Neuron-Glia Cell Adhesion Molecule Interacts with Neurons and Astroglia via Different Binding Mechanisms

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Abstract. The neuron–glia cell adhesion molecule (Ng-CAM) is present in the central nervous system on postmitotic neurons and in the periphery on neurons and Schwann cells. It has been implicated in binding between neurons and between neurons and glia. To understand the molecular mechanisms of Ng-CAM binding, we analyzed the aggregation of chick Ng-CAM either immobilized on 0.5-μm beads (Covaspheres) or reconstituted into liposomes. The results were correlated with the binding of these particles to different types of cells as well as with cell–cell binding itself. Both Ng-CAM-Covaspheres and Ng-CAM liposomes individually self-aggregated, and antibodies against Ng-CAM strongly inhibited their aggregation; the rate of aggregation increased approximately with the square of the concentration of the beads or the liposomes. Much higher rates of aggregation were observed when the ratio of Ng-CAM to lipid in the liposome was increased. Radioiodinated Ng-CAM on Covaspheres and in liposomes bound both to neurons and to glial cells and in each case antibodies against Ng-CAM inhibited 50–90% of the binding. Control preparations of fibroblasts and meningeal cells did not exhibit significant binding.

Adhesion between neurons and glia within and across species (chick and mouse) was explored in cellular assays after defining markers for each cell type, and optimal conditions of shear, temperature, and cell density. As previously noted using chick cells (Grumet, M., S. Hoffman, C.-M. Chuong, and G. M. Edelman. 1984 *Proc. Natl. Acad. Sci. USA.* 81:7989–7993), anti-Ng-CAM antibodies inhibited neuron–neuron and neuron–glia binding. In cross-species adhesion assays, binding of chick neurons to mouse astroglia and binding of mouse neurons to chick astroglia were both inhibited by anti-Ng-CAM antibodies. To identify whether the cellular ligands for Ng-CAM differed for neuron–neuron and neuron–glia binding, cells were preincubated with specific antibodies, the antibodies were removed by washing, and Ng-CAM-Covasphere binding was measured. Preincubation of neurons with anti-Ng-CAM antibodies inhibited Ng-CAM-Covasphere binding but similar preincubation of astroglial cells did not inhibit binding. In contrast, preincubation of astroglia with anti-astroglial cell antibodies inhibited binding to these cells but preincubation of neurons with these antibodies had no effect. Together with the data on Covaspheres and liposome aggregation, these findings suggested that Ng-CAM-Covaspheres bound to Ng-CAM on neurons but bound to different molecules on astroglia. The combined results therefore indicate that Ng-CAM binds homophilically to Ng-CAM on neurons and heterophilically to different molecules on astroglial cells. The homophilic mechanism is likely to mediate the fasciculation of neurites but the role of the heterophilic mechanism, whether for cell–cell adhesion or other neuron–glia interactions, remains to be determined. Each of these distinct binding functions appears to have been evolutionarily conserved.

THE use of different immunologically based assays for cell adhesion has led to the identification of several cell adhesion molecules (CAMs)[1] that are differentially expressed on cells in defined sequences during development (14–16). Different CAMs with independent molecular mechanisms of cell–cell adhesion may function simultaneously between pairs of cells. In the nervous system, three different CAMs have been characterized so far: the calcium-independent neural CAM (N-CAM) (34), the neuron–glia CAM (Ng-CAM) (21), and the calcium-dependent N-cadherin (28) or A-CAM (59, 60). N-CAM (15) and N-cadherin (29) are expressed on various nonneural cells at many morphogenetic sites as well as on neural cells and their precursors. In contrast, Ng-CAM is expressed mainly on post-mitotic central neurons and on neurons and Schwann cells in the periphery (11, 12, 22, 56). Ng-CAM is structurally and immunologically very similar to the mouse L1 antigen (22) and it has been shown by biochemical means to be identical to the nerve growth factor inducible large external

1. *Abbreviations used in this paper*: CAM, cell adhesion molecule; Ng-CAM, neuron–glia cell adhesion molecule; SME, Eagle's minimal essential medium with Spinner's salts.

glycoprotein (NILE) in PC12 cells (20). Previous studies have indicated that Ng-CAM is involved in neuron–neuron adhesion (22, 24), in fasciculation of neurites in explant cultures (33), in migration of neurons along Bergmann glia in tissue slices (7, 33), and in neuron–glia adhesion (21–24).

In addition to binding homotypically, neurons also bind heterotypically to developing myotubes and astroglia in vitro (17, 25, 26, 53). The presence of multiple adhesion molecules on different cells each with different specificities and ion dependencies and each having different contributions at different times of development complicates the attempt to relate in vitro functions to in vivo functions of CAMs. A fundamental requirement is to carry out a series of careful analyses using various binding assays and tests for specificity on well characterized cells. N-CAM binding is homophilic, i.e., N-CAM on one cell binds to N-CAM on an apposing cell, and the rate of N-CAM-mediated adhesion depends nonlinearly upon the surface density of the molecule (32). Both N-CAM and Ng-CAM mediate adhesion among neurons and contribute differentially to cell–cell adhesion depending on their local distribution and binding efficacy (22, 32, 33). In the chick, antibodies against Ng-CAM have been found to inhibit both neuron–glia and neuron–neuron adhesion (22). The fact that Ng-CAM is expressed on neurons and not on astroglia from the central nervous system prompted the hypothesis that Ng-CAM may bind neurons to neurons and Schwann cells to neurons by a homophilic mechanism, while mediating binding of neurons to some other molecule on astroglia by a heterophilic mechanism. These observations emphasize the need for further analysis of Ng-CAM-binding mechanisms.

In this paper, we describe extensive studies on the molecular mechanisms of Ng-CAM binding designed to test this hypothesis. We used Ng-CAM-coated beads and Ng-CAM reconstituted into liposomes as probes, and we also analyzed neuron–glia adhesion in vitro as a function of a number of physical variables. Strict criteria were established to identify the chick or mouse brain cells used in the assays as either neurons or glia. We found that Ng-CAM can bind to itself, that it functions as a homophilic ligand in neuron–neuron adhesion, and that it acts as a heterophilic ligand in neuron–glia adhesion within and across species.

Materials and Methods

Cells

Chicken glial cells were isolated from 9–10 d embryos by their ability to bind selectively to collagen-coated tissue culture dishes as described (21, 26). Astroglial cells were isolated from 3–5 d mouse brains by a similar procedure except that collagenase was used in isolating the cells. Briefly, brain tissue was dissected free of meninges, cut in 1-mm pieces, and incubated in calcium- and magnesium-free medium containing 0.25% trypsin/0.05% collagenase/0.1 mg/ml deoxyribonuclease I/1 mM EDTA for 30 min at 37°C. The tissue was washed and dissociated by trituration in DME containing 10% FCS and the cell suspension was incubated on collagen-coated dishes with intermittent agitation (21, 26). Glia adhered to the dishes while neurons, which aggregated in suspension, were removed by washing. Before use in cell adhesion assays, any residual phase-bright stellate cells (putative neurons) that were present on the monolayers were removed by vigorous washing. Using the criteria described in Table II, >95% of the monolayer cells were identified as astroglia. Cells from meninges and fibroblasts from the skin were isolated by incubating the appropriate tissue in 0.25% trypsin/1 mM EDTA for 30 min at 37°C, and then dissociating the tissue by trituration. The cells were grown on tissue culture dishes in the same medium as the glia. For binding assays, neurons were prepared in suspension from 9–10 d chick embryo brains or from 15–17 d embryonic mouse brains by trypsinization (20 μg/ml) at 37°C for 20 min in Eagle's minimal essential medium with Spinner salts containing 0.02 mg/ml deoxyribonuclease I (SME) and 1 mM EDTA as described (9, 26) (>90% of the cells were identified as neurons using the criteria described in Table II). N2A neuroblastoma cells were grown on dishes in DME containing 10% FCS and prepared in suspension from monolayers by treatment with 20 μg/ml trypsin/1 mM EDTA/SME.

Proteins

Ng-CAM (24) and N-CAM (34) were purified from detergent extracts of 14 d chicken embryo brains by immunoaffinity chromatography using monoclonal antibodies against each CAM and 1 mM PMSF was added to retard proteolysis. Human plasma fibronectin was obtained from The New York Blood Center (New York, N. Y.), BSA was from Miles Laboratories (Naperville, IL) and ovalbumin was from Sigma Chemical Co. (St. Louis, MO). Polyclonal rabbit antibodies and monoclonal mouse antibodies against chicken and mouse Ng-CAM (24, 50) and N-CAM (9, 34) and monovalent Fab' fragments were prepared from the IgG fraction as described (6). Polyclonal antibodies against both chicken (Fig. 1 *a*) and mouse (Fig. 1 *c*) Ng-CAM recognized components of M_r 200,000, 135,000, and 80,000, and monoclonal anti-Ng-CAM antibody 3G2 immunoblotted only chick components of M_r 200,000 and 135,000 (22). Anti-N-CAM polyclonal antibodies and monoclonal antibody no. 1 specifically recognized N-CAM (34). Anti-glial cell antibodies were obtained by immunizing rabbits at 2-w intervals with 10^7 chick glia in PBS; rabbits were bled 2 w after the third injection. Immunoblotting experiments indicated that the anti-glial antibodies recognized many components in extracts prepared from glia but did not recognize Ng-CAM. Monoclonal antibody GA3 was prepared from mice that had been immunized with 9 d chick embryo glia (33). Monoclonal antibody R5, which recognized cytoskeletal components in glia, was kindly provided by Dr. U. Drager (13). Antibodies against galactocerebroside were prepared as described (45). Polyclonal antibodies against glial fibrillary acidic protein (GFA) were kindly provided by Dr. D. Dahl (4). Polyclonal antibodies against human plasma fibronectin were obtained from rabbits that had been immunized at 2-w intervals with 0.1 mg of the protein.

Preparation of Covaspheres

To couple proteins covalently to Covaspheres, 100-μl aliquots of Covaspheres (Duke Scientific, Palo Alto, CA) were separately incubated for 2 h at 37°C with 50 μg of Ng-CAM, 50 μg of N-CAM, 100 μg of ovalbumin, or 100 μg of BSA. To remove the uncoupled molecules, the Covaspheres were washed twice by centrifugation in a microfuge for 5 min at 4°C and the pellet was resuspended in PBS/5 mg/ml BSA/10 mM NaN_3 by sonication using a bath sonicator (Branson Instruments, Inc., Mellville, NY) to dissociate aggregates. For radioactive-binding experiments, proteins were labeled by the chloramine T method (42) and iodinated proteins were mixed with an equal amount of unreacted protein and coupled to Covaspheres; after reaction with proteins, the Covaspheres were resuspended in their original volume.

