Evidence that an RGD-Dependent Receptor Mediates the Binding of Oligodendrocytes to a Novel Ligand in a Glial-derived Matrix

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Abstract. A simple adhesion assay was used to measure the interaction between rat oligodendrocytes and various substrata, including a matrix secreted by glial cells. Oligodendrocytes bound to surfaces coated with fibronectin, vitronectin and a protein component of the glial matrix. The binding of cells to all of these substrates was inhibited by a synthetic peptide (GRGDSP) modeled after the cell-binding domain of fibronectin. The component of the glial matrix responsible for the oligodendrocyte interaction is a protein which is either secreted by the glial cells or removed from serum by products of these cultures; serum alone does not promote adhesion to the same extent as the glial-derived matrix. The interaction of cells with this glial-derived matrix requires divalent cations and is not mediated by several known RGD-containing extracellular proteins, including fibronectin, vitronectin, thrombospondin, type I and type IV collagen, and tenascin.

THE surface interactions of cells with neighboring cells and, in some cases, with the extracellular matrix are among the most critical determinants of form and function in a developing tissue. The mechanisms of cell adhesion and the biochemistry of numerous components of extracellular matrix and of basement membrane implicated in these adhesive interactions is being actively examined (13, 18, 38, 41, 42, 49). Among noncollagenous proteins with cell-binding functions, fibronectin has been the most thoroughly characterized (16, 49). Specific-binding domains for various extracellular ligands have been mapped within the molecule, including a domain implicated in cell binding. Activity within the cell-binding domain has been further localized to a tripeptide of arginine-glycine-aspartic acid (RGD in single letter code) (31, 32, 51). When immobilized on a surface, synthetic peptides which contain the RGD sequence mimic fibronectin's cell-binding properties. When presented in solution, these same peptides competitively inhibit the binding of fibronectin to its cell surface receptor.

The mechanism defined for binding of cells to fibronectin appears to be of general significance. The RGD sequence has been found in the primary sequence of at least five other extracellular proteins with cell-binding functions, suggesting the existence of a family of proteins which bind cells via a common mechanism (40, 41). RGD-dependent interactions have been described in *Dictyostelium* (10), *Drosophila* (28), and *Xenopus* (3) as well as in mammalian and avian cells (40), suggesting that the interactions are highly conserved across species boundaries. Studies with synthetic competitors will be useful in discovering new functional members of the RGD-family of proteins even before direct purification and sequence data confirms their existence.

The importance of cell surface interactions in tissue development is probably commensurate with the complexity of the organ, therefore, these interactions would seem to play a crucial role in developing brain. Of the many biosynthetic events which take place in brain development, one of the most dramatic is myelination of neuronal axons by oligodendrocytes. Since the brain contains many process-bearing cells, including some neurons, which are not myelinated, specific recognition and adhesion events between certain neurons and oligodendroglia are likely to be critical in determining the ability of oligodendrocytes to form a stable myelin sheath.

We and others have shown that highly purified cultured oligodendrocytes produce a myelin-like membrane that is strikingly similar in morphology and biochemistry to the sheath seen in vivo (4, 39, 46). Unlike myelin-forming cells of the peripheral nervous system (Schwann cells), cultures of oligodendrocytes will produce a myelin-like membrane in the absence of neurons and are, therefore, ideally suited for experiments on the control of myelinogenesis. In this study, we have examined oligodendrocyte adhesion to various surfaces with the long term aim of assessing the contribution of these interactions to oligodendrocyte maturation and to the development of the cell's myelin-like properties. We have also examined the possibility that an RGD-dependent interaction might be an early step in the sequence of events leading to formation of the myelin sheath (5).

Materials and Methods

was obtained from Hyclone (Logan, UT). The Labline Junior Orbit orbital shaker used in both cell culture and adhesion assays was purchased from American Scientific Products (McGaw Park, IL). [^{35}S]Translabel was purchased from ICN (Cleveland, OH). Bovine plasma was purchased from Irvine Scientific (Santa Ana, CA). Rat fibronectin was purchased from Calbiochem (La Jolla, CA). Human vitronectin and rabbit antibodies to human vitronectin were a gift of Dr. Erkki Ruoslahti of the La Jolla Cancer Research Foundation (La Jolla, CA). Antibodies to mouse fibronectin were the gift of Dr. Judy Berliner of the Department of Anatomy, UCLA. Antibodies to human thrombospondin were a gift of Dr. David Roberts of the National Institutes of Health (NIH, Bethesda, MD). Antibodies to glial fibrillary acidic protein were the gift of Dr. L. Eng of the V.A. Medical Center (Palo Alto, CA). Antibodies to rat type IV collagen were a gift of Dr. John Fessler of the Department of Biology, UCLA. Antibodies to chick tenascin were a gift of Dr. Douglas Fambrough of the Department of Biology, Johns Hopkins University (Baltimore, MD). Preparation of antibodies to galactocerebroside have been described elsewhere (39). All other chemicals were from standard commercial suppliers.

Methods

Cell Culture. Purified oligodendrocytes were prepared from neonatal rat cerebral cortex by a modification of the method of McCarthy and de Vellis (25), as described (39). This method is based on the differential adhesion of oligodendrocytes and astrocytes in mixed glial cultures. Oligodendrocytes are released into the culture medium upon extended shaking, while astrocytes remain attached to the plastic surface.

Astroglial Matrix Preparation. Media was removed from confluent mixed glial cells and 1 ml water per well or 10 ml per 75 cm² flask was added. After 2 h at room temperature the plates or flasks were swirled briefly and the lysed cell material removed. The surfaces were washed two times with PBS and then stored in PBS at 37°C until use. Surfaces treated in this way contained no microscopically detectable cell debris or membranous structures. The material remaining on the culture surface after water lysis and wash is referred to as the astroglial matrix (AGM)[1]. A typical AGM preparation contained 0.75–1.0 µg protein/cm² of culture surface. AGM was removed from the plastic surface for analysis by solubilization in 62.5 mM Tris, pH 6.8, containing 3% SDS, 10% glycerol, and 5% β-mercaptoethanol for 1 h at room temperature.

Adhesion Assay. Oligodendrocytes released into the media upon shaking mixed glial cultures (39) were collected by centrifugation in a clinical centrifuge. The cell pellet was suspended by trituration in 10 ml of methionine- and glutamine-free Modified Eagle's Medium supplemented with 1% calf serum and 100–200 µCi [^{35}S]Translabel. The cells were labeled for 6–8 h at 37°C on an orbital shaker at 150 rpm. This gentle agitation prevented the oligodendrocytes from adhering to the surface of the flask and elaborating processes, thus obviating the need for chemical or enzymatic treatment of the cells to release them from the flask after labeling.

After labeling, the media was removed and cells were pelleted as above and the pellet was washed with PBS. The cells were washed once more with DME containing 0.2% BSA (DME/BSA) and the final pellet was resuspended in DME/BSA. The suspension was then filtered through a 20-µM mesh stainless steel sieve (Cellector, Bellco, Vineland, NJ) to remove cell aggregates. The filtered suspension was diluted with the same media to ∼2 × 10⁵ cells/ml.

