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An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension

(neuron–neuron adhesion/axon guidance)

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ABSTRACT **The 8D9 antigen, a cell surface protein isolated from chicken brain that is related to the L1 class of cell adhesion molecules, is shown to contain an activity that promotes the attachment of neurons and the outgrowth of neurites from chicken tecta and mouse cerebellum. When purified 8D9 antigen is attached to a nitrocellulose-coated substrate, neurons rapidly attach and extend unfasciculated neurites. Little or no attachment of astroglia, oligodendroglia, and fibroblast-like cells to the 8D9 antigen is observed. We propose that a function of the 8D9 antigen is that of a neurite extension-promoting substrate in axon fascicles and in regeneration of peripheral nerves.**

Axon fasciculation is an important developmental mechanism in guiding axons to their targets. Two types of cell adhesion molecules (CAMs) have been described that appear to be involved in neurite fasciculation in vertebrates. One type is represented by neural cell adhesion molecule (N-CAM) (1). The second is represented by nerve growth factor-inducible large external glycoprotein (NILE) (2) and closely related proteins L1 (3), neuron-glial CAM (Ng-CAM) (4), G4 (5), and 8D9 (6). In both cases, the involvement of these molecules with axon fasciculation was suggested by experiments in which explants were allowed to extend neurites in the presence or absence of monovalent Fab fragments of polyclonal antibodies directed against the respective molecules (1, 2, 5).

We have previously identified a cell surface antigen designated 8D9 that was detected in regions of the developing chicken optic pathway that were rich in fasciculated axons (6). We further demonstrated that the 8D9 antigen was immunologically related to L1 (6); others have demonstrated that L1 is immunologically related to NILE (7) and that NILE is immunologically related to Ng-CAM (8). Additionally, all of these molecules share similar anatomical distribution and molecular weight, strongly suggesting that all are identical or very closely related. In this report we describe an assay of 8D9 antigen function in neurite outgrowth that provides direct evidence that one of the 8D9 antigen's functions on axons may be to provide a substrate for the outgrowth of subsequent axons.

MATERIALS AND METHODS

Materials and Animals. Nitrocellulose was obtained from Schleicher & Schuell (type BA 85). Laminin was obtained from GIBCO; poly(L-ornithine) was from Sigma and bacteriological Petri plates were obtained from Falcon. Fertilized chicken eggs were obtained from Sachs and Sons Poultry Farms (Evans City, PA) and incubated and staged as described (6). Mice were outbred CD-1 obtained from Charles River Breeding Laboratories (Wilmington, MA).

Cell Culture. Cell culture substrates were prepared by coating Petri plates with nitrocellulose. This was done by dissolving 5 cm^2 of nitrocellulose in 6 ml of methanol. Aliquots of 0.5 ml of this solution were rapidly spread over the surface of 60-mm Petri plates and allowed to dry under a laminar flow hood. Test protein samples were applied in 1- to 5-μl droplets containing 0.1–1.0 mg/ml, as specified. After $\approx$1 min, the droplets were removed by aspiration and the substrate plates were then blocked by washing twice with either Dulbecco's modified Eagle's medium (DMEM)/1% bovine serum albumin or culture medium [DMEM with 10% horse serum for chicken tecta; basal Eagle's medium (Earle's) with Hanks' salts and 10% horse serum for mouse cerebellum]. Freshly dissociated tectal cells from embryonic day 10 chicken embryos or cerebellar cells from postnatal day 6 mice were prepared as described (6, 9) and added to substrate test plates in concentrations of 1×10^6 per ml in 2 ml of culture medium. For some experiments, granule cells were purified from mouse cerebellum using Percoll (Pharmacia) gradients (10). Mouse cerebellar explants were prepared by mechanical dissociation with fire-polished Pasteur pipettes and cultured as described above.

