Heterotypic Binding Between Neuronal Membrane Vesicles and Glial Cells Is Mediated by a Specific Cell Adhesion Molecule

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ABSTRACT By means of a multistage quantitative assay, we have identified a new kind of cell adhesion molecule (CAM) on neuronal cells of the chick embryo that is involved in their adhesion to glial cells. The assay used to identify the binding component (which we name neuron-glia CAM or Ng-CAM) was designed to distinguish between homotypic binding (e.g., neuron to neuron) and heterotypic binding (e.g., neuron to glia). This distinction was essential because a single neuron might simultaneously carry different CAMs separately mediating each of these interactions. The adhesion of neuronal cells to glial cells in vitro was previously found to be inhibited by Fab' fragments prepared from antisera against neuronal membranes but not by Fab' fragments against N-CAM, the neural cell adhesion molecule. This suggested that neuron-glia adhesion is mediated by specific cell surface molecules different from previously isolated CAMs. To verify that this was the case, neuronal membrane vesicles were labeled internally with 6-carboxyfluorescein and externally with ^{125}I-labeled antibodies to N-CAM to block their homotypic binding. Labeled vesicles bound to glial cells but not to fibroblasts during a 30-min incubation period. The specific binding of the neuronal vesicles to glial cells was measured by fluorescence microscopy and gamma spectroscopy of the ^{125}I label. Binding increased with increasing concentrations of both glial cells and neuronal vesicles. Fab' fragments prepared from anti-neuronal membrane sera that inhibited binding between neurons and glial cells were also found to inhibit neuronal vesicle binding to glial cells.

The inhibitory activity of the Fab' fragments was depleted by preincubation with neuronal cells but not with glial cells. Trypsin treatment of neuronal membrane vesicles released material that neutralized Fab' fragment inhibition; after chromatography, neutralizing activity was enriched 50-fold. This fraction was injected into mice to produce monoclonal antibodies; an antibody was obtained that interacted with neurons, inhibited binding of neuronal membrane vesicles to glial cells, and recognized an $M_r = 135{,}000$ band in immunoblots of embryonic chick brain membranes. These results suggest that this molecule is present on the surfaces of neurons and that it directly or indirectly mediates adhesion between neurons and glial cells. Because the monoclonal antibody as well as the original polyspecific antibodies that were active in the assay did not bind to glial cells, we infer that neuron-glial interaction is heterophilic, i.e., it occurs between Ng-CAM on neurons and an as yet unidentified CAM present on glial cells.

The brain and other neural tissues are composed mainly of two different types of cells, neurons and glia. Interactions between these cells are assumed to be essential both for the complex organization of neurons during development and for the mechanical and biochemical support of adult neural tissue (reviewed in reference 1). Evidence for various interactions between neuronal and glial cells was first provided by Ramon y Cajal (2); more recent studies involving three-dimensional reconstruction of serial sections of neural tissues have revealed the existence of an intimate relationship between the surfaces

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of neuronal and glial cells both in the adult and during development (3). The molecular nature of the binding interaction between these cells remains to be determined but the close apposition of neurons to radial glial cells during development raises the possibility that specific adhesion between these cells is the mechanism that ensures directed migration of neurons through many layers of cells. In tissue culture systems, neurons in suspension have been found to bind both to neurons (see reference 4 for review) as well as to monolayers of glial cells (5, 6); the binding to glial cells was inhibited by Fab' fragments prepared from antibodies against neuronal cell surface determinants (6). Antibodies to the neural cell adhesion molecule, N-CAM (4),[1] which inhibit interactions between neurons, do not inhibit adhesion of neurons to glial cells (6). It is therefore likely that the adhesion between neuronal and glial cells is mediated by specific cell surface molecules other than N-CAM.