Preparation of Liposomes

To reconstitute liposomes, a mixture of lipids containing phosphatidyl choline (20 mg) and phosphatidyl serine (2 mg) was added to 1–2 mg of Ng-CAM or N-CAM in PBS/0.5% Nonidet P-40/1 mM EDTA, the detergent was removed with 1/3 vol of Bio-Beads SM-2, and the liposomes were collected by centrifugation at 100,000 *g* for 30 min; 20–40% of the protein became stably associated with the liposomes. For radioactive binding experiments, 10% of the protein was labeled with ^{125}I by the chloramine T method before incorporation into liposomes.

Covasphere and Liposome Aggregation

Covaspheres and liposomes were dissociated by sonication (5–20 s) and the appearance of superthreshold particles was monitored using a Coulter Counter, (model ZBI) fitted with a 100-μm aperture set at amplification = 1/4, aperture current = 1/4, threshold = 5–100; these settings allowed detection of particles greater than 3.84 μm^3, equivalent to an aggregate of ~60 Covaspheres (31). Samples (20 μl) were removed at intervals, diluted to 20 ml PBS, and counted. The superthreshold particle volumes were plotted vs. time and the maximal slope of this curve was defined as the initial rate of aggregation. Aggregation of inherently fluorescent Covaspheres was

qualtitatively monitored under a fluorescence microscope equipped with filters to discriminate between differently labeled green-fluorescing and red-fluorescing Covaspheres. Pretreatments of Covaspheres and liposomes with antibodies were performed at 4°C for 30 min and the antibodies were removed by sedimenting the particles in a microfuge for 10 min and washing the pellets in PBS/5 mg/ml BSA.

Cell-Cell Adhesion Assays

The binding of neurons in suspension (labeled internally with diacetyl fluorescein) to confluent monolayers of glia was performed in 35-mm dishes in SME as described (25, 26). Unless otherwise indicated, adhesion was performed at 25°C for 30 min using 5×10^6 neurons with shaking at 55 rpm in a New Brunswick environmental incubator shaker (New Brunswick Scientific Co., New Brunswick, NJ). Aggregation of neurons in suspension in the 35-mm dishes (Fig. 6 *c*) was measured using a Coulter Counter after removing the cells in suspension and fixing them with glutaraldehyde (6, 24). Antibodies were preincubated with the neurons for 15 min at 4°C before addition to the monolayers. Duplicate dishes were measured and the average binding was calculated as described (26); small aggregates of neurons that bound to the monolayer were scored as a single binding event. Co-aggregation in suspension of 5×10^6 mouse N2A neuroblastoma cells with 5×10^6 9 d chick neurons (labeled internally with fluorescein diacetate) was performed as described (25, 30) in the presence of 1 mg of Fab' fragments of antibodies.

Cell-binding Assays

Binding of green-fluorescing Covaspheres (3–5 μl) to monolayer cells in 35-mm dishes was performed in 3 ml SME for 1 h with gentle rocking. After removal of unbound Covaspheres by three successive washes in SME, the cultures were photographed under an inverted microscope equipped with fluorescence optics. To obtain cells in suspension from monolayers, the cultures on 100-mm dishes were washed twice with PBS and incubated in 3 ml PBS containing 0.02 mg/ml 2 × recrystallized trypsin (Cooper Biomedical, Malvern, PA) at 37°C. After 10–20 min the trypsin was neutralized by addition of 0.1 ml FCS and the cells were then removed from the dish by gentle trituration, washed in SME, centrifuged through 8 ml of SME containing 3.5% BSA, and resuspended in SME. The concentration of cells in suspension was measured using a Coulter Counter and 2×10^6 cells were used in each determination (except for neurons which were smaller in which case 5×10^6 were used). Cells were incubated with Fab' fragments of different antibodies, and in certain experiments the antibodies were removed by sedimentation of the cells in a clinical centrifuge at 2,000 rpm for 2 min and the cells were resuspended in SME. For measurement of particle interactions, Covaspheres (1–3 μl) or liposomes (50–150 μg of lipid) were mixed with cells in 1 ml SME containing 1 mg/ml BSA and incubated at room temperature for 30 min. The cells were separated from the unbound Covaspheres or liposomes by centrifugation in a clinical centrifuge at 2,000 rpm for 2 min, the pellet was resuspended in 0.2 ml SME and centrifuged again through 1 ml of SME containing 3.5% BSA. The supernatant was discarded and the binding of Covaspheres or liposomes to the cells in the pellet was quantitated by gamma spectroscopy; the average binding in duplicate samples was calculated. To quantitate binding of fluorescent Covaspheres to cells, cells containing three or more fluorescent foci were scored as positive; 200 cells were scored. Binding of native fluorescent neuronal membrane vesicles to cells was measured as described (21).

Immunofluorescence and Analytical Procedures

Unfractionated cultures from postnatal 5 d mouse cerebella containing neurons and glial cells were grown on coverslips, fixed, and stained with antibodies (24). For staining with antibodies against cytoskeletal elements (anti-GFA and R5), the cultures were fixed and then extracted at −20°C with 5% acetic acid/ethanol. To identify cells after neuron-to-glial cell adhesion assays, the cells in the 35-mm dishes were fixed for 10 min with 3.7% formaldehyde, extracted, and stained with the appropriate antibodies. SDS-polyacrylamide (6%) gels were stained with Coomassie Blue (37) or immunoblotted (24, 58). Protein was determined by the method of Lowry (41).

Results

To elucidate potentially different molecular mechanisms of Ng-CAM binding, we used immobilized Ng-CAM to study binding of Ng-CAM to itself, to other molecules, and to different types of cells. The in vitro adhesion between neurons and glia, which were first discriminated from each other using a panel of antibodies as markers, was then analyzed under a variety of experimental conditions including cross-species binding and the results were correlated with those obtained with Covaspheres and liposomes.

Molecular Mechanism of Ng-CAM Binding

Preparations of the highly purified molecules from chick containing the previously identified components (Fig. 1 *a*) were coupled to 0.5-μm fluorescing beads (Covaspheres). Ng-CAM-Covaspheres rapidly self-aggregated (Fig. 2 *a*) and eventually formed very large particles which precipitated from the suspension. The aggregates could be dissociated by sonication and multiple cycles of aggregation-dissociation could be carried out. The ability of Ng-CAM-Covaspheres to aggregate was destroyed by boiling or treating the derivatized beads with 0.25% trypsin for 0.5 h at 37°C, indicating that binding was mediated by the attached protein. Aggregation of the beads was completely blocked when they were preincubated with Fab' fragments of specific antibodies against Ng-CAM (Fig. 2 *b*). Fab' fragments from nonimmune Ig and of antibodies against other molecules including N-CAM did not inhibit aggregation. Fab' fragments of monoclonal antibody 3G2 (anti-Ng-CAM), previously found to inhibit cell-cell adhesion (33), strongly inhibited Ng-CAM-Covasphere aggregation, confirming that Ng-CAM specifically mediated the Covasphere binding.

The specificity of Ng-CAM in the binding was examined further by testing the ability of different populations of Covaspheres coated with other molecules to co-aggregate with Ng-CAM-Covaspheres. Red-fluorescing Covaspheres were derivatized with Ng-CAM, N-CAM, fibronectin, BSA, and ovalbumin and tested for co-aggregation with green-fluorescing Ng-CAM-Covaspheres. Only those Covaspheres derivatized with Ng-CAM bound to Ng-CAM-Covaspheres; the Covaspheres derivatized with the other molecules did not self-aggregate and did not bind to Ng-CAM-Covaspheres. (Although N-CAM aggregated when in a lipid membrane (32) it did not aggregate when immobilized to the surface of Covaspheres). The observation that beads coated with highly purified Ng-CAM self-aggregated specifically raised the possibility that Ng-CAM can bind homophilically, i.e., to itself.

To test this idea further, Ng-CAM-Covaspheres were dis-

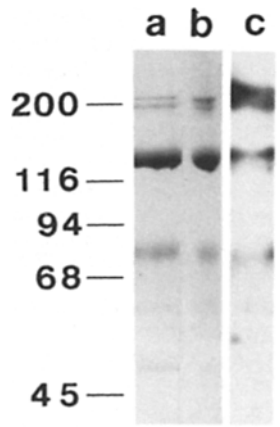

Figure 1. Electrophoretic analysis of Ng-CAM on SDS-polyacrylamide gels. Ng-CAM (10 μg of protein; lane *a*) and Ng-CAM liposomes (containing 5 μg of protein; lane *b*) were resolved on SDS-polyacrylamide gels and stained with Coomassie Blue. Lane *c* is an autoradiograph of an immunoblot with rabbit anti-mouse Ng-CAM (50 μg) of a Nonidet P-40 extract of neonatal mouse brain (100 μg of protein). The numbers on the left represent the molecular mass ($\times 10^{-3}$) of standards that migrated to the indicated positions.

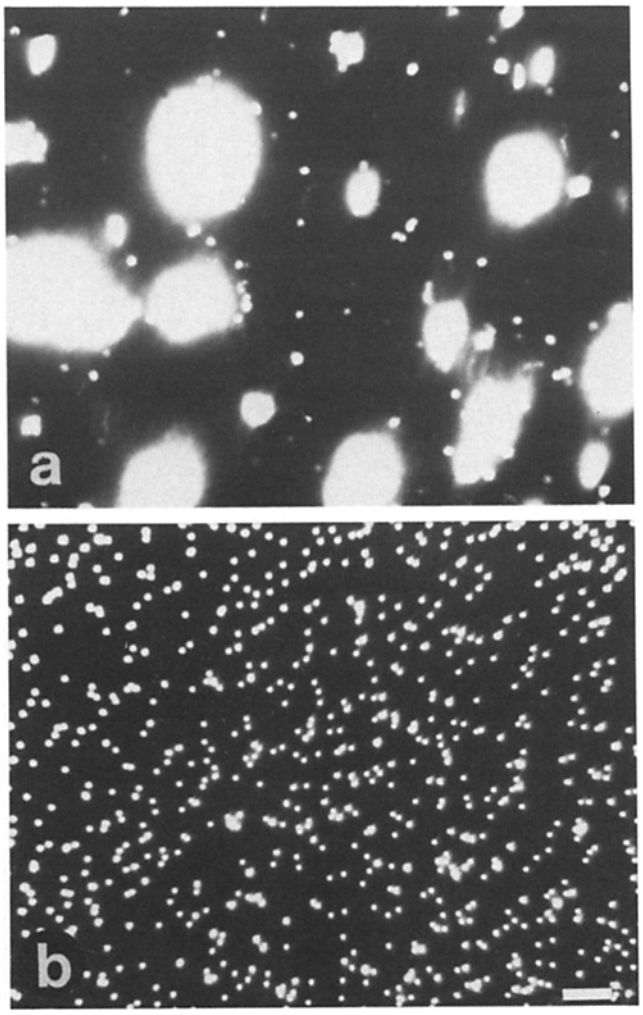

Figure 2. Aggregation of fluorescent Ng-CAM-Covaspheres. Ng-CAM-Covaspheres (green-fluorescing) were incubated in PBS with 1 mg/ml Fab' fragments of nonimmune antibodies (*a*) or anti-Ng-CAM antibodies (*b*) for 2 h at 25°C and photographed under a fluorescence microscope. Bar, 20 μm.

sociated by sonication and the kinetics of aggregation were measured as a function of the Ng-CAM-Covasphere concentration using a Coulter Counter (Fig. 3 *a*). As shown previously for N-CAM reconstituted into liposomes (32), the rate of aggregation in a particle assay is proportional to the square of the particle concentration. We found that this was a suitable model for the aggregation of Ng-CAM Covaspheres and Ng-CAM reconstituted in liposomes (see below). The initial rate of appearance of aggregates was plotted vs. Covasphere concentration on a log–log scale (Fig. 3 *b*); after a short lag phase the rate of aggregation increased approximately with the square of the Covasphere concentration (slope = 2.1). In accord with the conclusion that the binding was homophilic, a mixture of two equivalent aliquots of Ng-CAM-Covaspheres, one that had been preincubated with anti-Ng-CAM and the other with control nonimmune antibodies, aggregated at a rate that was no greater than the rate for a single aliquot of Covaspheres that had been pretreated with the nonimmune antibodies (Table I). In contrast, a double aliquot of Ng-CAM-Covaspheres aggregated 3.5 times faster than a single aliquot. The results clearly suggested that Ng-CAM on one bead binds to Ng-CAM on other beads.