Quantification of Neurite Length. The measurement techniques used are based on those developed by Chang *et al.* (11). Culture dishes were examined with a Leitz Dilux inverted phase microscope equipped with a Dage-MTI-65 video camera that was interfaced to an IBM-XT computer equipped with Bioquant Image Analysis system. Neurite length was measured as the distance between the center of the cell soma and the tip of its longest neurite. Neurites were only counted if (*i*) the neurite emerged from a cell in isolation (not in a clump of cells), (*ii*) the neurite did not contact other cells or neurites, (*iii*) and the neurite was longer than the diameter of the cell body. All cells with neurites in an area of $\approx$4.8 mm^2 were measured. The Mann–Whitney U test was used to determine whether different substrates produced significantly different amounts of neurite outgrowth. In addition, the percentage of single cells with neurites and the total number of cells in an area of 1.6 mm^2 were determined.

Purification of the 8D9 Antigen and N-CAM. These two CAMs were affinity purified from embryonic chicken brain using Affi-Gel 10 (Bio-Rad) coupled to either monoclonal antibody (mAb) 8D9 (6) or mAb 224-1A6, which binds to N-CAM (12) as described, except that membranes were solubilized in 1% sodium deoxycholate. Antigen was eluted with 0.1 M diethylamine (pH 11.5) and immediately neutralized with solid Tris·HCl to use in the substrate test. To examine the purity of the 8D9 antigen and to compare it with other related molecules, two-dimensional gels were prepared

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Abbreviations: CAM, cell adhesion molecule; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; mAb, monoclonal antibody; N-CAM, neural CAM; Ng-CAM, neuron-glial CAM; NILE, nerve growth factor-inducible large external glycoprotein.

using isoelectric focusing in the first dimension (using Servalyte 5-8 as ampholytes) and $NaDodSO_4$/PAGE for the second dimension as described by O'Farrell (13).

Immunocytochemistry. Mouse cerebellar astroglial cells were identified by staining with rabbit anti-glial fibrillary acidic protein (anti-GFAP) antiserum (Dako); chicken tectal glial cells were identified by staining with mAb 3A7 (14). Rabbit anti-GFAP was detected with goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Cappel Laboratories, Cochranville, PA); HRP was detected with 4-chloro-1-naphthol (Sigma). mAb 3A7 was detected with goat anti-mouse IgG conjugated with fluorescein (Cappel Laboratories) and observed with a Leitz Ortholux phase-epifluorescence microscope.

RESULTS

Assay for Promotion of Neurite Outgrowth. We wished to develop an assay system that would allow us to assess the ability of individual membrane-derived molecules to support attachment of neural cells. Since nitrocellulose is a convenient substrate for rapid noncovalent attachment of proteins, we coated sterile Petri plates with nitrocellulose dissolved in methanol. Six substances were compared for their ability to promote neurite outgrowth: laminin, poly(L-ornithine), N-CAM, 8D9 antigen, mAb 8D9, and mAb 224-1A6. The 8D9 antigen has been shown to be immunologically related to mouse L1 (6). To further characterize the 8D9 antigen and more rigorously compare it to chicken G4 (5), two-dimensional gels using isoelectric focusing and $NaDodSO_4$/PAGE were run. The resulting pattern shown in Fig. 1 is very similar to that of G4 (see ref. 5, figure 2A), a chicken molecule of which the N-terminal sequence has a 50% homology to that of mouse L1 (5). To test the ability of nitrocellulose-coated plates to attach substances that were known to act as cell attachment substrates, 1–5 μl of poly(L-ornithine) (0.1 mg/ml) and laminin (1 mg/ml) was spotted on the coated plates with micropipettes and the plates' remaining binding capacity was blocked by washing with tissue culture medium containing 10% horse serum. Both substances were effective in attaching cells, but the morphology of the cells differed greatly. Laminin was very effective in supporting neurite outgrowth (see Figs. 2*B*, 3*B*, and 5 *C* and *D*), in accordance with the findings of others (15); neurites on laminin were often fasciculated, although this was not the exclusive mode of outgrowth. Additionally, laminin promoted rapid spreading of astrocytes, oligodendrocytes, and fibroblast-like cells. In contrast, poly(L-ornithine) was relatively poor in supporting neurite outgrowth (see Figs. 3*A* and 5 *A* and *B*). In preliminary experiments with poly(L-ornithine), a concentration of 0.1 mg/ml was found to give optimum neurite outgrowth and was used for all subsequent experiments. Nitrocellulose coated only with serum showed relatively few fibroblast-like cells without attached neurons (Fig. 1*D*).