All surfaces on which adhesion was to be measured were preincubated with DME/BSA for 30 min before the addition of cells. The assay was begun by rapidly aliquoting 0.1 ml of cell suspension (∼2 × 10⁴ cells) to wells containing 0.4 ml of DME/BSA and placing the plates at 37°C. The incubation time was 30 min, unless otherwise noted. After incubation the plates were placed on an orbital shaker at 200 rpm for 10 s and the media was quickly aspirated. The adherent cells were disrupted with 0.5 ml of PBS containing 1% Triton X-100 and transferred to scintillation vials.

Substrates, except for collagen, were applied to the plastic surface, (not tissue culture treated 24-well plates) in a final volume of 0.4 ml at a concentration of 7.5 µg/ml in PBS containing 1 mM Ca²⁺ and 1 mM Mg²⁺ and incubated at 37°C for 2 h. Substrate solutions were removed and the surfaces washed once with PBS. Collagens (Sigma Chemical Co., St. Louis, MO) were applied to the plastic surfaces following the method of Kleinman et al. (19). For protein substrates the amount of material retained on the plastic surface was estimated by extracting the protein retained on the surface and analyzing the sample on SDS-PAGE. A sample representing the total

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1. *Abbreviations used in this paper:* AGM, astroglial matrix; CHAPS, 3-([3-cholamidopropyl]dimethyl-ammonio)-1-propanesulfonate).

amount of protein applied to each well was run on the same gel and the silver stained patterns were compared. For fibronectin and vitronectin, ∼50% of the protein applied to a well could be recovered from the plastic surface.

In experiments using antibodies to disrupt adhesion, wells were incubated for 1 h at 37°C in DME/BSA which contained a 1:200 dilution of the appropriate antisera. The media containing antibody was removed and the surface was washed once with PBS. In experiments using peptides to inhibit cell binding, 0.02 ml of a 25× peptide stock in DME/BSA solution was aliquoted to each well immediately before addition of cells.

Fibronectin Purification. Fibronectin was purified from citrate-treated bovine plasma by gelatin affinity chromatography (50). The column was prepared by coupling gelatin (Janssen, Beerse, Belgium) to Affigel-10 (Bio-Rad, Richmond, CA) in PBS at room temperature according to the Bio-Rad protocol. The coupled resin contained 25 mg gelatin/ml. The column was run at room temperature by applying 25 ml of filtered plasma to a 20-ml column. The bound material was washed extensively wtih PBS, followed by 2 column volumes of 1 M NaCl in PBS before a final wash with 2 column volumes of 4 M urea. The material eluted with urea was dialyzed against PBS at room temperature. Roughly 90–95% of the protein purified by this procedure migrated as a doublet of M_r 220,000.

SDS-PAGE. Polyacrylamide slab gels were prepared with SDS using the buffer system of Laemmli (21). Samples to be analyzed were prepared in buffer containing 62.5 mM Tris, 3% SDS, 10% glycerol, 5% β-mercaptoethanol, pH 6.8. Samples were heated at 100°C for 5 min and run on either 8 or 15% gels with a 4.5% stacking layer. Gels were silver stained by the method of Merril (27).

Immunoblotting and Immunochemistry. Western blot analysis was carried out by electrophoretic transfer to nylon membranes (Biotrace RP, Gelman) according to the method of Towbin (47). The membranes were blocked overnight at room temperature in 1% nonfat dry milk in PBS containing 0.02% sodium azide, pH 7.5. Primary antibodies were diluted 1:200 in 1% nonfat dry milk in PBS, and the membranes were incubated at 37°C for 1–2 h and washed with PBS. A horseradish peroxidase-conjugated secondary antibody (Bio-Rad) was diluted 1:750 in 1% milk/PBS and the membranes were incubated at 37°C for 45 min. After washing the membranes with PBS, antibody complexes were visualized with PBS containing 20% methanol, 0.5 mg/ml 4-chloro,1-naphthol, and 0.03% H₂O₂. Immunostaining was performed on oligodendrocyte cultures by fixing cells in freshly prepared 3.7% paraformaldehyde in PBS for 15 min followed by 15 min in 3.7% paraformaldehyde in PBS containing 0.2% Triton X-100. Fixed cells were incubated at 37°C for 2 h with a 1:100 dilution in PBS of antisera to either glial fibrillary acidic protein or to galactocerebroside or with nonimmune rabbit serum. The cells were washed with PBS several times over a period of 30 min and incubated with a 1:200 dilution of FITC-conjugated goat anti-rabbit IgG (Cappel) at 37°C for 1 h. Both primary and secondary antibodies contained 1:25 dilution of normal goat serum to reduce the nonspecific staining. The cells were washed thoroughly with PBS, mounted and visualized on a Nikon Diaphot-TMD inverted microscope.

Synthetic Peptides. Peptides were prepared by Dr. Janice Young of the UCLA Peptide Synthesis Facility in the Department of Biological Chemistry. The peptides were synthesized on an Applied Biosystems, Inc. (Foster City, CA) 430A Peptide Synthesizer using version 1.3 software.

Protein Determination. Protein values were estimated by the bicinchoninic acid method using a kit supplied by Pierce (Rockford, IL) with BSA as standard (44).