To investigate the binding mechanism of Ng-CAM in a lipid bilayer where the molecule may more closely approximate its orientation in a native membrane, we reconstituted Ng-CAM in artificial membranes containing phosphatidyl choline and phosphatidyl serine and studied their binding properties. Liposomes containing 0.041 mg of Ng-CAM protein/mg lipid rapidly formed superthreshold aggregates (Fig. 3 *c*). Liposomes reconstituted without protein did not aggregate and liposomes containing lower levels of Ng-CAM aggregated at much lower rates. For example, a threefold decrease in the Ng-CAM to lipid ratio resulted in a >30-fold decrease in the initial rate of liposome aggregation (cf. Fig. 3, *c* and *d*) and the rate of aggregation of Ng-CAM liposomes increased approximately with the square of the liposome concentration in a fashion similar to the kinetics of Ng-CAM-Covasphere aggregation. Preincubation of Ng-CAM liposomes with anti-Ng-CAM antibodies blocked their ability to self-aggregate and to co-aggregate with other Ng-CAM liposomes (Fig. 3 *c*). Whereas a double aliquot of Ng-CAM liposomes aggregated at approximately four times the rate of a single aliquot, a mixture of two aliquots of liposomes, one pretreated with anti-Ng-CAM and the other pretreated with nonimmune antibodies, aggregated at a rate no greater than that for the single aliquot of the control liposomes, a result similar to the one found using Ng-CAM-Covaspheres.

As a further control, and to determine whether Ng-CAM in one membrane might bind to different molecules (such as N-CAM) in another membrane, we measured the aggregation of mixtures of different liposomes containing N-CAM and Ng-CAM. We found that the individual rates of aggregation for liposomes containing either Ng-CAM or N-CAM increased exponentially with the concentration of the liposomes, e.g., a twofold increase in the liposome concentration gave approximately a fourfold increase in the rate of aggregation (Fig. 3 *d*). In contrast, when Ng-CAM liposomes were mixed with N-CAM liposomes the rate of aggregation of the mixture was approximately equal to the sum of the individual rates suggesting that there was no co-aggregation between Ng-CAM liposomes and N-CAM liposomes. The combined results strongly support the idea that Ng-CAM in one membrane specifically binds to Ng-CAM and not to N-CAM in another membrane.

Previous studies showed that antibodies against Ng-CAM blocked neuron–neuron adhesion (22, 24) suggesting an adhesive function for the molecule at the intact cell surface. To determine directly whether Ng-CAM on cells could act as a ligand for particles containing only Ng-CAM protein, we tested the binding of fluorescently labeled Ng-CAM Covaspheres to neurons. Neurons bound Ng-CAM-Covaspheres strongly during a 30-min incubation period and the binding was inhibited in the presence of anti-Ng-CAM but not of anti-N-CAM antibodies (Fig. 4). Of particular significance was the finding that anti-Ng-CAM antibodies also inhibited Ng-CAM-Covasphere binding after preincubating either the cells or the Covaspheres (data not shown) with the antibodies and then removing the unbound antibodies by washing. In control experiments, Covaspheres derivatized with BSA or ovalbumin did not bind. Consistent with the observations on liposomes and Covaspheres alone, the

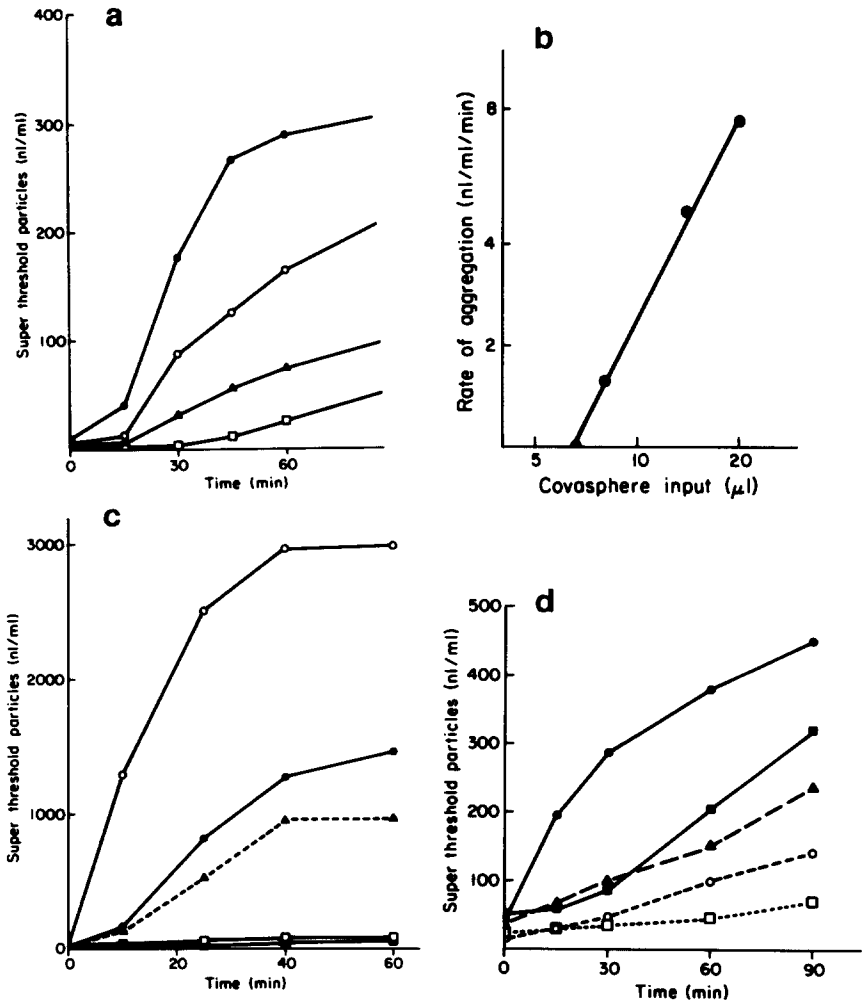

Figure 3. Kinetics of aggregation of Ng-CAM-Covaspheres and liposomes. Covaspheres (*a* and *b*) were derivatized with Ng-CAM, resuspended in their original volume, and aggregation was analyzed as described in Materials and Methods. In *a*, the concentration of superthreshold particles was plotted as a function of time using four different amounts of Covaspheres diluted in a final volume of 150 μl of PBS; (●) 20 μl; (○) 13 μl; (▲) 7.6 μl; (□) 6.7 μl. Using the data from *a*, the maximal rate of aggregation as a function of the Covaspheres input was plotted on a log-log scale (*b*). (*c*) Ng-CAM liposomes (0.041 mg protein/mg lipid) were preincubated with Fab' fragments of nonimmune or anti-Ng-CAM antibodies (50 mg antibody/mg Ng-CAM protein) for 30 min at 4°C and the liposomes were washed to remove unbound antibodies. Then the antibody-treated liposomes were mixed in a final volume of 150 μl using liposomes containing the following amounts of Ng-CAM protein; (○) 32 μg, nonimmune-treated; (●) 16 μg, nonimmune-treated; (□) 32 μg, anti-Ng-CAM-treated; (■) 16 μg, anti-Ng-CAM-treated; (▲) 16 μg, nonimmune-treated + 16 μg, anti-Ng-CAM-treated, and the appearance of superthreshold particles was measured as described in Materials and Methods. (*d*) Aggregation of Ng-CAM liposomes (0.013 mg protein/mg lipid) and N-CAM liposomes (0.030 mg protein/mg lipid) in a final volume of 150 μl; Ng-CAM liposomes (■, 32 μg; □, 16 μg protein); N-CAM liposomes (●, 72 μg; ○, 36 μg protein); ▲, a mixture of Ng-CAM liposomes (16 μg protein) and N-CAM liposomes (36 μg protein).

results strongly suggested that Ng-CAM on neurons is a homophilic ligand.

Because Ng-CAM is not present on astroglia (23, 24), we turned to cellular assays to analyze the molecular mechanisms of Ng-CAM binding for neurons and glia in further detail. To correlate the results with those on binding of appropriately labeled particles to cells, it was necessary to identify the cell types by strict criteria and to develop careful quantitative assessments of the variables affecting cell-cell binding.

Table I. Aggregation of Mixtures of Ng-CAM Covaspheres after Preincubation with Antibodies

Covaspheres (μl)			Initial rate of appearance of superthreshold particles ([nl/ml] mm)
Nonimmune	Anti-Ng-CAM	Buffer	
		μl	
50	0	50	1.75
100	0	0	6.06
0	50	50	0.07
0	100	0	0.11
50	50	0	1.48

45 μl of Ng-CAM-Covaspheres were preincubated with 450 μl of Fab' fragments of nonimmune or anti-Ng-CAM antibodies for 30 min and the antibodies were removed by washing the Covaspheres in PBS containing 5 mg/ml BSA. The Covaspheres were resuspended in 250 μl of the same buffer, sonicated to dissociate aggregates and mixed as indicated. The appearance of superthreshold particles was measured and the rates were calculated as described in Materials and Methods.