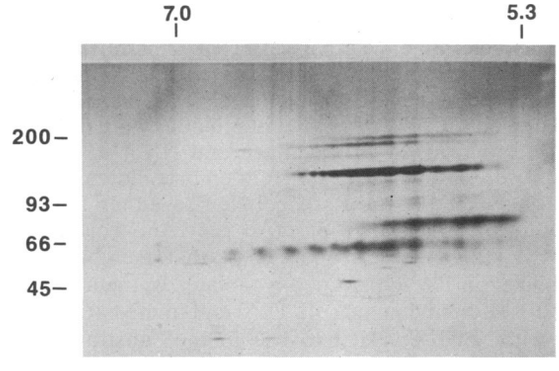

FIG. 1. Two-dimensional gel analysis of the 8D9 antigen. Affinity-purified 8D9 antigen was first prepared by isoelectric focusing in the pH 5–8 range and then run on a 5–15% gradient gel by $NaDodSO_4$/PAGE. The positions of molecular weight standards are indicated at the left and pH values are shown at the top. The proteins were visualized by silver staining.

Test of Membrane-Derived Molecules. Two solubilized purified cell surface molecules that have been implicated in cell adhesion, the 8D9 antigen and N-CAM, were tested as substrates for cell attachment. The concentration of the 8D9 antigen spotted on the dishes was 0.11 mg/ml and that of N-CAM was 0.14 mg/ml. Since it was possible that the purified antigens contained traces of antibody from the affinity columns, antibodies were also tested as substrates. The results of experiments with dissociated chicken tecta are shown in Fig. 2. Chicken neurons attach rapidly (within 1–2 hr of plating) and extend long unfasciculated neurites on the 8D9 antigen (Fig. 2*A*). The mAb 8D9 also attached neurons and induced some neurite outgrowth (Fig. 2*C*). In contrast, the N-CAM was unable to support cell attachment of chicken neural cells (data not shown). The ability of the 8D9 antigen to support unfasciculated neurite outgrowth was further explored with the use of explant cultures of mouse cerebellum. As shown in Fig. 3, microexplants produced extremely thick neurite fascicles on laminin or poly(L-ornithine) but showed virtually no fasciculation when in contact with the 8D9 antigen-coated substrate. In cases in which neurites exited the explants above the surface of the plate, fasciculation was observed up to the point of contact of the neurites with the 8D9 antigen-coated substrate. mAb 8D9 and mAb 224-1A6 (which reacts with chicken N-CAM) supported attachment of chicken retinal cells but were unable to attach mouse cerebellar cells to the substrate (data not shown). These results suggest that the activity associated with 8D9 antigen is in fact due to interaction of mouse cells with the antigen and not trace amounts of antibody that were released from the affinity column.

