml collagen IV (top) or 5 µg/ml laminin (bottom) without antibodies (A), with preimmune serum (B), with affinity purified anti-collagen IV antibodies (C), or with anti-laminin antiserum (D). Wells coated with poly-D-lysine were used as a positive control in the calculation of percent of total cells attached. Error bars show standard error of the mean.

Figure 4. Phase photomicrographs of E6 neural retinal cells plated on extracellular matrix protein substrates. E6 neural retinal cells were plated onto tissue culture plastic wells that had been coated with 5 µg/ml laminin (A), 300 µg/ml fibronectin (B), or 2 µg/ml collagen IV (C). The cells were maintained in culture for 24 h as for neurite outgrowth assay (see Materials and Methods). Cells were fixed with 2% fresh glutaraldehyde in cold PBS and then photographed. Compare the abundant and long neurites from cells plated on laminin (A) with the sparser and shorter neurites seen on either fibronectin (B) or collagen IV (C) substrates. Bar, 20 µm.

Neurite outgrowth by neural retinal cells on each of the three substrates was quantified (Fig. 5). At optimal concentrations of laminin as many as 70% of the neural retinal cells developed neurites longer than two cell diameters by 16–24 h. In contrast, on poly-D-lysine substrates, only 1–5% of the neurons developed neurites. Neurite outgrowth on laminin was concentration-dependent (Fig. 5). Maximal neurite outgrowth required coating plates with 5–10 µg/ml laminin; half-maximal neurite outgrowth required about 2–3 µg/ml laminin. As indicated above, similar concentrations of laminin were required for maximal and half-maximal attachment of neural retinal cells. Only a low level of neurite outgrowth was seen on fibronectin or collagen IV substrates (Fig. 5). Additionally, neurite outgrowth on collagen IV did not appear to be dependent on the amount of adsorbed ligand. On collagen IV, 20–30% of the cells developed neurites at concentrations within the range that was effective for cell attachment (compare to Fig. 1). When the effects of different concentrations of fibronectin were measured, the lowest concentration of fibronectin used in the attachment assay (Fig. 1) induced neurite outgrowth by ~15% of the cells. Increasing concentrations of fibronectin induced a higher percentage of neurons to extend neurites. At 300 µg/ml of fibronectin, a concentration that permitted attachment of 70% of the cells in the attachment assay, ~40% of the cells extended neurites. While neither collagen IV nor fibronectin promoted profuse neurite outgrowth, both were more effective than poly-D-lysine.

Effects of the CSAT and JG22 Antibodies on Retinal Cell Attachment and Neurite Outgrowth

CSAT and JG22 antibodies have been shown to bind an apparent complex of three glycoproteins that includes a functional fibronectin receptor (Horwitz et al., 1985; Greve and Gottlieb, 1982; Akiyama et al., 1986) and has some of the properties expected of a laminin receptor (Horwitz et al., 1985). We used these antibodies to determine whether glyco-
proteins recognized by the CSAT antibody were important in
mediating the response of neural retinal cells to extracellular
matrix glycoproteins. Attachment of neural retinal cells to
laminin, fibronectin, and collagen IV was inhibited in the
presence of CSAT antibody (Fig. 6). However, the CSAT an-
tibody had no effect on cell attachment to poly-D-lysine or
concanavalin A (data not shown), a finding which suggests
that the antibodies interfered specifically with attachment to

Figure 5. Quantitation of neurite outgrowth by embryonic neural
retinal cells cultured on laminin (solid circles), fibronectin (solid
triangles), or collagen IV (solid squares) substrates. E6 retinal cells
were plated on tissue culture wells coated with either laminin,
fibronectin, or collagen IV and maintained in culture in serum-free
medium for 16-24 h. Cells were fixed with 2% glutaraldehyde in
PBS and percent of cells bearing neurites was determined as de-
scribed in Materials and Methods. Each point is the average of at
least three separate determinations. Percent of cells bearing neu-
rites vs. protein coating concentration is shown. The error bars
show standard error of the mean.

Figure 6. CSAT antibody inhibition of embryonic neural retinal cell
attachment and neurite outgrowth on laminin, fibronectin, and col-
lagen IV. E6 neural retinal cells plated on wells coated with 5 μg/ml
laminin, 300 μg/ml fibronectin, or 2 μg/ml collagen IV were as-
sayed separately for attachment and neurite outgrowth as described
in Materials and Methods. Measurements were done without anti-
body (open bars), with 10 μg/ml mouse IgG (stippled bars) or with
10 μg/ml CSAT (solid bars). Attachment to poly-D-lysine in the
presence and absence of CSAT antibody is also shown. Error bars
show the standard error of the mean.