Identification of Neuronal and Glial Cells

In previous studies on cell-cell adhesion using chick embryo brain cells, we used a combination of cell morphology and antibody markers to identify the cells. In the present study, these procedures were extended to cells of mouse to identify unequivocally the developing neurons, astroglia, and meningeal cells in culture that would be used in cellular assays (Table II). Both round neurons in suspension before culture and stellate neurons in culture were recognized by antibodies against N-CAM and Ng-CAM (24). The major non-neuronal population of cells isolated from 10 d chick embryo brains have a flat, cobblestone morphology in culture (21, 26) and are specifically recognized at the cell surface by monoclonal antibody GA3 (33), and monoclonal antibody R5 which stained cytoskeletal elements in these cells (23). In comparison with the level of staining observed on neurons, antibod-

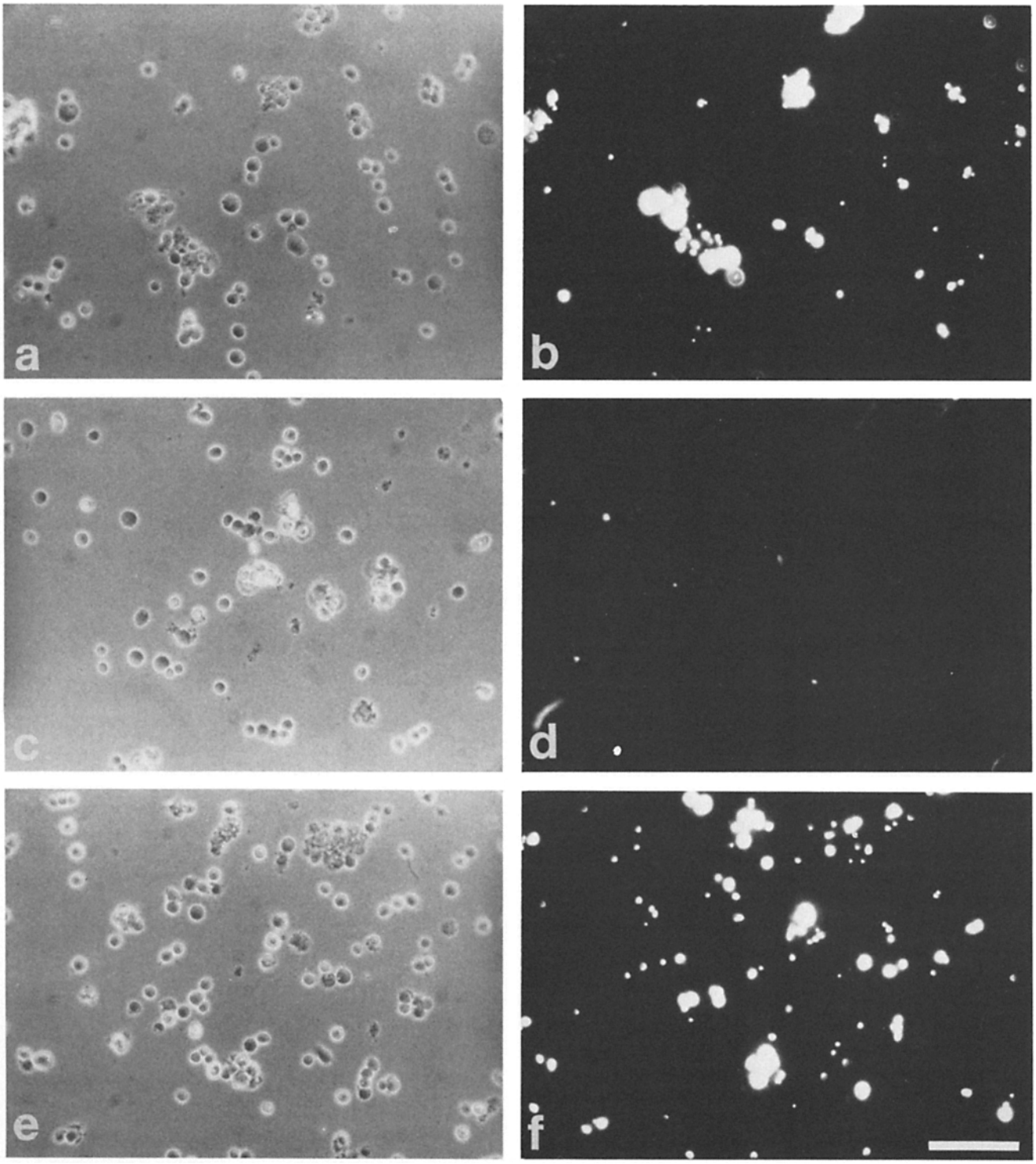

Figure 4. Binding of fluorescent Ng-CAM-Covaspheres to neurons. Neurons in suspension were preincubated with 1 mg of Fab' fragments of antibodies and binding of Ng-CAM-Covaspheres was performed as described in Materials and Methods. Nonimmune antibodies (*a* and *b*), anti-Ng-CAM antibodies (*c* and *d*), and anti-N-CAM (*e* and *f*). Phase-contrast micrographs (*a*, *c*, and *e*) and fluorescence micrographs (*b*, *d*, and *f*) of the same respective fields. Bar, 50 μm.

ies against N-CAM stained glia weakly (23, 24). Cells isolated from the meninges of the brain were morphologically distinct from the glia and were stained by monoclonal antibody R5 but not by the other antibodies.

We identified cells from postnatal mouse cerebella in culture with a similar set of antibodies but also included antibodies against GFA, a recognized marker for mature astrocytes (10, 18). This marker is not detected in immature chick cells (1, 18). Unfractionated cultures containing both neurons and glia were analyzed by immunofluorescence. Mouse neurons were stained brightly by rabbit antibodies against mouse Ng-CAM (Fig. 5 *a*) and N-CAM (Fig. 5 *b*). Monoclo-

Table II. Cell Identification in Culture with Immunological Markers

Type of cell	GA3	R5	Anti-N-CAM	Anti-Ng-CAM	Anti-GFA	Anti-GC
Chicken neurons	−(33)	−(23)	+(24)	+(24)	−	ND
Chicken astroglia	+(33)	+(23)	±(24)	−(24)	−	ND
Chicken meningeal cells	−	+	−	−	−	ND
Mouse neurons	−	−	+	+	−	−
Mouse astroglia	−	+	±	−	+	−
Mouse oligodendrocytes	ND	ND	ND	−	−	+

Summary of immunofluorescent staining of brain cells in culture prepared from chicken embryos and postnatal mice with monoclonal antibodies GA3 (33), R5 (23), anti-N-CAM (9, 23), and anti-Ng-CAM (24), and with polyclonal antibodies against N-CAM (9, 34), Ng-CAM (24, 50), GFA (4), and galactocerebroside (GC; 45). Numbers in parentheses are references. Anti-GFA did not react with chick cells and monoclonal antibody GA3 did not react with mouse cells. See Fig. 5 for staining data on mouse cells.
ND, Not determined.
+, Strong staining.
±, Weak staining.
−, No staining.

nal antibody GA3, which specifically recognized the chick glial cells, did not react with cells from the other species, but the polyclonal antibodies raised against chick glial cells bound strongly to mouse astrocytes and not to neurons (Fig. 5 *c*). Mouse astrocytes were stained brightly with antibodies against GFA (Fig. 5 *d*) but were not stained by anti-Ng-CAM (Fig. 5 *a*) and were only weakly stained by anti-N-CAM antibodies (Fig. 5 *b*). Astrocytes (Fig. 5 *e*) and meningeal cells were recognized by monoclonal antibody R5; double label immunofluorescence experiments showed that individual astrocytes were stained by anti-GFA but meningeal cells were not (data not shown). Oligodendrocytes which were infrequently found in these cultures were identified (45) by staining in a speckled pattern with antibodies against galactocerebroside (Fig. 5 *f*). To strengthen the case that characterized neurons bound to characterized astroglia via Ng-CAM, we used assays both within species and across species. As described below, care was also taken to establish optimal conditions for the cell-binding assays.

Characterization of the Neuron-to-Glial Cell Adhesion Assay

In previous studies (22, 24), the use of an assay for binding of neurons in suspension to monolayers of glial cells provided evidence for a cellular interaction involving Ng-CAM. To optimize this assay, we analyzed the effects of several key parameters on the adhesion of neurons to glial cells including shear, temperature, and cell concentration.

The level of neuronal adhesion to astroglia was increased only slightly as the shear forces were reduced by decreasing the speed of rotation (Fig. 6 *a*) indicating that the shear forces that were tested were not strong enough to reduce the level of adhesion significantly. Although adhesion was not detected at 4°C, the level of binding increased with temperature (Fig. 6 *b*). It is unlikely that the lack of adhesion at 4°C was due to an inhibition of metabolic processes because adhesion was not inhibited in the presence of 10 mM NaN_3; instead it seems likely that some aspect of cell shape was involved.

To evaluate the effect of neuron–neuron adhesion in the suspensions overlying the glial monolayers on the binding of neurons during the assay, we measured both types of adhesion in individual dishes (Fig. 6, *c* and *d*). The level of neuronal binding to the monolayer was directly dependent on the number of input neurons, but the percent of the input that bound decreased at higher input levels (Fig. 6 *d*). This decrease may be attributed partially to saturation of neuronal-binding sites on the astroglial monolayer with increasing levels of neuronal input. In addition aggregation of neurons in suspension (Fig. 6 *c*) reduced the apparent level of binding to the monolayer inasmuch as cell aggregates were not differentiated from single cells in the scoring analysis (see Materials and Methods). Given that the extent of neuronal aggregation increased with the neuronal input (Fig. 6 *c*), the number of neurons bound to glial monolayers was probably underestimated at higher input levels. To minimize this effect and to maintain a reasonable level of binding for antibody perturbation experiments, we performed the subsequent binding experiments using 5×10^6 cells at 25°C, and at a shaking speed of 55 rpm.

Ng-CAM-Mediated Binding within and Across Species

To determine whether Ng-CAM plays a role in neuron–glia adhesion in mammals, we measured the binding of fluorescently labeled mouse neurons in suspension to highly enriched monolayers of mouse astroglial cells and tested various antibodies against CAMs for their ability to inhibit the binding. Adhesion of neurons prepared from embryonic mouse brains to monolayers of postnatal mouse brain astrocytes was inhibited more than 50% with anti-mouse Ng-CAM antibodies (Table III). In contrast, anti-N-CAM antibodies produced little or no inhibition.

In cross-species adhesion assays, binding of chick neurons to mouse astroglia and binding of mouse neurons to chick astroglia were both inhibited by antibodies against chick or mouse Ng-CAM (Table III). Whereas antibodies against N-CAM did not inhibit adhesion, in several experiments the antibody slightly increased the level of neuron binding to the astroglial monolayer. This enhancement of binding was previously observed (22) and may be attributed to the fact that, in the absence of antibody, the neurons in suspension self-aggregated, thereby reducing the number of single neurons available for binding to the monolayer (Fig. 6, *c* and *d*). In the presence of anti-N-CAM antibodies, however, neuronal aggregation was inhibited allowing a greater number of single neurons to bind to the monolayer.