Results

Oligodendrocyte Adhesion to Various Substrates

A simple adhesion assay was used to test the ability of oligodendrocytes to adhere to various substrates. To determine the viability of adherent cells and to ensure that cells bound in the assay were indeed oligodendrocytes, adhesion was carried out on AGM for 30 min as described in Materials and Methods. Rather than extracting the adherent cells immediately for quantitation, though, culture media was added to the wells and the cells were grown for 2 d. As shown in Fig. 1, adherent cells display a morphology typical of cultured oligodendrocytes; the elaborate processes are particularly characteristic of this cell type. Immunofluorescence staining using anti–galactocerebroside, a specific marker for
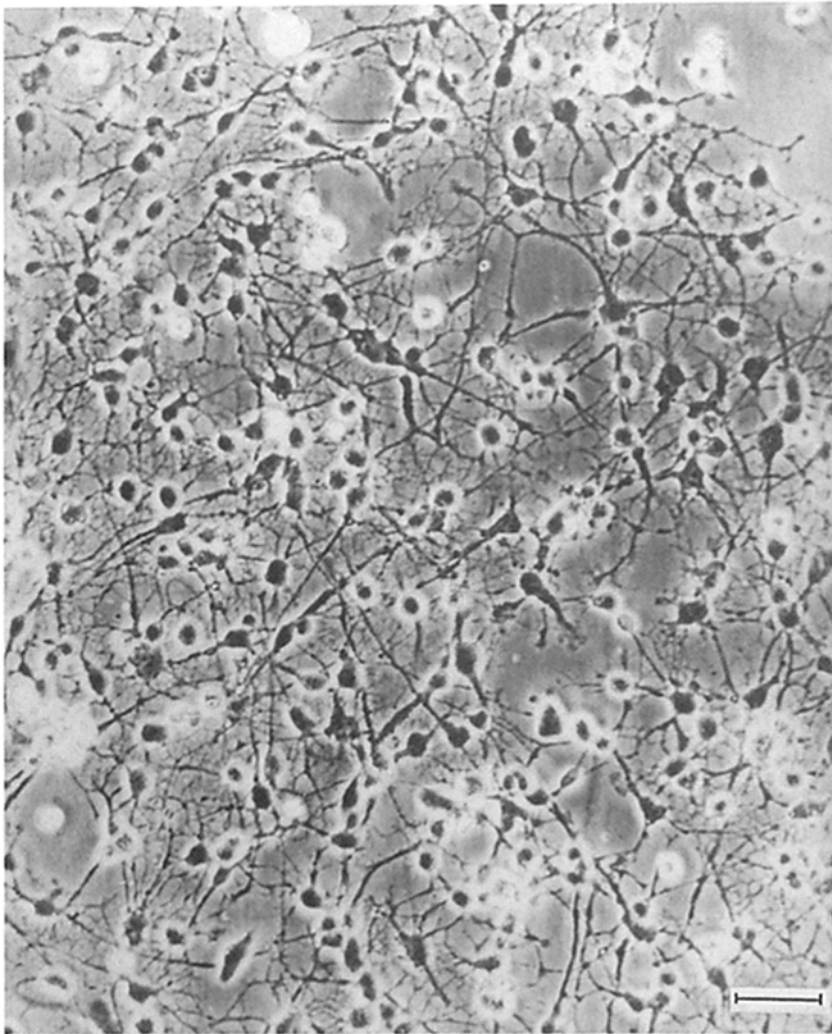
Figure 1. Oligodendrocytes bound in the adhesion assay. Oligodendrocytes were purified from mixed glial cultures, labeled in suspension and washed as described in Materials and Methods. The cells were plated in DME/BSA on AGM and incubated for 30 min. The 24-well plates were shaken and the media was aspirated as described in Materials and Methods. The adherent cells were maintained in culture for 48 h in DME/F12 containing 10% calf serum. Cells were viewed on a Nikon-TMD inverted microscope equipped with phase optics and a 20× objective. The cells were photographed on Tri-X film before fixation for immunofluorescence. Bar, 75 µm.

the oligodendrocyte, revealed positive staining in >80% of the cells. Only 7% of cells present after 2 d in culture were stained with anti–glial fibrillary acidic protein, a specific marker for astrocytes. Most of those cells which were scored as negative for galactocerebroside nonetheless possessed a typical oligodendrocyte morphology and were weakly reactive with galactocerebroside antibody. We chose to immunolabel the cells as soon as it was technically possible (i.e., when the cells had become attached well enough to withstand the numerous wash steps required) so that the population of cells closely reflected that used in adhesion experiments and was minimally effected by cell proliferation. Since the synthetic rate of galactocerebroside develops with time in culture (39), many oligodendrocytes are likely not detected by immunofluorescence after only 2 d in culture. The value of 80% is therefore a low estimate of the purity of oligodendrocytes used in these experiments. While fewer cells adhered to an untreated plastic surface, the percentage of galactocerebroside-positive cells was similar to the percentage of galactocerebroside-positive cells that adhered to AGM (data not shown).

As shown in Fig. 2, the initial rate of oligodendrocyte adhesion varied considerably depending on the nature of the substrate. If the incubation time was increased to 90 min,

all surfaces, including untreated plastic, were essentially equally effective in promoting the adhesion of oligodendrocytes (data not shown). 30 min was chosen as the incubation time for future assays, since the effects of different surface treatments on the initial rate of adhesion were readily apparent at this time. Among surfaces examined for the ability to promote oligodendrocyte adhesion, AGM, bovine plasma fibronectin, rat plasma fibronectin, and chondroitin sulfate all promoted the attachment of oligodendrocytes to a similar extent, with a 2.5–3-fold greater adhesion than seen for untreated plastic. Of all surface treatments examined, poly-L-lysine was most effective in the 30-min assay, increasing binding of cells nearly fivefold over the plastic level. Virtually 100% of the radioactivity associated with trichloroacetic acid precipitable material adheres to poly-L-lysine in a 15–30-min incubation. In a 90-min incubation essentially all of the radioactivity associated with TCA precipitable material adhered to all culture surfaces, indicating that given enough time all cells are competent to form stable adhesive interactions with a wide variety of substrates.

An RGD-containing Peptide Inhibits Oligodendrocyte Adhesion to Fibronectin, Vitronectin, and AGM

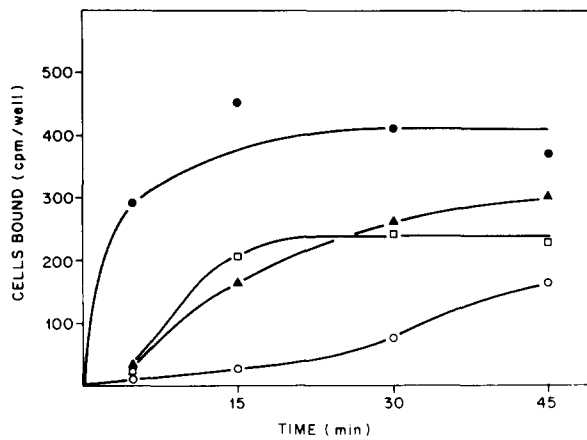Fibronectin and vitronectin are among the best characterized

Figure 2. Time course of oligodendrocyte adhesion. ³⁵S-labeled oligodendrocytes were incubated on surfaces treated with poly-L-lysine (●), bovine fibronectin (▲), AGM (□), prepared as described in Materials and Methods, or on untreated plastic (○). After the indicated times the plates were shaken, the media aspirated, and the adherent cells extracted and counted as described in Materials and Methods. Each point represents the average of three wells and variation between wells was less than 10%.

adhesion proteins. Different cell surface receptors for each of these proteins have been purified and, in each case, shown to recognize a defined sequence of the fibronectin or vitronectin primary structure (1, 35, 36, 45). To examine the specificity of the oligodendrocyte's interaction with fibronectin and vitronectin we used, as an inhibitor of adhesion, a synthetic hexapeptide (GRGDSP) derived from the known sequence of the fibronectin cell-binding domain. At concen-
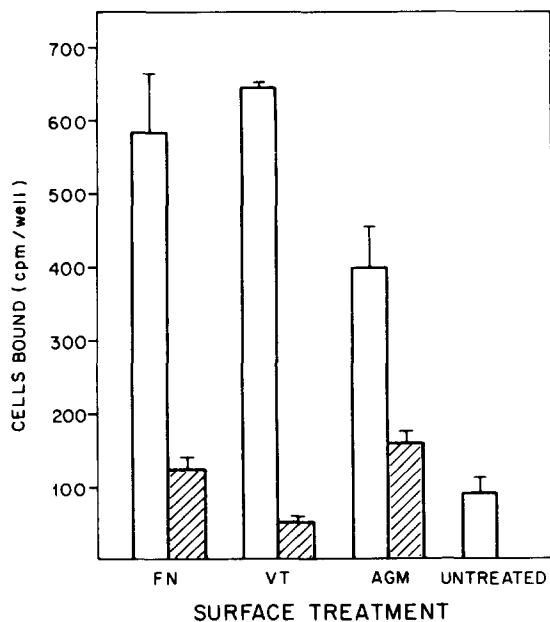