To test this hypothesis and to isolate these molecules, a quantitative assay suitable for determining adhesion between different cell types (heterotypic adhesion) is required. In this paper, we describe such an assay and apply it to the identification of a new cell adhesion molecule on neuronal cells of chick embryos. The basic characteristics of the assay are (*a*) the use of doubly labeled membrane vesicles from neurons, (*b*) the binding of these vesicles to intact glial cells, (*c*) the addition of Fab' fragments of anti-(N-CAM) in sufficient amounts to suppress homotypic vesicle aggregation, (*d*) the use of Fab' fragments from antibodies against other neuronal determinants to inhibit the binding of neuronal vesicles to glial cells, and (*e*) neutralization of this inhibition by extracts of neuronal membranes that contain the putative neuron-glia CAM, Ng-CAM. To identify antigens related to a putative Ng-CAM, cell surface antigens that had neutralizing activity were released from neuronal membrane vesicles in a soluble form by trypsin treatment and were fractionated by gel filtration and lentil lectin affinity chromatography. A monoclonal antibody obtained after injection of an enriched neutralizing fraction was found to inhibit binding of neuronal membrane vesicles to glial cells. Immunoblots of extracts prepared from chick brains with this antibody showed reactivity to material in a band of M_r = 135,000. Neuronal cells but not glial cells depleted the inhibitory antibody activity from solution, suggesting that this protein is present on neurons but not on glia. The antigenic and chemical properties of this protein suggest that it constitutes part of a specific adhesion system mediating heterotypic cell binding.

MATERIALS AND METHODS

Reagents and Media: Dulbecco's modified Eagle's Medium (DME), Eagle's minimal essential medium (MEM), fetal calf serum, and 0.25% porcine trypsin were from Gibco Laboratories (Grand Island, NY). Soybean trypsin inhibitor, HEPES, and EDTA were from Sigma Chemical Co. (St. Louis, MO). Deoxyribonuclease I (DNase), collagenase, and twice crystallized trypsin were from Worthington Biochemical Corp. (Freehold, NJ), and 6-carboxyfluorescein was from Eastman Laboratory and Specialty Chemicals (Rochester, NY). Tissue culture plates were Costar (Data Packaging, Cambridge, MA) or Falcon (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA), BSA was from Armour Pharmaceutical Co. (Tarrytown, NY), Nonidet P-40 was from BDH Chemicals Ltd. (Poole, England), Bio-Beads SM-2 were from Bio-Rad Laboratories (Richmond, CA), trasylol was from Mobay Chemical Corp. (Pittsburgh, PA), and Na ^{125}I was from New England Nuclear (Cambridge, MA). PBS contained 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, and 1.15 g Na_2HPO per liter. Calcium-magnesium-free medium (CMF) contained 8.0 g NaCl, 0.3 g KCl, 0.0575 g $NaH_2PO_4 \cdot H_2O$, 0.025 g KH_2PO_4, 1.0 g $NaHCO_3$, 2.0 g D-glucose, 20 mg DNase, and 10 mg phenol red per liter. Collagen was prepared by extraction of rat tail tendons with 0.1% acetic acid for 48 h at 4°C.

Preparation of Fluorescent Brain Vesicles: Brains from three 14-d chick embryos were homogenized with a Dounce homogenizer (A pestle) in 30 ml PBS containing 100 μg/ml DNase and 5 mg/ml 6-carboxyfluorescein. The homogenate was spun at 10,000 *g* for 10 min and the pellet was resuspended in 3 ml PBS containing 100 μg/ml DNase. This suspension was mixed with 30 ml of PBS containing 2.25 M sucrose and overlayed in a centrifuge tube with 20 ml of PBS containing 0.8 M sucrose. The discontinuous sucrose gradient was centrifuged at 100,000 *g* for 45 min and the fluorescent vesicles that migrated to the interface were collected and washed three times by centrifugation (10,000 *g* for 10 min) with PBS. The final pellet was resuspended in MEM to yield a ~10% vesicle suspension. All procedures were performed at 4°C.