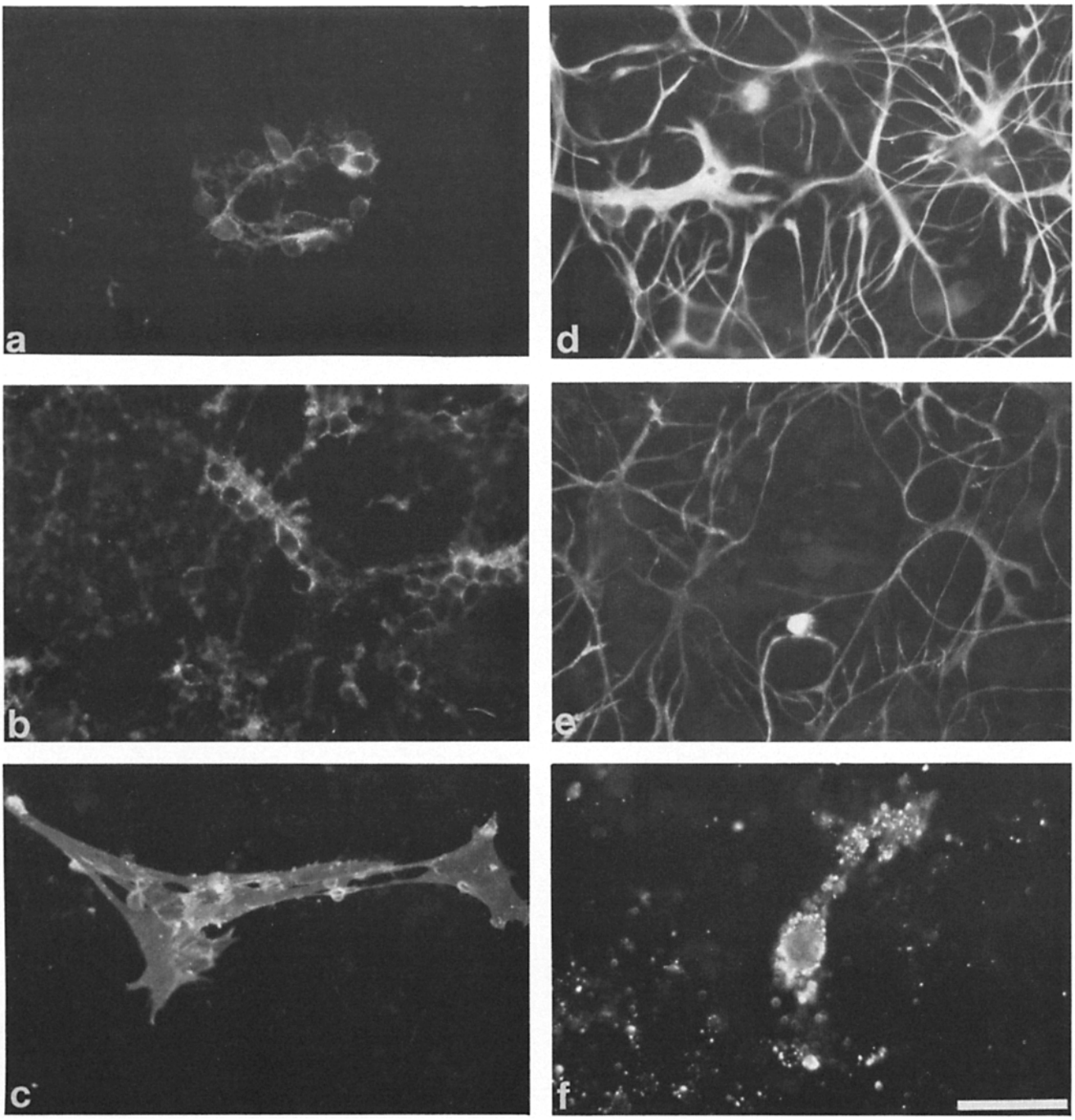

Figure 5. Immunofluorescence staining of mouse cerebellar cultures containing both neurons and astroglial cells. Unfractionated cultures were stained with rabbit antibodies against *a*, mouse Ng-CAM (0.1 mg/ml Ig); *b*, mouse N-CAM (0.1 mg/ml Ig); *c*, chick glial cells (0.1 mg/ml Ig); *d*, GFA (1:100 dilution of serum); *e*, monoclonal antibody R5 (0.05 mg/ml Ig); *f*, galactocerebroside (0.1 mg/ml Ig). Bar, 50 μm.

To verify the identity of the cells participating in the adhesion, the cultures were fixed at the conclusion of the adhesion assay and antibodies were used as markers to identify the cells (see Fig. 7, for example). After binding of chick neurons to mouse astroglia, the glia in the monolayers were stained brightly by anti-GFA and R5 (Fig. 7, *a* and *b*) antibodies. In contrast, the phase bright neurons that bound to the monolayers were not recognized by the astroglial markers and were stained brightly by anti-N-CAM (Fig. 7, *c* and *d*) and anti-Ng-CAM antibodies. The data indicate that the cells participating in the binding are neurons and astroglia.

To determine whether cell adhesion also occurs between chick and mouse neurons, fluorescein-labeled chick brain neurons and unlabeled mouse neuroblastoma cells were coincubated in the presence of nonimmune, anti-Ng-CAM, or anti-N-CAM Fab′ fragments. Microscopic observation after shaking at 90 rpm for 30 min using phase contrast and fluorescence optics clearly showed that composite aggre-

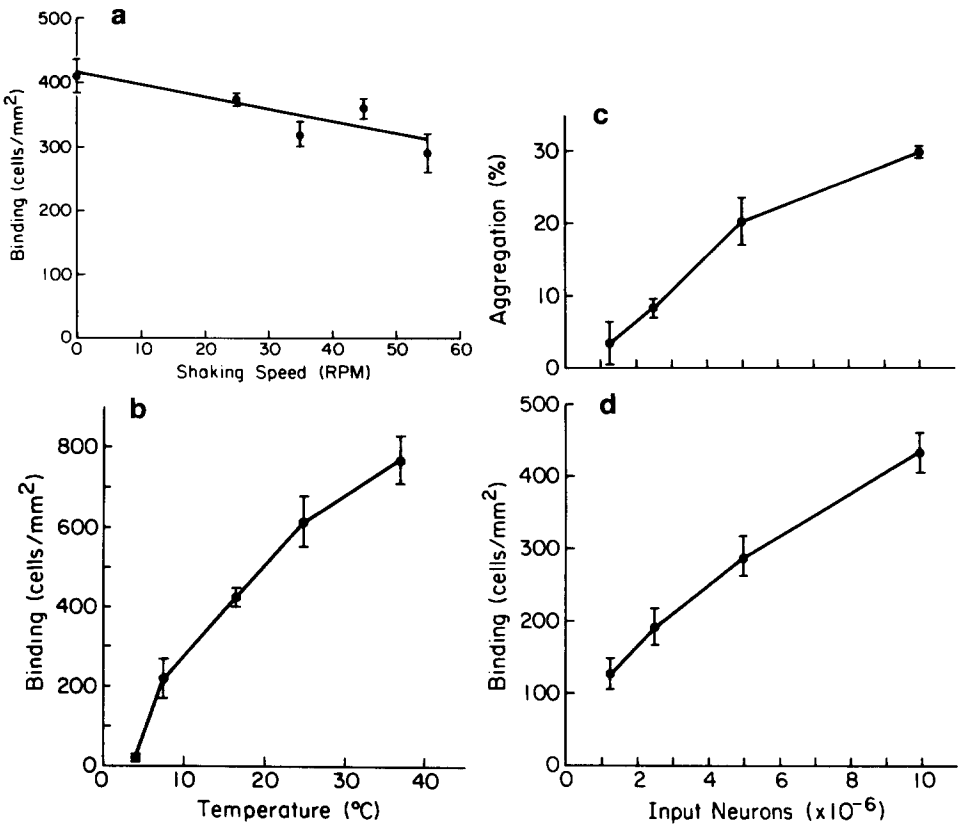

Figure 6. Characterization of cell–cell adhesion assays. Adhesion of fluorescent chick neurons in suspension to monolayers of secondary cultures of 9 d chick brain astroglial cells and aggregation of the cells in suspension were measured as described in Materials and Methods. (*a*) Binding of 5 × 10^6 neurons at 25°C was performed at different speeds of rotation. (*b*) Binding of 10^7 neurons was performed at 55 rpm at different temperatures. The effect of neuronal concentration on binding to astroglia and aggregation of the neurons in suspension was measured at 55 rpm and 25°C (*c* and *d*). Neuronal aggregation (*c*) as a function of input cell number was measured using a Coulter Counter and the percent aggregation was calculated as described (see reference 6); (*d*) neuronal binding as a function of input to the astroglial monolayers was also measured in the same cultures.

gates containing both mouse and chick cells were formed in the presence of non-immune Fab'. In the presence of anti–Ng-CAM or anti–N-CAM Fab', however, aggregation was inhibited (data not shown). The results provide qualitative evidence that mouse and chick neurons can co-aggregate by an Ng-CAM-dependent mechanism in addition to an N-CAM-dependent mechanism (30).

Comparison of Modes of Ng-CAM Binding with Neurons and Glial Cells

To analyze the ligands for Ng-CAM on neurons and glia, we tested the binding of the immobilized forms of Ng-CAM to each of these different cell types. Ng-CAM-coated Covaspheres bound strongly both to neurons (Fig. 4) and to astroglial cells (Fig. 8) but not to meningeal cells (see Fig. 9). After short periods of incubation, the unbound Covaspheres were removed by washing and Ng-CAM-Covaspheres were seen to be bound to monolayers of 9 d chick embryo glia by fluorescence microscopy (Fig. 8). The Covaspheres bound in large numbers to glial cells and not to the collagen-coated substrate. Whereas binding was observed after incubation in the presence of Fab' fragments of nonimmune antibodies, it was inhibited by monoclonal and polyclonal antibodies against Ng-CAM and also by the polyclonal anti-

Table III. Neuron–Glia Adhesion Within and Across Species

Heterotypic binding between				
Glial cell in monolayer	Neuronal cell in suspension	Fab' present during binding	Number of cells bound to monolayer	Inhibition by Fab'
				%
Mouse astroglia	Mouse brain	Nonimmune	369 ± 46	0
Mouse astroglia	Mouse brain	Anti-mouse N-CAM	346 ± 14	6
Mouse astroglia	Mouse brain	Anti-mouse Ng-CAM	154 ± 31	54
Mouse astroglia	Chick brain	Nonimmune	363 ± 10	0
Mouse astroglia	Chick brain	Anti-chick N-CAM	378 ± 26	0
Mouse astroglia	Chick brain	Anti-chick Ng-CAM	213 ± 18	41
Mouse astroglia	Chick brain	Anti-mouse N-CAM	399 ± 5	0
Mouse astroglia	Chick brain	Anti-mouse Ng-CAM	150 ± 59	59
Chick astroglia	Mouse brain	Nonimmune	199 ± 4	0
Chick astroglia	Mouse brain	Anti-mouse N-CAM	258 ± 23	0
Chick astroglia	Mouse brain	Anti-mouse Ng-CAM	87 ± 15	56

Binding to 1 mm^2 of the monolayer was measured in duplicate samples as described in Materials and Methods using 1 mg of Fab' fragments of antibodies.