Figure 3. Inhibition of oligodendrocyte adhesion by GRGDSP. Cells were incubated on bovine fibronectin (B-FN), vitronectin (VT), AGM, or untreated plastic. Hatched bars indicate the addition of 0.1 mg/ml of GRGDSP in the incubation media. Values given are the average of 3 wells and the error bars indicate SD.

Table I. Inhibition of Oligodendrocyte Adhesion with Synthetic Peptides

| | Cells Bound (percentage of control) | |
Treatment	AGM	Plastic
GRGDSP	53	100
GRADSP	106	101
SDGR	101	104
VYPNGA	103	113

The specificity of oligodendrocyte adhesion by GRGDSP was examined by testing several other peptides for inhibitory activity. All peptides were used at a final concentration of 0.1 mg/ml. The percent cell binding to the plastic surface was determined by calculating the ³⁵S radioactivity retained in wells containing peptide relative to wells which received no peptide. The percent cell binding to AGM was calculated by first subtracting the background level of ³⁵S radioactivity bound to untreated plastic from the values determined for binding to AGM.

trations of 0.1 mg/ml, the peptide decreased binding of cells to fibronectin by 79% and to vitronectin by 92% (Fig. 3). Increasing the concentration of peptide above this level did not increase the specific inhibition. Interestingly, the peptide also inhibited oligodendrocyte binding to AGM, decreasing adhesion by 60% at 0.1 mg/ml (Fig. 3). The RGD-containing hexapeptide had no effect on cell binding to plastic, poly-L-lysine or chondroitin sulfate (data not shown).

The specificity of this inhibition was examined by screening a group of peptides, both related and unrelated to GRGDSP. As shown in Table I, these peptides had no effect on adhesion of the oligodendrocyte to either AGM or to an untreated plastic surface. One of these peptides differed from the active peptide by the single substitution of alanine for glycine within the RGD sequence. Another inactive peptide, a tetramer with the sequence SDGR, represents the inverse sequence of the active region within the GRGDSP peptide, which has been shown to interfere with cell spreading of both baby hamster kidney cells and chick embryo fibroblasts on fibronectin, vitronectin, collagen, and laminin coated surfaces (52). The peptide GRGES has been shown to inhibit the spreading of certain cell types on fibronectin coated surfaces (15). This peptide, however, did not inhibit the adhesion of oligodendrocytes to either fibronectin or AGM (data not shown).

RGD-dependent Component of AGM Is Produced by Mixed Glial Cells and Is Not Derived from Serum

Since AGM is prepared from cells that have been grown in media containing calf serum, the RGD-dependent binding of oligodendrocytes to AGM might be explained by the presence of serum-derived proteins in the AGM which can bind to cells in a RGD-inhibitable manner. Fibronectin and vitronectin are the most abundant and the best characterized proteins of this family. The binding of cells to both fibronectin and vitronectin is known to require divalent cations. As shown in Fig. 4, binding of oligodendrocytes to either fibronectin, vitronectin, or AGM, is diminished to the level of untreated plastic by addition of 2 mm EDTA, consistent with the notion that cells are binding to each of these proteins by a related mechanism.

To further assess the role that serum-derived factors may play in the adhesive properties of AGM, tissue culture wells
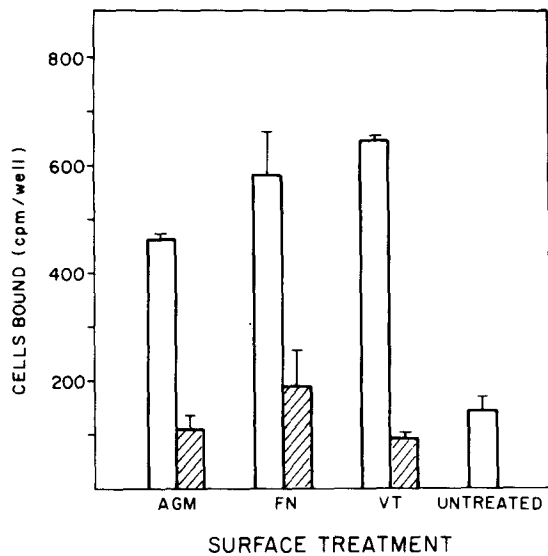
Figure 4. Effect of EDTA on oligodendrocyte adhesion. Cells were incubated on bovine fibronectin *(FN)*, vitronectin *(VT)*, AGM *(AGM)*, or untreated plastic. Hatched bars indicate the addition of 2 mM EDTA to the incubation media. Each bar represents the average of three wells and the error bars indicate the SD.

without cells were incubated with media containing 10% serum at 37°C. The wells were maintained in serum-containing media for 7 d and the media was replenished on the same schedule as parallel plates containing mixed glial cells. All wells were then treated with water lysis and subsequent washes by the same procedure used to prepare AGM from
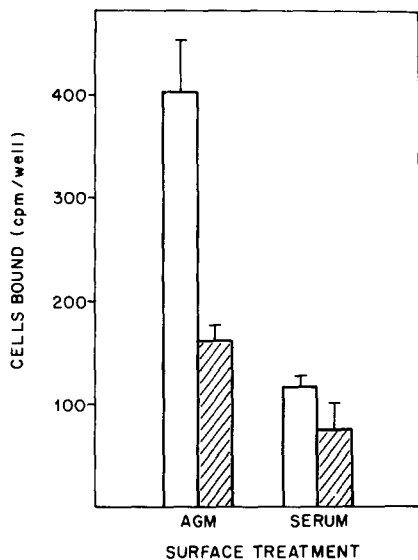