Preparation of Glial Cells: Neural tissues were dissected from white Leghorn chick embryos. Forebrain, hindbrain, or retina from embryos (10 or 14 d) were dissected under sterile conditions in CMF (brains were prepared free of meninges). The tissue was gently teased into fragments of ~2 mm and incubated at 37°C in CMF containing 0.1% trypsin and 1 mM EDTA for 45 min with shaking at 70 rpm. The suspension was centrifuged in a clinical centrifuge at 2,000 rpm for 3 min, the pellet was resuspended in DME containing 10% fetal calf serum, and triturated 20 times with a Pasteur pipette. The cell suspension was layered on a solution of PBS containing 3.5% BSA and centrifuged at 2,000 rpm for 3 min, and after dispersion, cells from the pellet were washed twice in the same medium. The pellet was resuspended at 10^6 cells/ml in DME supplemented with 10% fetal calf serum, 5 g/liter D-glucose, and 25% conditioned medium (see below) and cultured on tissue culture dishes that were coated with collagen. To prepare these dishes, the tissue culture surfaces were covered with a solution of collagen at 0.5 mg/ml which was removed and the surfaces were air-dried under ultra-violet irradiation for 2 h. To obtain monolayer cultures of glial cells, the cell suspension was incubated (75 ml per roller bottle) at 37°C. The bottles were rotated at 30 rph which allowed neurons to aggregate in suspension, while the glia adhered to and spread on the collagen-coated substrate. After 24–36 h the medium was removed and the bottles were gently washed twice with PBS. The medium was centrifuged to remove cells, passed through a 0.8-μm filter, and reapplied to the roller bottles. After 2–3 d in culture, the medium was removed (conditioned medium) and then replaced with fresh medium (DME containing 10% fetal calf serum and 5 g/liter D-glucose). After 5–7 d in culture, the flat cells grew to confluence and any residual neurons were removed by vigorously washing the monolayer with PBS.

Several considerations indicated that these cells were glial cells. The cultured cells bound a monoclonal antibody that stained cells resembling glia in sections of central and peripheral neural tissues; this monoclonal antibody did not bind to neurons or fibroblasts in culture (J.-P. Thiery, M. Grumet, and G. M. Edelman, unpublished results). In addition, these cells had morphologies and patterns of monoclonal antibody staining similar to flat glial cells that were isolated from 10–14-d chick embryo brains by the procedures of Partlow and co-workers (7, 8) and Adler et al. (9). Finally, the flat cells from brain obtained in our procedure did not stain with anti-(N-CAM); antibodies to N-CAM specifically stain the surfaces of neuronal cells in tissue sections and in culture (10, 11), but do not stain chick glial cells in tissue sections and in culture (11, 12). Based on their method of isolation, morphology in culture, and surface determinants, it is likely that these cells are related to astroglia (5–9), and in this report we will refer to them as glial cells. It should be noted that a completely adequate set of specific criteria distinguishing chick neuronal and glial cells at the tissue culture level remains to be established.

Preparation of Neuronal and Other Cells: Neuronal cells were prepared in suspension by trypsinization of tissue from brain and retina in calcium-free medium as described (13, 14). Suspensions of cells were prepared from embryonic livers by trypsinization in the presence of calcium (15). Fibroblasts were prepared from the skin of 10–12-d chick embryos by trypsinization as described above for brain tissue and cultured on collagen-coated tissue culture surfaces in DME supplemented with 10% fetal calf serum and 5 g/liter D-glucose. Meninges were dissected from 10-d chick embryo brains, trypsinized, and cultured similarly. All these cultures were free of neurons and had cells with a fibroblast-like morphology (for an example, see reference 6).