Figure 7. Phase photomicrographs of embryonic neural retinal cells
plated on antibody-coated substrates; 75 μg/ml JG22 (A), 100 μg/ml
anti-N-CAM (B), or 75 μg/ml anti-P34 (C). E6 neural retinal cells
were plated onto wells coated with antibodies and maintained in
culture for 16-24 h then fixed with 2% glutaraldehyde and pho-
tographed. Polygonal, well-attached, and spread cells with neurites
are seen on JG22 antibody substrates (A). Cells on the anti-N-CAM
antibody substrate appear well attached and spread but have few
neurites (B). The arrow in B shows a broad lamellodipodial region.
Loosely attached cell aggregates are observed in cultures on anti-
P34 antibody substrates (C). Bar, 26 μm.
the protein ligands. Cells were also unable to initiate neurites when cultured overnight in the presence of CSAT antibodies (Fig. 6). The dramatic inhibitory effects of the CSAT antibody on retinal cell attachment and neurite outgrowth were also seen using the JG22 antibody (data not shown), which binds the same glycoprotein triplet (Greve and Gottlieb, 1982; Chapman, 1984).

If, as indicated above, the proteins bound by the CSAT and JG22 antibodies are present on the surfaces of neural retinal cells, these antibodies should promote cell attachment when adsorbed onto tissue culture plates (MacLeish et al., 1983; Chen et al., 1985b). Results in Fig. 7 show that plates coated with CSAT (JG22) antibody or anti-N-CAM antibody promoted cell attachment while those coated with antibodies specific for the precursor to beta-NGF did not. Substrates of CSAT and JG22, but not anti-N-CAM IgG, supported neurite outgrowth (Fig. 7, data for CSAT not shown). Neurite outgrowth on CSAT antibody-coated substrates was concentration dependent (data not shown). Cells plated on CSAT or JG22 antibodies were similar in appearance to those plated on laminin. They attached readily, assumed a polygonal shape, and extended neurites within several hours after plating. The neurites were frequently long and often individual, rather than fasciculated as on collagen IV or fibronectin. The results in this section provide two types of evidence that neural retinal cells possess surface proteins bound by CSAT and JG22 antibodies. First, CSAT and JG22 antibodies disrupted both cell attachment and neurite outgrowth on extracellular...
Developmental Changes in the Responsiveness of Neural Retinal Cells to Extracellular Matrix Proteins

Neural retinal cells undergo many developmental changes between E6 and E12 in vivo. It seemed possible that these would be accompanied by changes in their properties in vitro. To examine this, neural retinal cells were isolated from embryos of different ages and assayed for the capacity to respond to laminin, fibronectin, collagen IV, and CSAT antibody substrates. Attachment and neurite outgrowth were measured in separate quantitative assays. The results, presented in Fig. 8, show that adsorbed CSAT antibodies were effective at promoting the attachment of neural retinal cells, regardless of the age of the embryo (E6 through E12). Collagen IV was also an effective substrate for cell attachment throughout this period. In contrast, while 60% of the E6 cells plated attached to fibronectin and 90% to laminin, the ability of cells to attach to these substrates declined sharply with age and was absent by E11 (Fig. 8). Interaction with laminin was examined in greater detail by determining the concentration dependence of attachment for cells of different ages (Fig. 9). Half-maximal attachment of E7 cells required only 1–2 μg/ml of laminin, while twofold and threefold more laminin was needed to obtain equivalent attachment with E8 and E9 cells, respectively. With cells from E12 embryos, even 40-fold higher concentrations of laminin failed to promote equivalent attachment (Fig. 9).

The morphology of cells grown overnight on extracellular matrix glycoproteins also varied with embryonic age. Within several hours after plating, E6 embryonic retinal cells assumed a characteristic polygonal shape, and extended neurites on laminin (Fig. 10). Although retinal cells of all ages have a tendency to aggregate, most E6 cells attached directly to the substrate. In contrast, many of the cells from E8 embryos adhered to each other in aggregates that were loosely attached to the substrate. Some E8 cells appeared well attached and had a polygonal shape similar to the E6 cells. In general, polygonal cells appeared to be well attached to the culture substrate, and these cells extended neurites (Fig. 10 B). Few E12 cells attached to the laminin substrate, even after 24 h of culture. A trend toward more cell aggregation and less substrate interaction was also observed for cells plated on either fibronectin or collagen IV after 24 h of culture (data not shown). Aggregates of unattached E12 cells that had been in culture for 16 to 24 h were viable since they transported MTT, indicating mitochondrial function (data not shown, Manthorpe et al., 1986).