Figure 5. Inhibition by GRGDSP of oligodendrocyte adhesion to serum-treated plastic and to AGM. Wells were plated with mixed glial cells or were "mock-treated" by the addition of DME/F12 containing 10% calf serum, but no cells. All wells were maintained on the same feeding schedule. After 13 d in culture, wells were treated with water lysis and washed resulting in AGM (from the wells which contained cells) and serum-treated plastic (from those wells which contained no cells). These surfaces were then used to compare adhesion in the presence *(hatched bars)* and absence *(open bars)* of 0.1 mg/ml of GRGDSP.
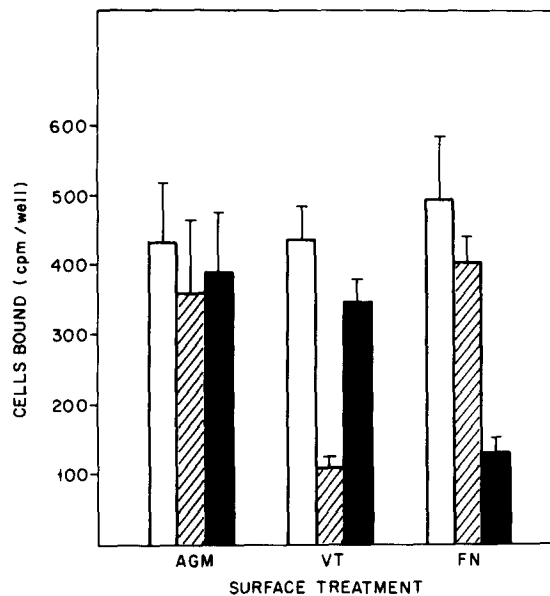

Figure 6. Inhibition of oligodendrocyte adhesion by antibodies to fibronectin and vitronectin. AGM *(AGM)*, vitronectin-*(VT)*, and fibronectin-*(FN)* coated surfaces were preincubated with antibodies to fibronectin *(solid bars)*, vitronectin *(hatched bars)*, or with nonimmune rabbit serum *(open bars)*, as described in Materials and Methods. The antibody treated surfaces were washed with PBS before measuring oligodendrocyte adhesion. Each bar represents the average of three wells and the error bars indicate SD.

mixed cells. These surfaces were then used to compare RGD-dependent adhesion. As shown in Fig. 5, the mock-treated surface exhibited only 16% of the RGD-dependent adhesion of AGM.

Thus it appears that serum-derived factors are insufficient to account for the behavior of AGM. It is conceivable, however, that serum proteins such as fibronectin and/or vitronectin could combine with constituents of the AGM to show adhesion beyond that seen for either protein alone. However, if this were the case, antibodies which specifically inhibit adhesion to purified serum proteins might be expected to inhibit adhesion to the AGM as well. As shown in Fig. 6, pretreatment of wells with antibodies to fibronectin inhibited adhesion of cells to fibronectin-coated plastic, but were ineffective against either vitronectin, or AGM. Similarly, antibodies to vitronectin inhibited adhesion to vitronectin-coated surfaces but were ineffective against either fibronectin or AGM.

The antibodies against mouse fibronectin are equally effective in inhibiting adhesion to both rat and bovine fibronectin excluding the possibility that fibronectin secreted by the glial cells is the active adhesion component of the AGM.

In a separate experiment, surfaces pretreated with antibody to either fibronectin, vitronectin or both were tested for RGD-inhibitable adhesion. As shown in Fig. 7, fibronectin antibodies inhibit the binding of oligodendrocytes to fibronectin-coated surfaces to the background value of untreated plastic and addition of RGD-containing peptide caused no further inhibition. The same is true of vitronectin-coated surfaces; antibodies to vitronectin decrease binding to back-
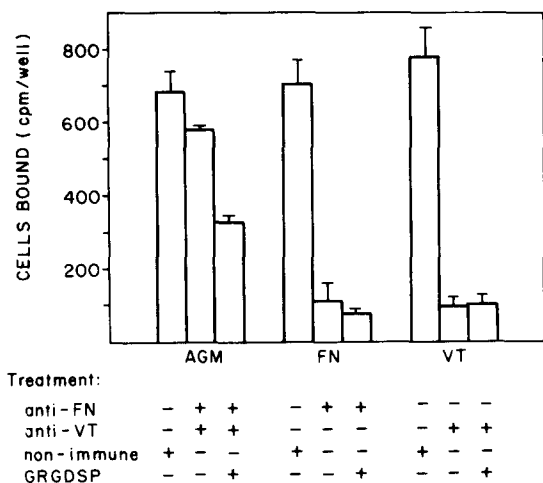
Figure 7. GRGDSP inhibition of oligodendrocyte adhesion to surfaces pretreated with antibodies to fibronectin and vitronectin. Fibronectin-(*FN*) coated surfaces were preincubated with nonimmune serum, or anti-fibronectin (*anti-FN*); vitronectin-(*VT*) coated surfaces were preincubated with nonimmune serum, or anti-vitronectin (*anti-VT*); AGM was preincubated with nonimmune serum, or with both anti-FN and anti-VT. Oligodendrocyte adhesion was measured in the presence and absence of 0.1 mg/ml GRGDSP. Each bar represents the average of 3 wells and the error bars indicate SD.

Figure 9. Effect of pronase digestion of AGM on oligodendrocyte adhesion. AGM and untreated plastic surfaces were incubated with PBS containing 10 µg/ml pronase B for 1 h at 37°C. The surfaces were washed with PBS and used to compare adhesion of oligodendrocytes in the presence (*hatched bars*) and absence (*open bars*) of 0.1 mg/ml GRGDSP.

ground levels and no RGD-inhibitable adhesion is evident on antibody treated surfaces. When AGM is treated with antibodies to both vitronectin and fibronectin, cell binding is diminished by 18%. However, 44% of the cells which bind to AGM treated with both antibodies are still inhibited by 0.1 mg/ml of RGD-containing hexapeptide, indicating that the RGD-inhibitable behavior of the AGM is imparted largely by a component(s) other than fibronectin or vitronectin.

To estimate the amount of fibronectin or vitronectin that would be needed to account for the properties of AGM, we carried out titration curves, applying variable amounts of purified proteins to the plastic wells and measuring adhesion of oligodendrocytes to these surfaces. Maximal adhesion oc-
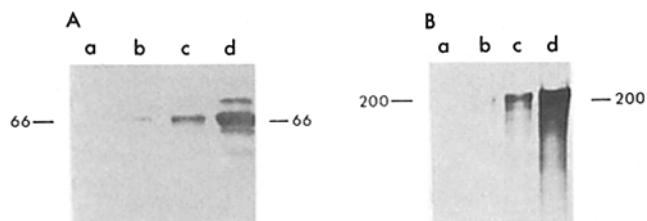