Antibodies: Antibodies to purified N-CAM were prepared as described (16). Monovalent Fab' fragments were prepared from the IgG fraction of rabbit serum (17). Polyspecific antibodies (anti-brain membrane) were obtained by intramuscular, intraperitoneal, and subcutaneous injections of rabbits with 1

[1] *Abbreviations used in this paper:* CAM, cell adhesion molecule; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DNase, Deoxyribonuclease I; CMF, calcium-magnesium-free medium; DME, Dulbecco's modified Eagle's medium; MEM, Eagle's minimal essential medium; N-CAM, neural CAM; Ng-CAM, neuron-glia CAM.

ml of membranes (50% suspension in PBS) from 10- or 14-d chick embryo brains. The first injection was in complete Freund's adjuvant, the second (2 wk later) was in incomplete Freund's adjuvant, and subsequent injections at 2 wk intervals were without adjuvant. Rabbits were bled weekly after the third injection.

Anti-(N-CAM) Fab' was labeled (18) with ^{125}I for use in the vesicle-to-cell binding assay. 18 ml of anti-(N-CAM) Fab' fragments (5 mg/ml) was incubated with 2 ml chloramine T (1 mg/ml) and 2 ml Na ^{125}I (5 mCi/ml) in PBS at 4°C for 10 min, and then 5 mg of sodium metabisulfite was added to quench the reaction. Unbound ^{125}I was removed by dialysis for 4 h at 4°C in PBS containing 20 mM KI, followed by successive dialyses in PBS. BSA was labeled with Na ^{125}I using the same procedure.

All protein concentrations were measured by the method of Lowry et al. (19).

Vesicle-to-Cell-Binding Assay: This multistage assay measures the binding of neuronal membrane vesicles to cells in suspension and was employed in three experimental contexts: (*a*) to compare the adhesion of the vesicles to different types of cells; (*b*) to measure the ability of specific Fab' fragments to inhibit vesicle binding to cells by blocking cell surface adhesion molecules; and (*c*) to test antigens obtained from embryonic brain membranes for their ability to neutralize inhibition of vesicle binding by the Fab' fragments; we define this as Ng-CAM neutralizing activity.

Glial cells were obtained in suspension from monolayers that were grown to confluence on roller bottles (850 cm^2, Corning Glass Works, Corning, NY) as described above, washed two times with PBS, and treated with 8 ml of 0.25% trypsin containing 1.5 mg/ml collagenase, 0.02% EDTA, and 100 μg/ml DNase. After enzyme treatment for 45 min at 37°C, 2 vol of DME containing 10% fetal calf serum and trasylol (100 U/ml) were added and the cells were gently triturated with a Pasteur pipette. The cell suspension was diluted to approximately 10^6 cells/ml with the same medium and incubated in a spinner bottle (Bellco Glass, Inc., Vineland, NJ) with gentle stirring (100 rpm) for 16–20 h at 37°C under 10% CO_2. The bottles were then placed in an ice bath for 10 min with gentle stirring. The cells were sedimented by centrifugation for 3 min at 2,000 rpm, resuspended in cold MEM containing 20 μg/ml DNase, centrifuged, and resuspended in 5 ml of MEM. This procedure yielded single cell with only a few aggregates. The number of cells was determined using a Cytograf (Bio/Physics Systems, Westwood, MA); 2–3 × 10^8 cells were obtained from 30 forebrains from 14-d embryos after 1 wk in culture on five roller bottles. In culture, these cells had a flat appearance and did not contain any neurons (detected as small, round, process-bearing cells) as judged by light microscopy. Cell viability was 85 ± 10% as judged by trypan blue exclusion.