Quantitation of neurite outgrowth revealed a decrease on all of the substrates tested with increasing embryonic age (Fig. 8). Neurite outgrowth on fibronectin and collagen IV, while never comparable to that observed on laminin, decreased to even lower levels with increasing embryonic age. This was also true of neurite outgrowth on CSAT antibody substrates (Fig. 8), which declined at approximately the same rate as the response to laminin. Neurite outgrowth on laminin-coated substrates remained concentration dependent through E9 (Fig. 9). In contrast to cell attachment, there was

Figure 10. Phase photomicrographs of neural retinal cells from embryos of different age plated on laminin-coated substrates. Neural retinal cells from E6 (A), E8 (B), or E12 (C) were plated on substrates coated with 5 μg/ml laminin and photographed after 24 h in culture. E6 neural retinal cells exhibit a well attached, polygonal morphology with many long neurites (A). Some E8 cells appear well attached and have neurites (B, arrow), while others are in large cell aggregates that are primarily out of the plane of focus (B, star). E12 neural retinal cells are primarily unattached, in large cell aggregates (C, arrow) with a few attached cells, but no neurites. Bar, 20 μm.
no clear shift in the amount of laminin required to induce a half maximal neurite outgrowth response.

Characterization of Surface Proteins on Neural Retinal Cells That Are Precipitated by CSAT Antibodies

Alterations in the composition or activity of molecules recognized by the CSAT antibody may be related to the observed changes in neuronal response to extracellular matrix proteins. To investigate this possibility, we examined the proteins bound by CSAT antibodies on retinal cells of different ages. Dissociated neural retinal cells were obtained from embryos of different ages, preplated to remove nonneuronal cells, and metabolically labeled. Chick embryo fibroblasts were used for comparison to previously described proteins precipitated by CSAT and JG22 antibodies (Horwitz et al., 1984; Hasegawa et al., 1985; Knudsen et al., 1985).

We found several small but reproducible differences between retinal cells from different aged embryos. As in previous reports (Horwitz et al., 1985; Hasegawa et al., 1985; Knudsen et al., 1985), surface proteins precipitated by CSAT antibodies from chick embryo fibroblasts consisted of two major bands, of Mr 160,000 and Mr 130,000-135,000, when fractionated under reducing conditions (Fig. 11). Proteins obtained by immunoprecipitation from embryonic neural retinal cells exhibited two major bands and one or more minor bands. The higher molecular mass retinal band was less diffuse than the fibroblast protein of similar molecular mass, and migrated in a slightly different position (Fig. 11). Proteins obtained by immunoprecipitation from embryonic neural retinal cells exhibited two major bands and one or more minor bands. The higher molecular mass retinal band was less diffuse than the fibroblast protein of similar molecular mass, and migrated in a slightly different position (Fig. 11). The lower molecular mass retinal protein band was diffuse, but unlike that obtained from chick embryo fibroblasts, resolved into several components as shown by the densitometric scans. E6 retinal cells had a distinct band of Mr 115,000, verified by densitometry, that was not present in fibroblast samples and was less prominent in the E8 or E11 retinal samples (Fig. 11).

Developmental differences in the retinal CSAT-precipitable proteins were more obvious upon nonreducing SDS PAGE, where the fibroblast CSAT glycoproteins were separated into four distinct components of Mr 145,000, 135,000, 125,000, and 110,000. Again, the bands present in the retinal samples were similar, but not identical to those of fibroblasts (Fig. 12). A band of Mr 145,000 was present both in chick embryo fibroblast and in retinal immunoprecipitates of each age. A middle band, of Mr 135,000, was present in all four samples, but migrated as a doublet in the fibroblast and E11 retinal samples (Fig. 12). Densitometry confirmed that this band migrated as a doublet. While a diffuse protein band of Mr 120,000 was also present in all four samples, E6 retinal cells had an additional band of Mr 110,000 that was less prominent in the other samples and could be seen as a shoulder on the densitometric scan. The nature of the relationship between these developmentally regulated structural differences and variation in response to extracellular matrix molecules clearly warrants further investigation.