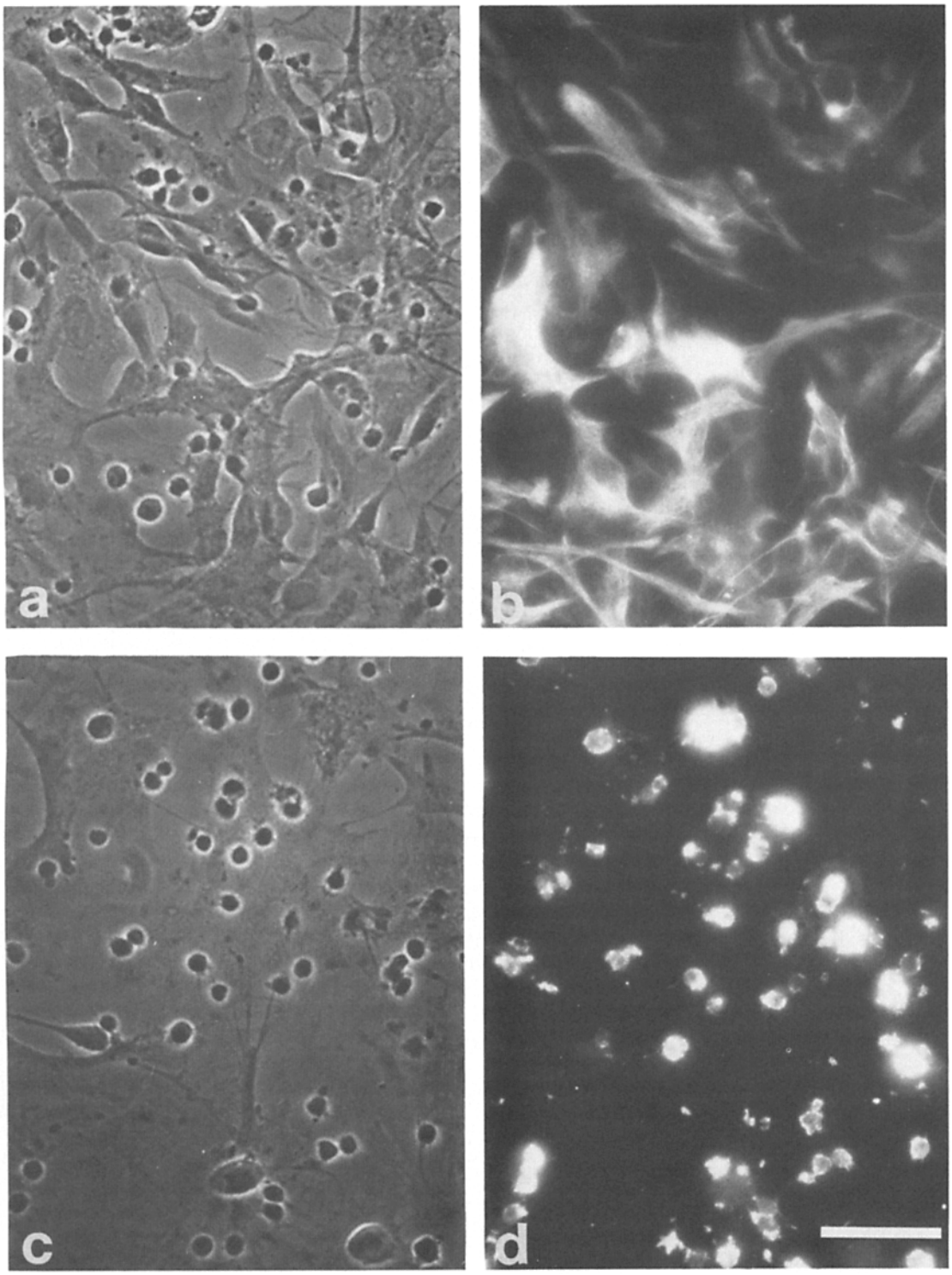

Figure 7. Identification of neurons and astroglia by immunofluorescence after cell–cell adhesion in vitro. Adhesion of chick neurons to monolayers of mouse astroglia was performed in the presence of 1 mg/ml Fab' fragments of nonimmune antibodies, the cultures were then fixed and stained with antibodies as described in Materials and Methods. Phase contrast (*a* and *c*) and fluorescence (*b* and *d*) micrographs of the same fields stained with monoclonal antibody R5 (*a* and *b*) and anti-chick N-CAM Ig (*c* and *d*). Note that the flat astroglia and not the round neurons were stained by R5 and the opposite pattern of staining was obtained with anti-N-CAM antibodies. Bar, 50 μm.

bodies against chick astroglial cells. Inhibition was strongest with anti-Ng-CAM antibodies and was somewhat less with the anti-astroglial antibodies. Treatment of the cells with anti-astroglial cell antibodies did not cause any significant change in cell shape, suggesting that these antibodies inhibited Covasphere binding by blocking specific sites on the surface of the cells without globally modulating the cells.

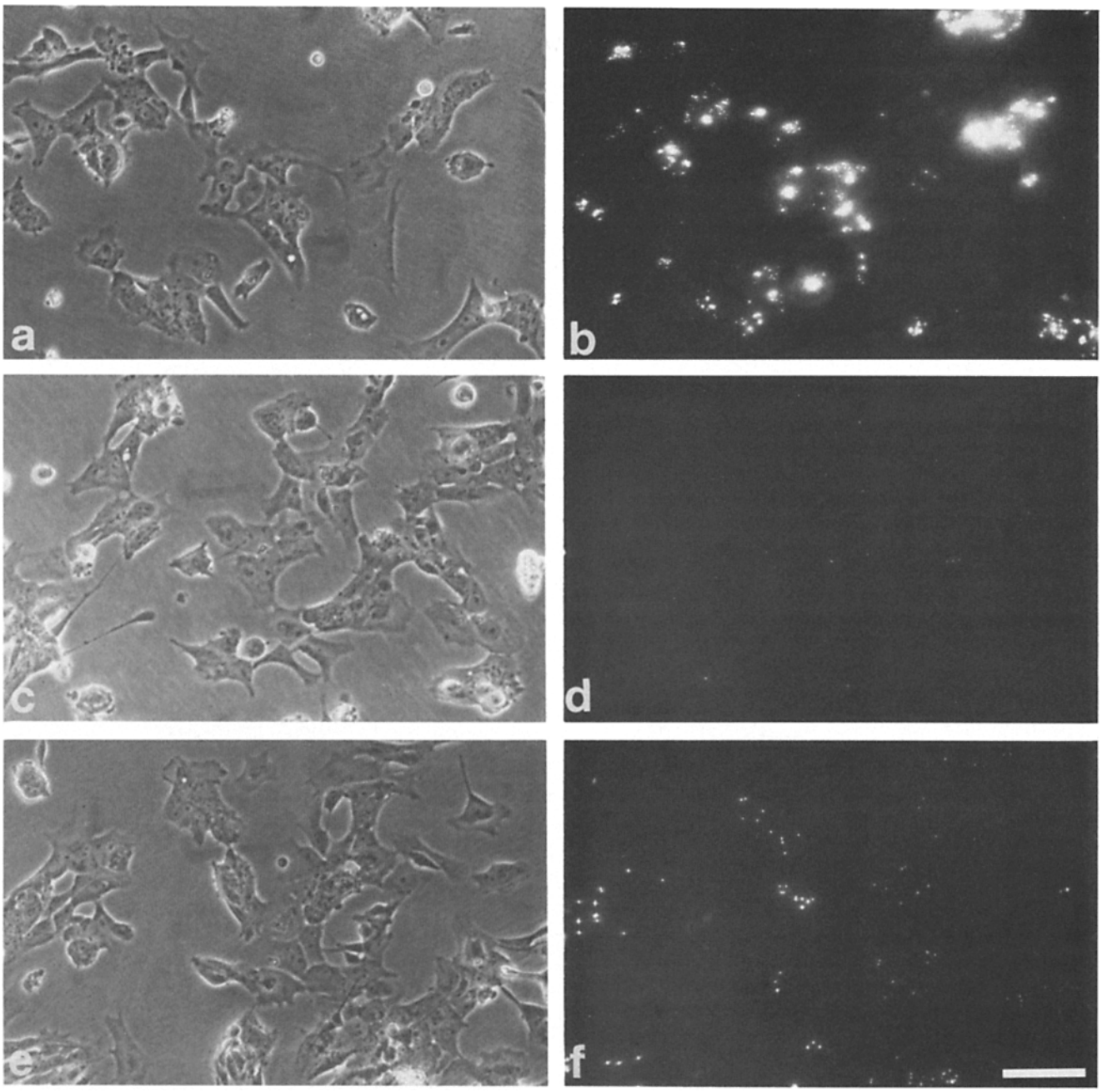

Figure 8. Binding of Ng-CAM-Covaspheres to astroglial cells in monolayers. Ng-CAM-Covaspheres and astroglial monolayers were each preincubated for 15 min with 0.5 mg of Fab' fragments of nonimmune antibodies (*a* and *b*), anti-Ng-CAM antibodies (*c* and *d*), and anti-astroglial cell antibodies (*e* and *f*) and then Covasphere binding was analyzed by phase contrast (*a*, *c*, and *e*) and fluorescence (*b*, *d*, and *f*) microscopy. Bar, 50 μm.

Because different types of cells have different morphologies in culture, it was difficult to compare Covasphere binding with cells on dishes. To circumvent this difficulty, cells were prepared in suspension and uniform assay conditions were developed to measure Covasphere binding. Binding of Ng-CAM-Covaspheres to glia obtained in suspension after treatment with 0.02 mg/ml trypsin was inhibited in the presence of anti-Ng-CAM antibodies. Meningeal cells treated in a similar manner had few or no cells that bound Ng-CAM-Covaspheres (Fig. 9). The results confirmed that glia both on a monolayer and in suspension bound Ng-CAM.