Figure 8. Western blot analysis of AGM using antibodies to fibronectin and vitronectin. AGM was extracted from the surface of flasks in SDS-PAGE sample buffer as described in Materials and Methods. Approximately 0.75–1.0 µg of AGM extract was run per lane which is the amount of material extracted per 1 cm² culture surface. In *A*, a 15% gel was run with AGM extract (lane *a*), 0.01, 0.10, and 1.0 µg vitronectin (lanes *b*, *c*, and *d*). In *B*, a 8% gel with AGM extract (lane *a*), 0.01, 0.1 and 1.0 µg bovine fibronectin (lane *b*, *c*, and *d*). The gels were transferred to nylon membranes and processed with anti-vitronectin antibodies (*A*) or anti-fibronectin antibodies (*B*).
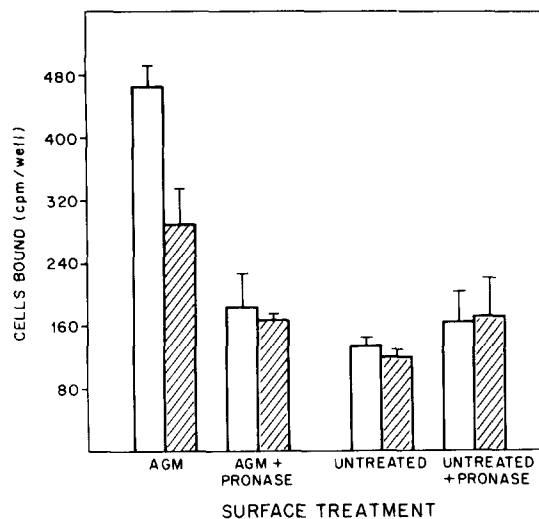
curred at >1.0 µg/well fibronectin and at >0.5 µg/well vitronectin. The material extracted from AGM was directly analyzed on SDS–PAGE for the presence of these serum proteins. Neither protein is detected by western blotting (Fig. 8). In other experiments using more concentrated AGM we were able to detect 20 ng/well of fibronectin. This amount is less than 5% of what would be required to explain the RGD-inhibitable properties of AGM.

The results summarized above suggest that the component of AGM responsible for its RGD-inhibitable behavior is not derived from serum. In particular, they show that the active component is neither fibronectin nor vitronectin. Three other proteins which bind to cells via RGD-dependent mechanisms, thrombospondin (22), Type I collagen (8) and tenascin (7, 9), were also considered as the AGM active component. Antibodies to human thrombospondin and chick tenascin were ineffective in inhibiting the adhesion of oligodendrocytes to AGM and neither tenascin nor thrombospondin were detected in AGM extracts by western blot (data not shown). Plastic surfaces coated with either type IV or type I collagen did not promote adhesion above the level for untreated plastic. Antibodies to type IV collagen, which contains an RGD-sequence (43), did not inhibit the binding of cells to AGM (data not shown).

The chemical nature of the active factor(s) in AGM was probed by digestion of AGM using protease and glycosidases. As shown in Fig. 9, digestion of AGM with pronase B reduces the binding of cells to the level of untreated plastic. The pronase digested AGM shows essentially no RGD inhibition. In contrast, digestion of AGM with heparinase and chondroitinase ABC (Miles, Lisle, IL) had little or no effect on cell adhesion (data not shown).

Discussion

The use of cells in primary culture to study the differentiated

properties which typify those cells in vivo, often requires the use of a specific substratum (11, 13, 38, 41). Schwann cells, the counterpart of the oligodendrocyte in the peripheral nervous system, when kept in culture under appropriate conditions, produce an extracellular matrix which is similar to the matrix formed in peripheral nerve endoneurium. In these cultures, basement membrane components have been shown to induce normal Schwann cell differentiation (6, 26). Our interest in the substratum produced by mixed glial cultures stems from the observations that oligodendrocytes plated on AGM, rather than plastic or poly-L-lysine, tend to more rapidly extend processes and develop morphologically. In addition, cells plated on AGM show a twofold higher rate of incorporation of [^{3}H]thymidine into trichloroacetic acid precipitable material than cells grown either on plastic or poly-L-lysine (Bullock, P. N., and L. H. Rome, unpublished observations). As a first step in quantifying the role that the AGM plays in development of oligodendrocytes in culture, we have begun to characterize the initial adhesive interaction between oligodendrocytes and components of this surface.

The results presented here show that oligodendrocytes, like many cell types, bind to fibronectin and vitronectin in an RGD-dependent manner. More interestingly, oligodendrocytes bind to a protein component present in an extracellular matrix produced by cultures of mixed glial cells. This interaction is also inhibited by an RGD-containing hexapeptide. The AGM interaction is not inhibited by antibodies to either vitronectin or fibronectin even though the same antibody treatment prevented oligodendrocyte adhesion to a surface coated with the purified proteins. Furthermore, the AGM component does not appear to be derived from serum.

Cultures of astrocytes from neonatal rat cerebral cortex have been reported to synthesize fibronectin (34). These cultures synthesize and secrete fibronectin in amounts comparable to fibroblasts, but the form of the protein produced does not assemble into a matrix. Fibronectin has also been immunolocalized to the surface of astrocyte cultures which were derived from various brain regions. However, astrocytes from neonatal cerebral cortex, as used in our study to form the AGM, were negative when stained for cell surface fibronectin (23). Since our fibronectin antibodies recognize rat as well as bovine fibronectin, it is unlikely that fibronectin secreted by the mixed glial cells, or derived from serum, is the active component of the AGM.

It was possible that the RGD-dependent behavior of AGM could be imparted by fibronectin derived from either the serum or the mixed glial cells which, though interactions with other products of the glial cultures, were not recognized by the antibodies we used. For example, the binding of proteoglycans to a site distinct from the cell-binding site has been shown to inhibit the cell-binding characteristics of fibronectin and of type I collagen (41). Presumably the proteoglycan exerts this effect indirectly by obscuring the cell-binding domain. A similar interaction with proteoglycans secreted by the glial cells might result in the 'masking' of fibronectin antigenic sites from antibodies. However, since we could not detect an appreciable level of either fibronectin or vitronectin in AGM extracts by western blot, it is unlikely that these proteins are components of AGM.

Thrombospondin is another serum protein which binds to cells in an RGD-dependent manner (22). That this protein might be a component of the AGM is suggested by the report that cultures of human astrocytes synthesize thrombospondin (2). However, antibodies to human thrombospondin had no effect on the ability of oligodendrocytes to adhere to the AGM. Additionally, we were unable to demonstrate its presence in extracts of AGM by western blot.

The extracellular protein tenascin (7, 9), previously known as chick myotendinous antigen, is apparently identical to several independently isolated extracellular proteins, including cytotactin. Cell binding to this protein is inhibited by RGD-containing peptides. Cytotactin has been shown to be produced by glial cells and has been implicated in neuron–glia interactions (12). While we did see material which reacted with tenascin antibodies in extracts of mixed glial cultures, antibodies to tenascin were ineffective in inhibiting adhesion to AGM and the protein was not detected in AGM by western blot.

The chemical basis of the oligodendrocyte/neuron interaction has been the subject of much speculation. Several molecules with cell adhesive properties have been implicated as mediators of this interaction. The neural cell adhesion molecule, NCAM, is widely distributed among neuronal types and is a component of oligodendrocytes both in vivo (24) and in vitro (29). In contrast to the mechanism of fibronectin binding to its receptor, NCAM mediated cell–cell interactions are homophillic and Ca^{2+} independent. The myelin associated glycoprotein, MAG, has been suggested to play a role in the interaction on the basis of its periaxonal localization within myelin and on its adhesive properties seen with in vitro assays (33). MAG, NCAM, and cytotactin are members of a group of adhesion proteins which share a common carbohydrate epitope which is itself implicated in the adhesive properties of these proteins (20). Interestingly, cytotactin has been shown bind to cells in an RGD-dependent manner and, while MAG has not been shown to have such binding properties, it has been reported to have an RGD region in a proposed extracellular domain. The precise role that these proteins play in oligodendrocyte function remains to be determined but the potential overlap between these two classes of adhesive interactions is intriguing.