Discussion

Neural Retinal Cells Respond Differently to Three Matrix Glycoproteins

The use of separate quantitative assays for cell attachment and neurite outgrowth has shown that neural retinal cells respond specifically but differently to collagen IV, laminin, and fibronectin. Attachment to all three substrates was concentration dependent (Fig. 1). Laminin and collagen IV were both effective substrates for cell attachment. Maximal attachment was seen with 0.7 ng of laminin and 0.12 ng of collagen IV to an area of 0.00028 cm² (bottom of well). Both of these were more effective substrates than fibronectin. Even when ~90 ng of adsorbed fibronectin was tested, only ~70% of the plated cells attached. Thus, retinal cells have a relatively low affinity for fibronectin, which corroborates previous work (Rogers et al., 1983). Similar results were obtained with serum fibronectin, suggesting that fibronectin, and not contaminants in the cellular fibronectin preparation, was responsible for cell attachment (data not shown). Others have demonstrated the specific interaction of neurons with fibronectin, in concentrations similar to those reported here.
These interactions were inhibited by anti-fibronectin antiserum (Akers et al., 1981; Rogers et al., 1983).

Stimulation of neurite outgrowth by laminin was also distinctive in several ways. First, a high proportion of cells, 70%, initiated neurites on laminin substrates, compared with <40% on either fibronectin or collagen IV. Second, the morphology of neurites on laminin substrates was unique among the substrates tested. Neurites on laminin were long, branched, and usually not fasciculated. In contrast, the neurites observed on either fibronectin or collagen IV were frequently short, straight, and fasciculated. A differential response to laminin and fibronectin substrates has previously been reported for retinal and spinal cord neurons. Peripheral neurons, in contrast, respond well to both fibronectin and laminin (Rogers et al., 1983).

Differences in the substrate requirements for cell attachment and neurite outgrowth suggest that while adherence to a substrate is a prerequisite for neurite outgrowth, it is not sufficient. Neurons need an appropriately adhesive substrate for neurite initiation and elongation (Letourneau, 1975, 1982). However, the present study and other recent work using defined substrates indicate that adhesivity may not be as tightly coupled to neurite outgrowth as previously thought (see Adler et al., 1985). In our experiments, <10% of the cells plated on poly-D-lysine, a very adhesive substrate, had neurites when examined after 16 to 24 h in culture. Collagen IV, also a very effective substrate for neural retinal cell attachment, supported only modest neurite outgrowth. In contrast, fibronectin, which was notably less effective than collagen IV for cell attachment, proved to be a somewhat better substrate for neurite outgrowth. Only laminin, among the extracellular matrix glycoproteins tested, promoted both efficient cell attachment and vigorous neurite outgrowth. Thus, while neural retinal cells are able to attach to a variety of substrates, different substrates have distinct consequences on neuronal differentiation.

The Role of Molecules Recognized by the CSAT and JG22 Antibodies in Mediating Neural Retinal Response to Matrix Molecules

The CSAT and JG22 monoclonal antibodies have been previously shown to disrupt cell interactions with many extracellular matrix macromolecules, including fibronectin and laminin (Greve and Gottlieb, 1982; Neff et al., 1982; Akiyama et al., 1986; Horwitz et al., 1985). Interactions of dorsal root ganglion neurons with laminin, fibronectin, and collagen I substrates are perturbed by the CSAT antibody (Bozyczko and Horwitz, 1986). In our experiments, low levels of CSAT antibodies (1-10 µg/ml) were shown to inhibit neural retinal cell attachment to laminin, fibronectin, and collagen IV but not to poly-D-lysine or concanavalin A. The lack of effect on attachment to poly-D-lysine indicates that these antibodies act to inhibit specific interactions with extracellular matrix proteins, and not in a general way to impede cell attachment. Furthermore, CSAT and JG22 antibodies do not inhibit neuron attachment to monolayers of nonneuronal cells (Cohen et al., 1986; Tomaselli et al., 1986) or to mixed substrates of laminin and anti-N-CAM antibodies (Tomaselli et al., 1986). The inhibition by CSAT antibodies of neural retinal cell interaction with collagen IV substrates extends the range of cell-substrate interactions perturbed by these antibodies.