Quantification of Neurite Outgrowth on Different Substrates. Dissociated mouse cerebellar cells or purified cere-

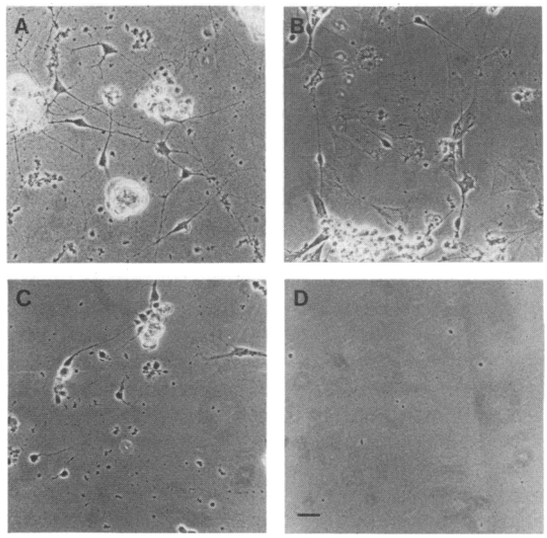

FIG. 2. Chicken tectal cells plated on various substrates. (*A*) Several neurons with long neurites on the 8D9 antigen. There are no flat epithelial cells present. (*B*) Cells plated on laminin. There are numerous epithelial-like cells and a few neurons with long neurites. (*C*) Cells plated on mAb 8D9. There are some neurons with relatively short neurites present. (*D*) Region of the nitrocellulose-coated dish that had no substrate other than serum proteins. Neither neurons nor flat cells find this to be an effective substrate for attachment. Cells were in culture for 2 days. (Bar = 20 μm.)

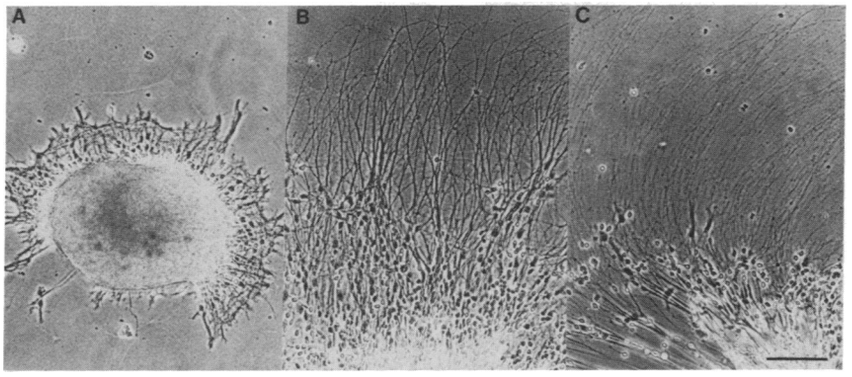

FIG. 3. Comparison of mouse cerebellar explant neurite outgrowth on differing substrates. All explants were prepared from postnatal day 6 mice by mechanical dissociation and maintained *in vitro* for 3 days. Prior to plating explants, 1 μl of various substrates was spotted on a nitrocellulose-coated plate. (*A*) Poly(L-ornithine) resulted in relatively short, highly fasciculated neurites. (*B*) Laminin produced long fasciculated neurites. (*C*) The 8D9 antigen produced long unfasciculated neurites. (Bar = 100 μm.)

bellar granule cells were plated on dishes containing nonoverlapping sectors of poly(L-ornithine), laminin, N-CAM, 8D9 antigen, and an uncoated sector. After ≈18 hr, the cultures were fixed; cell numbers and neurite lengths were then determined for each substrate as described in *Materials and Methods*. Fig. 4 shows the data from one experiment. The 8D9 antigen produced the longest neurites, with some >200 μm in length after 18 hr in culture. Laminin also produced cells with long neurites but the longest was <150 μm. Poly(L-ornithine) and N-CAM were much less effective at producing neurite outgrowth, although they were better than uncoated tissue culture plastic. As summarized in Table 1, the 8D9 antigen produced the longest neurites, although laminin was also an effective substrate for neurite outgrowth. The 8D9 antigen was significantly better than poly(L-ornithine), N-CAM, and tissue culture plastic coated only with nitrocellulose at the 0.01 level or better. Similarly, the 8D9 antigen produced the highest percentage of cells with neurites. Poly(L-ornithine) and laminin were better as cell attachment factors than the 8D9 antigen. N-CAM was a relatively ineffective substrate for either cell attachment or neurite outgrowth, being only slightly better than uncoated plates. Dilution of the 8D9 antigen indicated that below a concentration of 0.01 mg/ml it was ineffective as a substrate for cell attachment or neurite outgrowth.