Oligodendrocytes were able to adhere in an RGD-dependent manner to at least three different ligands; fibronectin, vitronectin, and a component in AGM. Several cell surface receptors which recognize RGD sequences have been isolated (1, 8, 10, 14, 17, 30, 35–37). Fibroblasts bind to both vitronectin and fibronectin via distinct receptor complexes, which show exclusive binding specificities. There is precedent, however, for a single receptor complex binding to several different proteins in an RGD-dependent manner. The glycoprotein IIb/IIIa complex of platelets binds to at least four different proteins in an RGD-dependent manner (9, 37). Integrin, previously called CSAT antigen, binds to fibronectin, vitronectin and to laminin (14). The binding of this receptor complex to all three of these proteins is inhibited by RGD-containing peptides. Our preliminary attempts to purify an oligodendrocyte cell surface protein by chromatography of detergent extracts of these cells on immobilized GRGDSP hexapeptide have so far been unsuccessful. However, affinity chromatography using an immobilized peptide has been generally less effective than chromatography with the intact protein or proteolytic fragments of the parent pro-

tein. For example, while both the vitronectin and fibronectin receptors from osteosarcoma cells can be affinity purified using the parent protein as affinity support, only the vitronectin receptor can be isolated using a peptide affinity column (35, 36).

The adhesive component of the AGM can be solubilized using detergents and chaotropic agents. We have not yet been able to reconstitute the adhesive activity of these extracts. A major obstacle to these studies has been the insolubility of the AGM under physiological conditions. For example, a buffer containing 4 M urea and 1% CHAPS completely removes the activity from the culture surface. Dialysis of this extract against PBS at room temperature results in the irreversible precipitation of most of the protein, and what remains in solution is inactive in promoting adhesion.

Recent work on the biology of the oligodendrocyte has suggested that nonspecific adhesion may play a role in the phenotypic development of these cells in culture (48, 53). Using a system in which mature oligodendrocytes can be maintained either floating or attached to a poly-L-lysine surface, it has been demonstrated that attached cells show increased levels of synthesis of the major myelin proteins (proteolipid protein and myelin basic protein), as well as increased phosphorylation of myelin basic protein. With our observation of a specific and easily inhibitable adhesive interaction as a starting point, we have begun to examine the contribution that this RGD-dependent interaction makes to development of the myelin-like properties of neonatal rat oligodendrocytes in culture (5).

References

1. Akiyama, S. K., S. S. Yamada, and K. M. Yamada. 1986. Characterization of a 140-kD avian cell surface antigen as a fibronectin-binding molecule. *J. Cell Biol.* 102:442-448.
2. Asch, A. S., L. L. K. Leung, J. Shapiro, and R. L. Nachman. 1986. Human brain glial cells synthesize thrombospondin. *Proc. Natl. Acad. Sci. USA.* 83:2904-2908.
3. Boucaut, J.-C., T. Darribere, T. J. Poole, H. Aoyama, K. M. Yamada, and M. P. Thiery. 1984. Biologically active synthetic peptides as probes of embryonic development: a competitive peptide inhibitor of fibronectin function inhibits gastrulation in amphibian embryos and neural crest cell migration in avian embryos. *J. Cell Biol.* 99:1822-1830.
4. Bradel, E. J., and F. P. Prince. 1983. Cultured neonatal rat oligodendrocytes elaborate myelin membrane in the absence of neurons. *J. Neurosci. Res.* 9:381-392.
5. Cardwell, M. C., and L. H. Rome. 1988. RGD-containing peptides inhibit the synthesis of myelin-like membrane by cultured oligodendrocytes. *J. Cell Biol.* 107:1551-1559.
6. Carey, D. J., M. S. Todd, and C. M. Rafferty. 1986. Schwann cell myelination: induction by exogenous basement membrane-like extracellular matrix. *J. Cell Biol.* 102:2254-2263.
7. Chiquet, M., and D. M. Fambrough. 1984. Chick myotendinous antigen. II. A novel extracellular glycoprotein complex consisting of large disulfide-linked subunits. *J. Cell Biol.* 98:1937-1946.
8. Dedhar, S., E. Ruoslahti, and M. D. Pierschbacher. 1987. A cell surface receptor complex for collagen Type I recognizes the arg-gly-asp sequence. *J. Cell Biol.* 104:585-593.
9. Erickson, H. P., and H. C. Taylor. 1987. Hexabrachion proteins in embryonic chicken tissues and human tumors. *J. Cell Biol.* 105:1387-1394.
10. Gabius, H.-J., W. R. Springer, and S. H. Barondes. 1985. Receptor for the cell binding sites of discoidin I. *Cell.* 42:449-456.
11. Gospodarowicz, D. J. 1984. Extracellular matrices and the control of cell proliferation and differentiation in vitro. *In* New Approaches to the Study of Benign Prostatic Hyperplasia. F. A. Kimball, A. E. Buhl, and D. B. Carter, editors. Alan R. Liss, Inc., New York. 103-128.
12. Grumet, M., S. Hoffman, K. L. Crossin, and G. M. Edelman. 1985. Cytotactin, an extracellular matrix protein of neural and non-neural tissues that mediates glia-neuron interaction. *Proc. Natl. Acad. Sci. USA.* 82:8075-8079.
13. Hay, E. D. 1984. Cell-matrix interaction in the embryo: cell shape, cell surface, cell skeletons, and their role in differentiation. *In* The Role of the Extracellular Matrix in Development. R. L. Trelstad, editor. Alan R. Liss, Inc., New York, 1-31.
14. Horwitz, A., K. Duggan, R. Greggs, C. Decker, and C. Buck. 1985. The cell substrate attachment (CSAT) antigen has properties of a receptor for laminin and fibronectin. *J. Cell Biol.* 101:2134-2144.
15. Humphries, M. J., S. K. Akiyama, A. Komoriya, K. Olden, and K. M. Yamada. 1986. Identification of an alternatively sliced site in human plasma fibronectin that mediates cell type-specific adhesion. *J. Cell Biol.* 103:2637-2647.
16. Hynes, R. O. 1981. Fibronectin and its relation to cellular structure and behavior. *In* Cell Biology of Extracellular Matrix. E. D. Hay, editor. Plenum Press, New York. 295-333.
17. Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48: 549-554.
18. Kleinman, H. K., M. L. McGarvey, J. R. Hassell, V. L. Star, F. B. Cannon, G. W. Laurie, and G. R. Martin. 1986. Basement membrane complexes with biological activity. *Biochemistry.* 25:312-318.
19. Kleinman, H. K., E. B. McGoodwin, S. I. Rennard, and G. R. Martin. 1979. Preparation of collagen substrates for cell attachment: effect of collagen concentration and phosphate buffer. *Anal. Biochem.* 94:308-312.
20. Kunemund, V., F. B. Jungalwala, G. Fischer, D. K. H. Chou. G. Keilhauer, and M. Schachner. 1988. The L2/HNK-1 carbohydrate of neural cell adhesion molecules is involved in cell interactions. *J. Cell Biol.* 106: 213-223.
21. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 277:680-685.
22. Lawler, J., and R. O. Hynes. 1986. The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins. *J. Cell Biol.* 103:1635-1648.
23. Liesi, P., T. Kirkwood, and A. Vaheri. 1986. Fibronectin is expressed by astrocytes cultured from embryonic and early postnatal rat brain. *Exp. Cell Res.* 163:175-185.
24. Martini, R., and M. Schachner. 1986. Immunoelectron microscopic localization of neural cell adhesion molecules (L1, N-CAM and MAG) and their shared carbohydrate epitope and myelin basic protein in developing sciatic nerve. *J. Cell Biol.* 103:2439-2448.
25. McCarthy, K. D., and J. de Vellis. 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J. Cell Biol.* 85:890-902.
26. McGarvey, M. L., A. Baron-Van Evercooren, H. K. Kleinman, and M. Dubois-Dalcq. 1984. Synthesis and effects of basement membrane components in cultured rat Schwann cells. *Dev. Biol.* 105:18-28.
27. Merril, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert. 1980. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science (Wash. DC).* 211:1437-1438.
28. Naidet, C., M. Semeriva, K. M. Yamada, and J. P. Thiery. 1987. Peptides containing the cell-attachment recognition signal Arg-Gly-Asp prevent gastrulation in *Drosophila* embryos. *Nature (Lond.).* 325:348-350.
29. Noble, M., M. Albrechtsen, C. Moller, J. Lyles, E. Bock, C. Goridis, M. Watanabe, and U. Rutishauser. 1985. Glial cells express N-CAM/D2-CAM-like polypeptides *in vitro. Nature (Lond.).* 316:725-728.
30. Parise, L. V., and D. R. Phillips. 1986. Fibronectin-binding properties of the purified platelet glycoprotein IIb-IIIa complex. *J. Biol. Chem.* 261: 14011-14017.
31. Pierschbacher, M. D., and E. Ruoslahti. 1984. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature (Lond.).* 309:30-33.
32. Pierschbacher, M., E. G. Hayman, and E. Ruoslahti. 1983. Synthetic peptide with cell attachment activity of fibronectin. *Proc. Natl. Acad. Sci. USA.* 80:1224-1227.
33. Poltorak, M., R. Sadoul, G. Keilhauer, C. Landa, T. Fahrig, and M. Schachner. 1987. Myelin-associated glycoprotein, a member of the L2/HNK-1 family of neural cell adhesion molecules, is involved in neuron-oligodendrocyte and oligodendrocyte-oligodendrocyte interaction. *J. Cell Biol.* 105:1893-1899.
34. Price, J., and R. O. Hynes. 1985. Astrocytes in culture synthesize and secrete a variant form of fibronectin. *J. Neurosci.* 5:2205-2211.
35. Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. A 125/115-kDa cell surface receptor specific for vitronectin interacts with the arginine-glycine-aspartic acid adhesion sequence derived from fibronectin. *Proc. Natl. Acad. Sci. USA.* 82:5766-5770.
36. Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. Identification and isolation of a 140 kd cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell.* 40:191-198.
37. Pytela, R., M. D. Pierschbacher, M. H. Ginsberg, E. F. Plow, and E. Ruoslahti. 1986. Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp-specific adhesion receptors. *Science (Wash. DC).* 231:1559-1562.