Neural retinal cells maintained overnight in the presence of CSAT antibody were unable to initiate neurites. Since these cells were not attached to the substrate, the experiments do not address whether CSAT antibody interfered directly
with neurite initiation and extension. Two types of evidence suggest that molecules recognized by the CSAT antibody have a direct role in neurite extension and adhesion of the neurite and growth cone. Neurons cultured on CSAT antibody substrates grew neurites, suggesting that under these artificial conditions, cell surface molecules recognized by the CSAT antibody acted to anchor not only the cell body but also the growth cone. In addition, ciliary neurons cultured in the presence of CSAT antibody on mixed substrates of either laminin and anti-N-CAM IgG or laminin and poly-D-lysine were found to attach, but not to initiate neurites (Tomaselli et al., 1986). Therefore, under conditions where the cells were attached, CSAT antibody still inhibited neurite extension. The presence of CSAT immunostaining on neurites and growth cones as well as on cell bodies (Bozyczko and Horwitz, 1986) and the ability of CSAT antibody to detach existing neurites from laminin substrates (Bozyczko and Horwitz, 1986) are also consistent with a direct role of molecules recognized by CSAT in neurite extension.

Substrate-attached CSAT antibodies mimic, to a degree, the effects of laminin on neural retinal cells (Fig. 4). Neuronal retinal cells attached to plates coated with anti-N-CAM or CSAT antibody but not to those coated with control antibodies. However, CSAT and JG22, but not anti-N-CAM antibodies, promoted vigorous neurite outgrowth when used as substrates (Fig. 4). These results are similar, in some respects, to effects on neural crest cell and fibroblast adhesion to JG22-coated substrates (Duband et al., 1986; Chen et al., 1985b). Substrate-adsorbed JG22 antibodies promoted attachment and spreading of both cell types (Chen et al., 1985b). Although neural crest cells normally migrate on fibronectin, they failed to migrate on JG22 antibody substrates, indicating that the antibodies did not completely mimic the effects of fibronectin (Duband et al., 1986). In our experiments, laminin was a more effective substrate for neurite extension than the CSAT antibody.

Retinal Cell Response to Matrix Molecules Is Developmentally Regulated

Results in this paper show that neuronal responsiveness to matrix macromolecules changes with age. Collagen IV was an effective substrate for cell attachment throughout the embryonic period examined, E6 through E12. Substrate-adsorbed CSAT antibody remained able to promote cell attachment by E12 neural retinal cells, demonstrating the persistence of surface molecules recognized by the CSAT antibody. The continued presence of molecules recognized by the CSAT antibody on E12 cells was confirmed by immunoprecipitation, discussed below. In contrast, neuronal responsiveness to laminin and fibronectin declines with age. Attachment to fibronectin, already comparatively low on E6, declines over the next 3 d and is absent by E9. Attachment to laminin remains high between E6 and E9, but declines by E11. Examination of dose-response curves shows that cells become progressively less responsive to laminin between E6 and E9 and that most become completely unresponsive by E11. Whether specific subpopulations of cells interact differently with individual substrates is unknown. This does not seem to be the case for E6 retinal cells, since a high proportion of the cells plated attached to the different substrates tested. However, changes in cell-substrate interactions seen at later times, may reflect, in part, changes in the composition of the neural retinal cell population.

The ability of neural retinal cells to initiate neurites on extracellular matrix protein substrates also varied markedly with the age of the neurons. Laminin, CSAT antibody, and, to a lesser extent, fibronectin and collagen were all effective substrates for neurite initiation by E6 neurons. Neurite outgrowth on fibronectin was decreased by E8. The ability to extend neurites persisted longer for cells plated on either laminin or CSAT antibody but was absent by E12. Since E11 neural retinal cells are able to extend neurites when cultured on monolayers of astrocytes, the lack of neurite extension on matrix substrates does not reflect a loss of the ability to extend neurites (Cohen et al., 1986; Tomaselli, K., unpublished results). Developmental changes in the responsiveness of ciliary ganglion neurons to substrates coated with heart cell-conditioned medium have also been shown (Collins and Lee, 1982). More recently, ciliary neurons have been shown to initiate neurites on monolayers of nonneuronal cells at a time when the cells did not respond to laminin-coated substrates (Tomaselli, K., unpublished results). Since the stimulation of neurite outgrowth by nonneuronal cells is not inhibited by the CSAT and JG22 antibodies (Cohen et al., 1986; Tomaselli et al., 1986), these interactions must be mediated by cell surface molecules other than those recognized by these antibodies.