Cell Class Specificity. To determine if the 8D9 antigen supported attachment of one class of neural cells in preference to others, dissociated mouse cerebellar cells were plated on the 8D9 antigen. After 3 days, the cells were stained with anti-GFAP to assess the relative numbers of astrocytes. For comparison, poly(L-ornithine)- and laminin-coated substrates that had been seeded with cells from the same dissociation were also stained for GFAP. Poly(L-ornithine) and laminin were effective in attaching neurons and glia, although laminin was a greatly preferred substrate for both cell types (Fig. 5 *A–D*). As shown in Fig. 5 *E* and *F*, the 8D9 antigen-coated substrate was almost free of astrocytes. In fact, most astrocytes that were detectable on the 8D9 antigen-coated substrate were observed in association with clumps of neurons, suggesting that their attachment was by means of the neurons and not as a result of interaction with the 8D9 antigen. Parallel studies using chicken cells and antibody 3A7 (14) for the detection of chicken glia gave similar results (data not shown).

DISCUSSION

An important feature of this work is the development of a previously unreported means of attaching purified proteins to cell culture dishes to facilitate identification of cell attach-

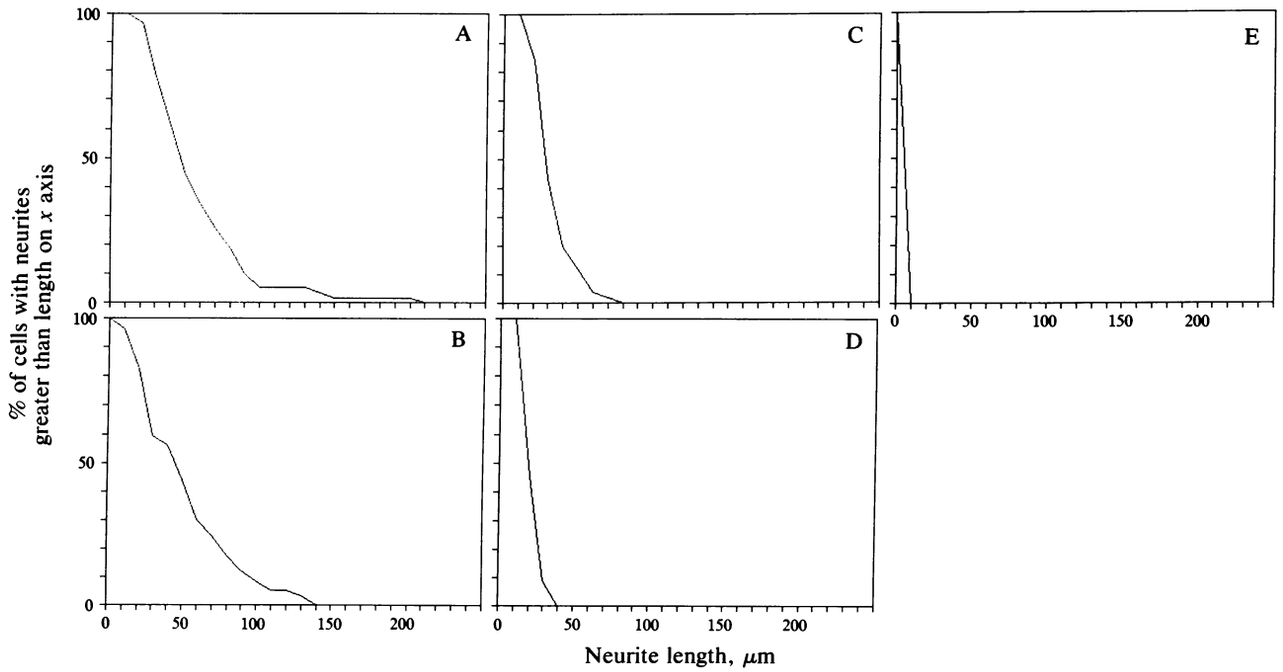

FIG. 4. Distribution of neurite lengths of mouse cerebellar granule cells grown on different substrates: percentage of neurons with neurites (*y* axis) longer than a given length (*x* axis). Distributions are shown for cells on the 8D9 antigen (*A*), laminin (*B*), poly(L-ornithine) (*C*), N-CAM (*D*), and a control region of the dish that received no substrate other than the bovine serum albumin blocking solution (*E*).

Table 1. Neurite growth on different substrates

Substrate	Granule cells				Cerebellar cells			
	Single cells with neurites, *n*	Neurite length,* μm	% single cells with neurites†	Total cells (single and clumped),† *n*	Single cells with neurites, *n*	Neurite length,* μm	% single cells with neurites†	Total cells (single and clumped),† *n*
	Experiment 1, 18 hr in culture				Experiment 1, 18 hr in culture			