38. Reid, L. M., and D. M. Jefferson. 1984. Cell culture studies using extracts of extracellular matrix to study growth and differentiation in mammalian cells, in *Mammalian Cell Culture*. J. P. Mather, editor. Plenum Press, New York. 239–280.

39. Rome, L. H., P. N. Bullock, F. Chiappelli, M. C. Cardwell, A. M. Adinolfi, and D. Swanson. 1986. Synthesis of a myelin-like membrane by oligodendrocytes in culture. *J. Neurosci. Res.* 15:49–65.

40. Ruoslahti, E., and M. D. Pierschbacher. 1986. Arg-Gly-Asp: a versatile cell recognition signal. *Cell.* 44:517–518.

41. Ruoslahti, E., E. G. Hayman, and M. D. Pierschbacher. 1985. Extracellular matrices and cell adhesion. *Arteriosclerosis.* 5:581–594.

42. Sanes, J. R. 1983. Roles of extracellular matrix in neural development. *Annu. Rev. Physiol.* 45:581–600.

43. Schuppan, D., R. W. Glanville, and R. Timpl. 1982. Covalent structure of mouse type-IV collagen. *Eur. J. Biochem.* 123:505–512.

44. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Analyt. Biochem.* 150:76–85.

45. Suzuki, S., A. Oldberg, E. G. Hayman, M. D. Pierschbacher, and E. Ruoslahti. 1985. Complete amino acid sequence of human vitronectin deduced from cDNA. Similarity of cell attachment sites in vitronectin and fibronectin. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2519–2524.

46. Szuchet, S., S. H. Yim, and S. Monsma. 1983. Lipid metabolism of isolated oligodendrocytes maintained in long-term culture mimics events associated with myelinogenesis. *Proc. Natl. Acad. Sci. USA.* 80:7019–7023.

47. Towbin, H., T. Staehelim, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

48. Vartanian, T., S. Szuchet, G. Dawson, and A. T. Campagnoni. 1986. Oligodendrocyte adhesion activates protein kinase C-mediated phosphorylation of myelin basic protein. *Science (Wash. DC).* 234:1395–1398.

49. Yamada, K. M. 1983. Cell surface interactions with extracellular materials. *Annu. Rev. Biochem.* 52:761–799.

50. Yamada, K. M. 1982. Isolation of fibronectin from plasma and cells. *In* Immunochemistry of the Extracellular Matrix. Volume I. H. Furthmayr, editor. CRC Press, 111–123.

51. Yamada, K. M., and D. W. Kennedy. 1984. Dualistic nature of adhesive protein function: fibronectin and its biologically active peptide fragments can autoinhibit fibronectin function. *J. Cell Biol.* 99:29–36.

52. Yamada, K. M., and D. W. Kennedy. 1987. Peptide inhibitors of fibronectin laminin and other adhesion molecules: unique and shared features. *J. Cell Physiol.* 130:21–28.

53. Yim, S. H., S. Szuchet, and P. E. Polak. 1986. Cultured oligodendrocytes: A role for cell-substratum interaction in phenotypic expression. *J. Biol. Chem.* 261:11808–11815.