The continued presence of molecules recognized by the CSAT antibody, demonstrated by attachment to CSAT antibody substrates and by immunoprecipitation, indicates that lack of cell attachment to laminin and fibronectin, and absence of neurite outgrowth by E12 cells cannot be attributed to the loss of such molecules from the cell surface. The proteins recognized by CSAT antibody on retinal neurons appear similar, but not identical, to those on fibroblasts (Figs. 2 and 4). There were, however, discernible differences in the pattern of proteins precipitated by the CSAT antibody from E6, E8, and E11 retinal cells. Two differences in the pattern of proteins from cells of different ages were seen consistently. The E6 sample had an additional component of Mr 110,000–115,000 that was not present in either fibroblasts or in the other retinal samples. On nonreducing gels, the 135-kD component migrated as a single band in the E6 and E8 retinal samples, but as a doublet in the E11 retinal and fibroblast samples. In contrast, the affinity purified antigen from sensory neurons may lack the high molecular mass band seen on nonreducing gels (Bozyczko and Horwitz, 1986). The significance of these differences is not known. Several lines of evidence from our and other laboratories suggest the possibility that molecules recognized by the CSAT antibody may belong to a family of receptors, of closely related structure, but with distinct ligand specificities (Leptin, 1986; Horwitz et al., 1985; Pytela et al., 1985; Ruoslahti and Pierschbacher, 1986; Pytela et al., 1986). One possibility is that retinal cells of different ages have structurally related but distinct cell surface molecules, all recognized by the CSAT antibody, that mediate interaction with different matrix glycoproteins.

Whether the loss of neuronal responsiveness to laminin and fibronectin reflects the loss of a specific external receptor or of an intracellular coupling event is unknown. It is clear from our data that molecules recognized by the CSAT anti-
body are present on EII retinal cells, that these cells retain the ability to attach to collagen IV, and that cell interaction with collagen IV is disrupted by the CSAT antibody. However, whether the subtle differences we observed in the pattern of proteins immunoprecipitated with the CSAT antibody from E6 and the EII cells are sufficient to explain the different ligand specificities of the cells at these two developmental times is unclear.

**Possible Roles for the Extracellular Matrix in Regulating Retinal Neuron Development**

In many developing systems, neuronal growth cones have been found in contact with basal laminae, which may be expected to contain collagen IV and laminin (Raper et al., 1983; Taylor and Roberts, 1983; Easter et al., 1984; Rogers et al., 1986; McLoon, 1984). In the retina, axonal growth cones have extensive contact with the inner limiting membrane and other recently formed axons, suggesting that both axon surface and extracellular matrix constituents may be important in directing growth cone behavior (Easter et al., 1984). Evidence suggests that molecules in the basement membrane stimulate axon initiation and provide directional cues to elongating axons (Halfter and Deiss, 1984; 1986). Our experiments suggest that the interactions with laminin and other basement membrane glycoproteins involve neuronal cell surface molecules recognized by the CSAT and JG22 antibodies.

Changes in the responsiveness of neural retinal cells to extracellular matrix constituents observed in vitro may be related to the in vivo development of retinal ganglion cells. In the chick, retinal ganglion cells are generated between E2.5 and E7 (Kahn, 1973). Because ganglion cell birth rate is exponential, many more cells become postmitotic (Kahn, 1973) and initiate axons toward the end of this period (Goldberg and Coulombre, 1972; Halfter et al., 1983). The growth of the first axons to appear is likely to involve the basement membrane, since no other axons are initially present (Halfter et al., 1985). Axon elongation within the eye, occurring for the greatest number of axons around days 6 and 7, is also likely to involve interactions with basement membrane (Halfter and Deiss, 1984), while elongation within the optic nerve and optic tectum may primarily involve interactions with cell surfaces (Thanos and Bonhoeffer, 1983). In this regard, it is particularly interesting that II retinal ganglion cells, the majority of which would have axons that had reached the optic tectum, retain the ability to extend axons on astrocyte surfaces but not on extracellular matrix molecules (Cohen et al., 1986). The growth of neurites on monolayers of astrocytes, Schwann cells, and skeletal myotubes is not inhibited by the CSAT antibody (Tomasselli et al., 1986). Thus, neurones have multiple receptors that can promote neurite outgrowth on different substrates. Our observations are consistent with the possibility that these receptors are developmentally regulated to ensure that axon growth is appropriately guided at different stages.

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