8D9	59	56 ± 4	56	64	53	66 ± 6	66	258
Poly(L-ornithine)	51	31 ± 2	19	213	52	35 ± 2	29	551
Laminin	57	49 ± 4	51	312	56	63 ± 7	47	334
N-CAM	11	23 ± 3	31	23	14	44 ± 9	30	28
Uncoated	—	—	0	4	—	—	0	6
	Experiment 2, 17.5 hr in culture				Experiment 2, 16.5 hr in culture			
8D9	58	62 ± 7	40	129	105	70 ± 5	60	110
Poly(L-ornithine)	10	29 ± 4	6	174	21	28 ± 3	2	248
Laminin	35	37 ± 4	33	342	19	36 ± 4	20	161
N-CAM	7	26 ± 4	5	68	12	24 ± 2	13	32
Uncoated	3	20 ± 4	3	53	9	22 ± 2	3	52

*Mean ± SEM.
†In 1.6 mm².

ment factors. The use of methanol-solubilized nitrocellulose has a number of advantages over (*i*) drying proteins on tissue culture plastic, (*ii*) attaching them by means of reactive cross-linkers, or (*iii*) growing cells on sheets of nitrocellulose. These include the fact that the resulting nitrocellulose coating is clear and permits excellent optical observation of cells. Plate preparation is very easy, taking <10 min from dissolving the nitrocellulose until it is dry on the dishes. The nitrocellulose has a high capacity for binding proteins. The proteins bind to the nitrocellulose in <1 min and the drops do not have to be evaporated to ensure adequate attachment. This method allows one to spot many samples on a single plate. Therefore it is possible to assay numerous fractions, such as those from a molecular sieve column, for cell attachment activity. Nitrocellulose does not appear to be toxic for neural cells and, when coated only with bovine serum albumin or serum, is a very poor substrate for neural cell attachment. This gives low levels of background attachment and makes identification of true cell attachment molecules relatively easy. Finally, we were unable to demonstrate cell attachment or neurite outgrowth on uncoated tissue culture plastic that was treated with the 8D9 antigen. Nitrocellulose may not be advantageous for molecules such as laminin, which bind to tissue culture plastic given enough time. However, there exist some molecules, such as the 8D9 antigen, that appear to have a low affinity for plastic that can be overcome by first coating it with nitrocellulose.