Quantitative measurement of the binding to cells in suspension was achieved using ^{125}I-labeled Ng-CAM covalently linked to Covaspheres. The Covaspheres aggregated rapidly indicating that the binding activity of the Ng-CAM was not inhibited by the iodination reaction. The radioactive-binding assay allowed rapid measurement of binding in many samples simultaneously and removed the subjectivity associated with the fluorescence assay. A comparison of binding of [^{125}I]Ng-CAM-Covaspheres with a variety of cells both by fluorescence and radioactive scoring methods gave similar results (Table IV). The level of binding to neurons and glia

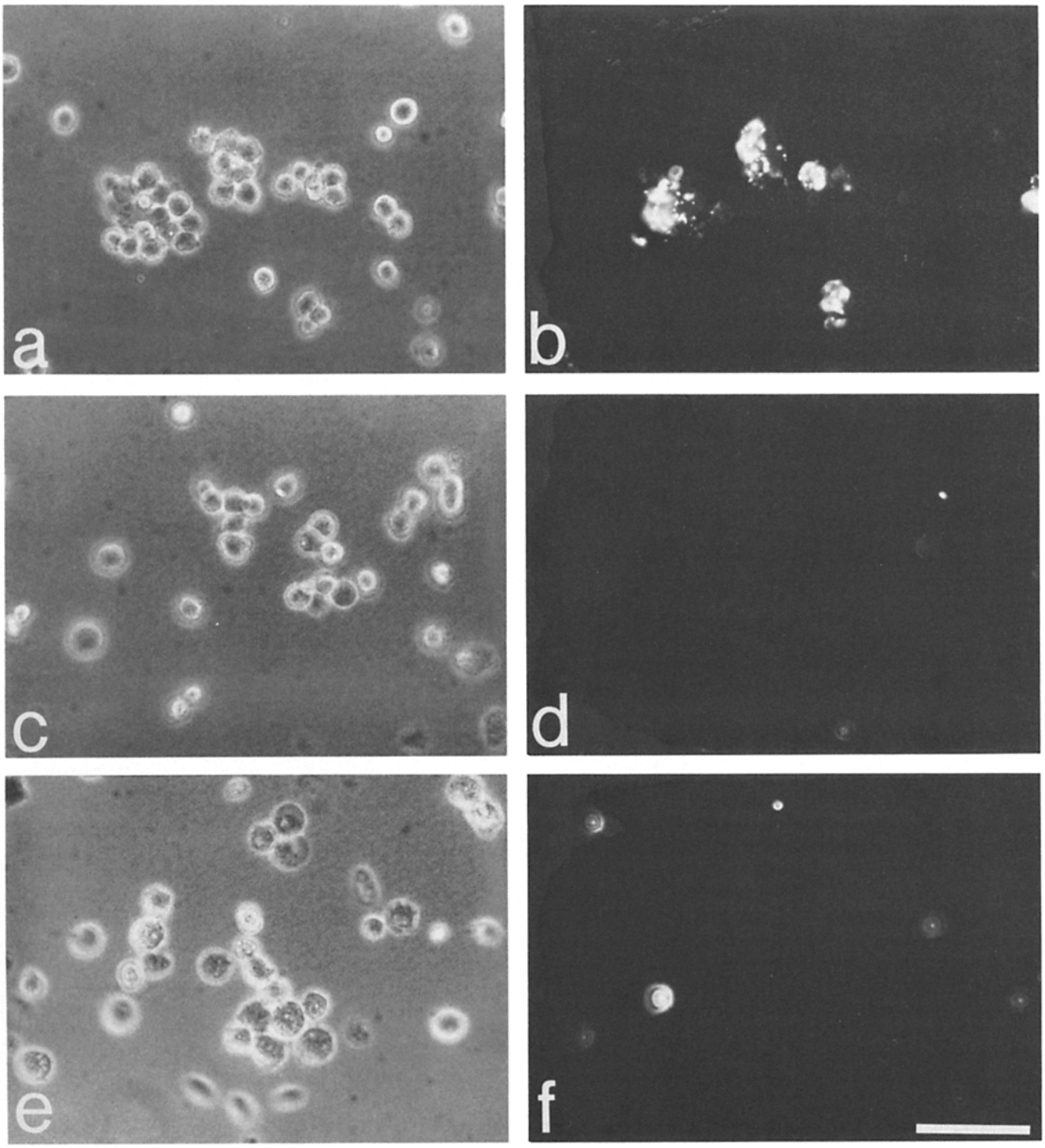

Figure 9. Binding of fluorescent Ng-CAM-Covaspheres to astroglial and meningeal cells in suspension. Cells in suspension were preincubated with 1 mg of Fab′ fragments of antibodies and binding of Ng-CAM-Covaspheres was performed as described in Materials and Methods. Binding was observed to astroglia in the presence of nonimmune antibodies (*a* and *b*) but not in the presence of anti–Ng-CAM antibodies (*c* and *d*), and not to meningeal cells in the presence of nonimmune antibodies (*e* and *f*). Phase-contrast micrographs (*a*, *c*, and *e*) and fluorescence micrographs (*b*, *d*, and *f*) of the same fields. Bar, 50 μm.

was at least 5–10-fold higher than the level of binding to other cells. Preincubation of the Covaspheres with Fab′ fragments of antibodies against Ng-CAM inhibited binding to neurons and glial cells by 50–90% (data not shown). Whereas anti–Ng-CAM antibodies inhibited the binding to neurons and antibodies against glia inhibited the binding to glia, neither of these antibodies inhibited the low levels of binding to fibroblasts and meningeal cells. As a control for nonspecific binding, we showed that the level of binding to cells of Covaspheres derivatized with ovalbumin and BSA was less than 0.6% of the input.

To determine whether different cell surface molecules

Table IV. Comparison of Binding of [^{125}I]Ng-CAM-Covaspheres to Cells by Two Different Methods and Effects of Preincubation with Antibodies

Cell type	Antibody preincubated with cells	Antibody removed by washing	Binding			
			Radioactivity		Fluorescence	
			Bound, cpm	Inhibition	Cells binding	Inhibition
			%	%	%	%
Glia	Nonimmune	yes	2.64 ± 0.08	0	50	0
Glia	Nonimmune	no	2.55 ± 0.15	4	–	
Glia	Anti-Ng-CAM	yes	2.18 ± 0.10	17	43	14
Glia	Anti-Ng-CAM	no	0.79 ± 0.30	70	–	
Glia	Anti-glia	yes	1.44 ± 0.05	45	21	58
Glia	Anti-glia	no	0.92 ± 0.13	65	–	
Neurons	Nonimmune	yes	3.45 ± 0.41	0	35	0
Neurons	Nonimmune	no	3.60 ± 0.40	0	–	
Neurons	Anti-Ng-CAM	yes	1.47 ± 0.14	57	17	51
Neurons	Anti-Ng-CAM	no	0.58 ± 0.12	83	–	
Neurons	Anti-glia	yes	3.27 ± 0.45	5	35	0
Neurons	Anti-glia	no	3.61 ± 0.10	0	–	
Fibroblasts	Nonimmune	yes	0.53 ± 0.10		0	
Meningeal cells	Nonimmune	yes	0.57 ± 0.04		2	

Cells were preincubated with 1 mg of Fab' fragments of antibodies for 30 min. To remove the antibodies, the cells were sedimented by centrifugation and after removal of the supernatant the cells were resuspended in medium. Then, Covaspheres (3 μl containing 100,000 cpm) were incubated with the cells for 30 min and the binding in individual samples was measured first by gamma spectroscopy and then under a fluorescence microscope as described in Materials and Methods. –, Not done.

were involved in the binding in each case, Ng-CAM-Covasphere binding was measured to cells either in the presence of antibodies or after pretreatment of the cells with antibodies and removal of the antibodies by washing (Table IV). When antibodies were present during the entire assay, anti-Ng-CAM antibodies strongly inhibited binding to both neurons and glial cells but anti-glial antibodies only inhibited binding to the glia (Table IV). Whereas the binding to neurons was inhibited by pretreatment of the cells with anti-Ng-CAM antibodies, anti-glial antibodies had little or no effect (Table IV). In contrast, the binding to glial cells was inhibited by pretreatment of the cells with anti-glial antibodies and only weakly inhibited by pretreatment with anti-Ng-CAM antibodies. These results indicate that (*a*) exposure of Ng-CAM-Covaspheres to antibodies against Ng-CAM inhibits their binding. (*b*) Ng-CAM on the surface of neurons is involved in binding of Ng-CAM on Covaspheres, and (*c*) glial cells, which do not contain Ng-CAM, bind Ng-CAM apparently via different molecules on glia but not detectable on neurons at the threshold of these tests.

To verify that Ng-CAM in a membrane binds specifically to brain cells, we tested the binding of radiolabeled Ng-CAM liposomes to cells. Similar to the results obtained with [^{125}I]Ng-CAM-Covaspheres, [^{125}I]Ng-CAM liposome binding to neurons and glial cells was inhibited strongly by antibodies against Ng-CAM (Table V). Other cells such as meningeal cells showed little binding, and the binding that occurred was not inhibited by antibodies against Ng-CAM, suggesting that it was nonspecific.

These results using artificial membrane vesicles prompted us to reexamine the binding of native neuronal membranes to glial cells, an assay that was originally used to identify and purify Ng-CAM (21). When glial cells in suspension were incubated with fluorescently labeled neuronal membrane vesicles, 45% of the cells showed strong binding (Table VI), approximately the same percentage of glia that bound Ng-CAM-Covaspheres (Table IV). Both the polyclonal antibodies against Ng-CAM and those against chick astroglia inhibited binding to the cells whereas antibodies against other cell surface proteins including N-CAM and fibronectin showed

Table V. Binding of Reconstituted Ng-CAM Liposomes to Cells and Effect of Treatment with Trypsin

Type of cell	Number of cells	Trypsin treatment	Bound (%)		
			Nonimmune	Anti-Ng-CAM	Inhibition
		mg/ml			%
Glia	2 × 10^6	0.02	3.61 ± 0.06	1.41 ± 0.12	61
Glia	2 × 10^6	0.10	1.32 ± 0.11	ND	
Glia	2 × 10^6	2.5	0.43 ± 0.24	ND	
Neurons	5 × 10^6	0.02	4.01 ± 0.03	1.12 ± 0.05	72
Meningeal cells	2 × 10^6	0.02	0.80 ± 0.05	0.73 ± 0.03	9

[^{125}I]Ng-CAM liposomes (50,000 cpm) were preincubated with 1 mg of Fab' fragments of antibodies for 15 min on ice and the binding to cells in suspension was measured in duplicate samples by gamma spectroscopy. Trypsin-treatment was for 30 min at 37°C. Bound (%) = specific binding (cpm)/total input (cpm). ND, not determined.

Table VI. Effects of Antibodies on Binding of Neuronal Membrane Vesicles to Glia in Suspension

Antibody	Cells binding	Inhibition
	%	%
Nonimmune	45.5 ± 2.5	0
Polyclonal anti-Ng-CAM	15.0 ± 4.0	67
Polyclonal anti-N-CAM	42.0 ± 7.0	8
Polyclonal anti-glial cell	18.7 ± 3.0	59
Polyclonal anti-fibronectin	49.0 ± 6.0	0
Monoclonal anti-Ng-CAM 3G2	19.5 ± 3.5	57
Monoclonal anti-N-CAM no. 1	44.5 ± 1.0	2

Fluorescently labeled neuronal membrane vesicles and glial cells were individually preincubated with 0.5 mg of Fab' fragments for 15 min on ice; the cells and vesicles were mixed, incubated at 37°C for 30 min and binding of the vesicles to 200 cells was scored in duplicate samples.

no significant inhibition (Table VI). Similarly, inhibition was found with monoclonal antibody 3G2 (anti-Ng-CAM) but not with monoclonal antibody anti-N-CAM no. 1. These results confirm that the binding of neuronal membranes to astroglial cells involves Ng-CAM.