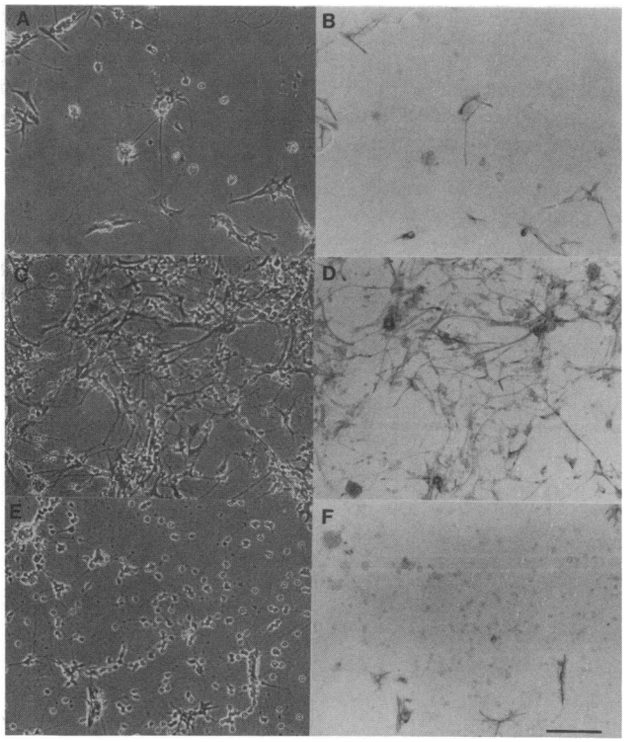

FIG. 5. Cell type specificity of cerebellar cell attachment and growth on different substrates. Cells were maintained *in vitro* for 3 days and then stained for GFAP to detect astrocytes. HRP and 4-chloro-1-naphthol were used to visualize the location of the anti-GFAP. (*A*, *C*, and *E*) Phase photographs to demonstrate the location of neurons and other cells. (*B*, *D*, and *F*) Transmitted light photographs to demonstrate the GFAP-containing cells. (*A* and *B*) Poly(L-ornithine) was an effective substrate for neurons and GFAP-positive cells. (*C* and *D*) Laminin was a very effective substrate for neurons and GFAP-positive cells. (*E* and *F*) The 8D9 antigen was a highly effective substrate for neuronal attachment and resulted in the production of numerous neurites. GFAP-positive cells were present in much lower numbers than on laminin and appeared to be present in aggregates with neurons. (Bar = 100 μm.)

The findings presented here identify an activity associated with the isolated 8D9 antigen. When attached to nitrocellulose-coated plates, the 8D9 antigen supports the attachment of embryonic chicken tectal and postnatal mouse cerebellar neurons and the rapid extension of neurites on this substrate. Quantitative measures of neurite length demonstrated that the 8D9 antigen promoted neurite outgrowth comparable to or better than laminin. The 8D9 antigen was not as effective at promoting cell attachment as laminin or poly(L-ornithine), perhaps indicating that the 8D9 receptor is predominantly localized to axons, as is the 8D9 antigen (6). Neurites from both sources appear to be unfasciculated when they grow on the 8D9 antigen. These results are in agreement with the proposed involvement of the 8D9 antigen-related molecules NILE and Ng-CAM in fasciculation. These results seem in conflict with those of Stallcup and Beasley (2), who have compared the effects of monovalent antibodies directed against NILE and N-CAM. These workers demonstrated that antibodies directed against NILE inhibited fasciculation in cultures of embryonic rat cerebellum but could not do so in cultures prepared from postnatal day 5 rats. They were, however, able to demonstrate inhibition of fasciculation in postnatal day 5 rat cerebellar cultures with antibodies directed against N-CAM. We believe that our results may differ for several reasons. It is possible that the receptor for the 8D9 antigen is maintained at high levels in early and later

development, whereas the 8D9 antigen is not; in this case, our assay, which provides exogenous, purified 8D9 antigen, would reveal unfasciculated outgrowth at both stages. Alternatively, immobilized 8D9 antigen may provide a more effective substrate than mobile N-CAM and the 8D9 antigen on other nearby axons. The 8D9 antigen-coated plates also present axons with a single choice of adhesive substrate without other possible adhesive (or repulsive) cell surface molecules. Although these differences may significantly effect the types of interactions that are possible between neurons and 8D9 antigen, this assay system does provide a straightforward method for analyzing the adhesive interactions of the 8D9 antigen with cells and other molecules.

A remarkable finding of these studies is that chicken and mouse neurons can attach and extend neurites on chicken-derived 8D9 antigen. This suggests that the site on the 8D9 antigen responsible for neurite extension has been highly conserved over evolution. Interestingly, poor immunological crossreactivity of the 8D9-related molecule G4 with mouse L1 antigen and N-terminal sequence homology studies (5) indicate only partial homology between the chicken and mouse molecules. This suggests that the portion of the 8D9 antigen that is involved in cell–cell binding has been selectively conserved. Though neurons are able to rapidly attach to the 8D9 antigen and send out neurites, no preferential attachment of glia was seen with either test culture system, although both contained many nonneuronal cells that could be cultured on laminin or poly(L-ornithine) bound to nitrocellulose. Although our findings clearly support the role of the 8D9 antigen in neuron–neuron and not neuron–glia adhesion, in agreement with others (5, 10, 11), it is possible that glial adhesion is weaker or that in the course of isolation of the 8D9 antigen, a glial binding site has been destroyed or obscured in binding to nitrocellulose.

To date two classes of cell surface molecules have been implicated in axon fasciculation: adhesion molecules related to the 8D9 antigen and those related to N-CAM. Fab fragments of antibodies directed against these molecules were demonstrated to interfere with fasciculated axon outgrowth from explants of neural tissue (2, 4). Our findings indicate that the role of the 8D9 antigen on a given axon is to promote the extension of other neurites along this axon, which, in turn, produces fasciculated axons. We were unable to demonstrate a similar activity with immunopurified N-CAM antigen. This may simply indicate that N-CAM is difficult to purify in an active form or that it was unable to bind to nitrocellulose-coated plates in an active orientation. Alternatively, these findings could indicate that N-CAM does not play a major role in fasciculated axon outgrowth at the stages we examined. Our results concerning the effectiveness of 8D9 and the ineffectiveness of N-CAM as substrates for neurite outgrowth are also in agreement with the results of Rathjen and associates (5, 11).

Studies of the 8D9-related adhesion molecule Ng-CAM indicate that binding is heterophilic (4), although the receptor for it has not been isolated. Data presented here indicate that the 8D9 receptor is present on both neurites and growth cones, because both structures attach readily to 8D9 antigen-coated dishes. Since the isolated molecule retains binding activity it should be possible to use the 8D9 antigen as an affinity ligand for identification of the 8D9 receptor.

The possibility that the 8D9 antigen functions in axon fasciculation and guidance suggests that its presence or absence may play a crucial role in the regeneration of damaged axonal pathways. The developmental expression of L1, NILE, and Ng-CAM has been investigated in rat, mouse, and chicken (16–19). Although there are some species-specific differences in the expression of these molecules, all are found in association with fascicles of axons during development; in mature peripheral nerves and the mature central nervous system, all are observed only on unmyelinated axons. It is possible that the absence of this molecule from mature myelinated central nervous system axon pathways could prevent the regeneration of axons to their targets. Strikingly, Niek and Schachner (16) found that L1 becomes strongly expressed on Schwann cells found in the distal stumps of transected sciatic nerves. Since peripheral nerves can support regeneration, it could be speculated that the reexpression of L1 represents the reestablishment of a permissive pathway for axon regeneration.

Based on the studies presented here, the 8D9 antigen appears to be a good choice for a substrate in regeneration experiments. Is it possible that purified 8D9 antigen could promote axon regeneration in peripheral nerves where suitable nerve grafts are unavailable? Could the purified 8D9 antigen be used as a substrate for regeneration of central nervous system axon pathways? *In vivo* experimentation is necessary to test these hypotheses.

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