The combined data from neuron to glial cell adhesion assays (22, 24), native plasma membrane vesicle-binding assays (Table VI; 21) and Ng-CAM-binding assays (Tables IV and V) strongly suggested the existence of surface molecules on glia differing from Ng-CAM that mediate binding via Ng-CAM on neurons. More than 50% of the binding of [^{125}I]Ng-CAM liposomes to glial cells was inhibited by preincubation of the cells with the antibodies against astroglial cells (data not shown), providing further support for this idea. As a first step to analyze the nature of the putative ligands for Ng-CAM on glia, we have tested the susceptibility of Ng-CAM liposome binding to protease treatment of the glial cells. After the cells were treated with different amounts of trypsin, the enzyme was neutralized, and then binding of Ng-CAM liposomes was measured. Treatment of glia in suspension with 0.10 mg/ml trypsin for 30 min eliminated most of the binding and more extensive treatment reduced the level of binding to background levels (Table V) suggesting that proteins on the surface of the glia are involved in binding of Ng-CAM. As shown by the experiments on liposome interaction, the protein is not likely to be N-CAM.

Discussion

The results reported here demonstrate that Ng-CAM binds specifically to the surface of neurons and astroglial cells by different molecular mechanisms and suggest that certain binding interactions of Ng-CAM have been conserved during evolution. Because of the potential complexity of these interactions, particular care was taken to use a variety of assays and to choose conditions only after quantitative assessment of physical variables in the cell-cell binding. The data prompt the conclusion that Ng-CAM binding to neurons is homophilic and that Ng-CAM binding to astroglia is apparently heterophilic (Ng-CAM on neurons binds to a different molecule on glia); the glial ligand remains to be identified.

The evidence supporting the homophilic-binding mechanism include the findings that: (*a*) Covaspheres coated with highly purified Ng-CAM self-aggregated specifically, (*b*) reconstituted liposomes containing only Ng-CAM protein self-aggregated specifically, (*c*) monoclonal and polyclonal antibodies against Ng-CAM specifically inhibited Ng-CAM-mediated binding, and (*d*) binding of Ng-CAM-Covaspheres to neurons was inhibited after pretreatment of the cells with anti-Ng-CAM antibodies and removal of the antibodies by washing. The facts that antibodies against Ng-CAM inhibited aggregation of neurons (22) and fasciculation among neurons (33) are consistent with a homophilic-binding mechanism for Ng-CAM. In view of the different thresholds of various binding mechanisms, however, we can not exclude the possibility that Ng-CAM may have other ligands on neurons.

Given the results of previous studies, we conclude that N-CAM and Ng-CAM each binds homophilically by unrelated specificities that are structurally and immunologically distinct (24). Despite the specificity difference, in both cases: (*a*) aggregation of liposomes containing the purified CAM was inhibited specifically by antibodies against the CAM, (*b*) the rate of aggregation was dependent on the square of the concentration of the liposomes in suspension, and (*c*) the rate of aggregation was exponentially related to the concentration of the CAM in the liposomes (Fig. 3; 32). Although N-CAM and Ng-CAM share at least one carbohydrate antigenic determinant (24) which has been postulated to be involved in cell-cell adhesion (36), Ng-CAM liposomes apparently did not bind to N-CAM liposomes. These results suggest that N-CAM on one cell is not a ligand for Ng-CAM on an apposing cell, although it has not been excluded that N-CAM and Ng-CAM may interact *cis*, i.e., in the same cell membrane (33). In addition to the function of these CAMs in homophilic binding between neurons, it is a reasonable hypothesis to suppose that interactions between peripheral glia (Schwann cells) and neurons proceed via Ng-CAM homophilic binding as well as via N-CAM homophilic binding.

In the present experiments, all of the components of chicken Ng-CAM (M_r 80,000, 135,000, and the doublet at M_r 200,000) were incorporated into liposomes (Fig. 1), suggesting either that they all have hydrophobic membrane-binding regions or that certain components interact with others. To understand the function of each of the components it will be necessary to isolate them and analyze their individual binding activities.

The hypothesis that the molecular mechanism of Ng-CAM binding to astroglia is heterophilic and that astroglia in vitro have distinct cell surface ligands for Ng-CAM is supported by the observations that: (*a*) Ng-CAM is found on neurons and not on brain astroglial cells (24), (*b*) Ng-CAM-Covaspheres and Ng-CAM liposomes bound to glial cells specifically, (*c*) binding of Ng-CAM-Covaspheres to glial cells (but not to neurons) was inhibited after preincubation of the cells with polyclonal anti-astroglial cell antibodies and removal of the antibodies by washing, and (*d*) the binding of Ng-CAM-Covaspheres to glial cells was sensitive to protease treatment of the cells. Consistent with this conclusion are the observations that binding of native neuronal membrane vesicles to glial cells and adhesion between neurons and glial cells was inhibited by anti-Ng-CAM and anti-astroglial cell antibodies. As emphasized previously (21), only when heterophilic glial ligands for Ng-CAM are purified can the binding mechanism for astroglia be considered as defined.

Previous perturbation studies using cells from chick em-

bryo brains suggested a role for Ng-CAM in neuron-glia adhesion (22, 24). Several morphological and immunological characteristics of the cells indicated that they are astroglia or astroglial precursors. The glial cells were isolated from chick embryo brains by their ability to adhere to collagen-coated surfaces. They had a flat, cobblestone appearance when they were grown at high density in culture, a morphology very different from the fibroblast-like morphology of cells isolated from the meninges of the brain (2, 18, 27). Glial cells isolated by very similar methods were shown to express glutamine synthetase which is expressed in increasing amounts as astroglia mature (57). In our studies, the chick glia were identified by monoclonal antibody GA3 which specifically recognized these cells (33) and monoclonal antibody R5 which recognizes astrocytes in rodents (13, 23).

Because developing chick glia did not react with a variety of antibodies against GFA (1, 18), we also examined the role of Ng-CAM using mouse astroglial cells which could be definitely identified using GFA. By methods similar to those applied to isolate glia from chick brains, GFA-positive astroglial cells were isolated from neonatal mouse brains. Adhesion of neurons from both mouse and chick brains to the mouse astroglia was inhibited by antibodies against Ng CAM, confirming a role for Ng-CAM in adhesion of neurons to astroglia. The results of the cross-species neuron–neuron and neuron–astroglia adhesion experiments suggest that functional binding mechanisms of Ng-CAM have been conserved in the evolution of birds and mammals. Immunoblotting data indicated the presence of Ng-CAM in chick (Fig. 1 *a*), mouse (Fig. 1 *c*), rat (20), amphibians, and primates (our unpublished observations) and it is likely that Ng-CAM, like N-CAM (30), will be seen in all vertebrate species.

In other studies (35), it was reported that antibodies against L1 antigen, which is immmunologically related to Ng-CAM (5, 20), did not inhibit neuron–astrocyte adhesion. To determine the source of the discrepancy between this finding and our results that anti-Ng-CAM antibodies inhibited neuron–astroglia adhesion in vitro, we have carefully analyzed the effects of changes in key parameters of the adhesion assays (see Fig. 6). Although there were probably differences between the shear rates in the different assays used (21, 26, 35), it is unlikely that this could account for the differences found because adhesion was minimally effected by changes in shear forces. Despite the fact that immunological and structural studies (5, 20, 51) have indicated that Ng-CAM, NILE, and L1 antigen are very similar or identical molecules, the relative ability of antibodies against each molecule to inhibit cell–cell adhesion may vary. Moreover, different species have different ratios of Ng-CAM components which may have important bearing on the effects of antibodies on cell adhesion; chick cells contain a major component of M_r 135,000 and lesser amounts of M_r 200,000 and 80,000 components (22, 38, 49), in contrast, rodent cells contain predominantly the M_r 200,000 component (Fig. 1, 44, 48, 52, 55).

Perturbation of cell–cell adhesion with specific antibodies indicates that, for a given pair of cells, more than one cell surface molecule may be involved in mediating adhesion. Neuron–astroglia adhesion apparently involves neuronal Ng-CAM binding to as yet unidentified ligands on glia in a calcium-independent mode and the binding of cytotactin, a matrix component that is secreted by glia to a neuronal proteoglycan which is not synthesized by astroglia (23). Reports that antibodies against an adhesion molecule on glia and against N-CAM inhibit neuron–glia adhesion by 25 and 29% (3), respectively, suggest that these molecules may also be involved. In our studies, however, the antibodies against N-CAM did not inhibit the binding of neurons to astroglia despite the fact that they strongly inhibited neuron–neuron and neuron–myoblast (25) adhesion. The fact that the rate of N-CAM-mediated adhesion is highly dependent on its surface density in the membrane (32) and that astroglia have very low levels of N-CAM on their surface (24) are consistent with our observations that N-CAM binding was not a major factor in neuron–glia adhesion in vitro. Cells in tissue and in culture may express different forms and amounts of N-CAM and variations in CAM expression may account for the differences observed using different populations of cells (8, 61). Thus, it is possible that N-CAM may be involved in adhesion between neurons and glia in vivo at sites where both cells express the molecule (54).

The physiological role of Ng-CAM binding in neuron–neuron and neuron–glia interactions must be interpreted cautiously until the glial ligands have been isolated, mapped, and analyzed for adhesive function. The available data on Ng-CAM binding, distribution, and perturbation in vitro and in vivo, however, can be correlated to some extent. In the developing cerebellum, granule cells continually express N-CAM and after they become postmitotic in the superficial layers they express Ng-CAM (22, 33). emit parallel fibers in the granule cell layer, and then migrate inward along Bergmann glial cells (43, 46). Perturbation studies with antibodies against specific molecules have indicated that migration of granule cells involves Ng-CAM (7, 33), L1 antigen (40), cytotactin (7), a glial-derived neurite promoting factor (39), an adhesion molecule on glia (3), and possibly N-CAM (7, 33, 40). Whereas antibodies against Ng-CAM strongly inhibited migration of granule cells in cerebellar explants, antibodies against N-CAM produced little or no inhibition (33, 40) suggesting a major role for Ng-CAM and not for N-CAM in the migration. Although antibodies against both CAMs inhibited aggregation of cerebellar neurons (22, 47) and fasciculation of fibers in cerebellar explants (19), only anti-Ng-CAM antibodies strongly inhibited granule cell migration across the molecular layer. It is possible that the inhibition of granule cell migration by antibodies resulted indirectly from an inhibition of fasciculation of granule cell processes as the cell body entered the molecular layer.

The conclusion that neuron–glia adhesion may be mediated by at least five or more molecules underscores the need for more detailed studies of mechanisms of binding in each case. It also suggests that caution be exercised in extrapolating conclusions even from quantitative in vitro adhesion assays such as those described here to the interpretation of in vivo events. Only when detailed mechanisms of binding, cell surface distribution, and degree of expression in vivo are all capable of being related can firm conclusions be reached on the exact contribution of each molecule to morphogenetic events.

We thank Andrew Flood and David Hewitt for excellent technical assistance.

This work was supported by United States Public Health Service grants NS-21629 and a Senator Jacob Javits Center for Excellence in Neuroscience

grant (NS-22789). M. Grumet is a recipient of an Irma T. Hirschl Career Scientist Award.

Received for publication 27 August 1987, and in revised form 14 October 1987.

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