The vesicles used in the assay were obtained from homogenates of embryonic brains as described above. Following centrifugation, the fluorescent membrane pellets were resuspended in 3 ml of PBS containing iodinated rabbit anti-(N-CAM) Fab' (4 mg/ml with ~10^8 cpm/ml) and 100 μg/ml DNase, incubated overnight at 4°C, and fractionated on discontinuous sucrose gradients as described. The vesicles, which were labeled internally with 6-carboxyfluorescein and externally with ^{125}I-(anti-[N-CAM] Fab'), were collected by centrifugation, washed three times with PBS, and resuspended in MEM as a 10% suspension. Aggregates of vesicles were removed by centrifugation at 2,000 rpm for 75 s and the supernatant fraction was used in the binding assay. Aliquots of vesicles (0.1 ml containing ~200,000 cpm) were added to 0.2–0.5 ml of cold PBS containing Fab' fragments, or to a mixture of Fab' fragments and brain antigens that had been preincubated together for 15 min at 4°C. After 15 min at 4°C, the volume was adjusted to 1.2 ml by addition of cold MEM containing 20 μg/ml DNase. Equal aliquots of cells (2–5 × 10^6) were added, the samples were transferred to glass scintillation vials (28 × 61 mm, Wheaton Scientific, Millville, NJ), and rotated at 70 rpm in a New Brunswick environmental incubator shaker at 37°C. After 30 min, the samples were transferred to 12 × 75 mm borosilicate glass tubes (Fisher Scientific Co., Pittsburgh, PA) and centrifuged at 2,000 rpm for 75 s. The supernatant fraction was removed with an aspirator and the pellets were resuspended in 1 ml MEM, layered onto 3.5 ml of PBS containing 3.5% BSA, and centrifuged at 2,000 rpm for 75 s. The supernatant fraction was removed with an aspirator and the pellets were counted in a gamma spectrometer. These conditions were selected to minimize the co-sedimentation of unbound vesicles with cells containing bound vesicles. The final pellets were gently resuspended in medium and, as determined by fluorescence microscopy, were essentially free of vesicles that were not bound to cells.

Preparation of Detergent Extracts of Membranes from Chick Embryo Brains: Extracts were prepared as described previously (20). Briefly, brains were removed from 14-d chick embryos and homogenized in a Dounce homogenizer containing CMF supplemented with 200 U/ml trasylol. The homogenate was centrifuged for 10 min at 18,000 rpm in a Sorvall SS34 rotor (E. I. DuPont de Nemours & Co., Inc., Sorvall Instruments Div., Newton, CT) and the pellets were resuspended in 2.25 M sucrose and fractionated on discontinuous sucrose gradients. The membranes that migrated to the interface were collected, washed twice with PBS as described above, extracted in PBS/0.5% Nonidet P-40/1 mM EDTA, and centrifuged for 40 min at 100,000 *g*. The clear supernatant was removed and mixed with one-third volume of Bio-Beads SM-2 for 2 h with vigorous shaking, and the unbound fraction was dialyzed against PBS. For gel filtration chromatography, 2.5 ml of a 50% suspension of membranes was extracted in 5 ml of 10 mM Tris, 1 mM EDTA, and 0.5% sodium deoxycholate, pH 8.2, and centrifuged at 100,000 *g* for 1 h. The supernatant fraction was chromatographed on a Sephacryl S-300 column (1.8 × 100 cm) in the same buffer and the eluted fractions (4 ml) were dialyzed extensively against MEM before being tested for Ng-CAM neutralizing activity. All procedures were performed at 4°C.

Fractionation of Neutralizing Activity from Trypsin-released Extracts of Chick Embryo Brain Membranes: Membranes (35 ml of a 50% [vol/vol] suspension in PBS) were prepared from 14-d chick embryo brains (20) and incubated with 3.5 mg trypsin for 75 min at 37°C. The enzyme was inactivated by addition of 6.3 mg of soybean trypsin inhibitor, and the membranes were pelleted by centrifugation at 100,000 *g* for 45 min. The supernatant fraction, the trypsin-released extract, was passed over an anti-(N-CAM) No. 1 affinity column (20) to deplete the extract of N-CAM antigenic determinants. The ionic strength of the unbound fraction was raised to 0.3 M salt by adding concentrated KCl and the extract was then passed over a 25-ml column of DEAE-cellulose which was equilibrated in 10 mM HEPES/0.3 M KCl, pH 7.4. The unbound fraction was dialyzed extensively in 5 mM HEPES, pH 7.0, and lyophylized; the sample was dissolved and dialyzed in PBS, and fractionated on a Sephacryl S-300 column (1.8 × 95 cm) in PBS containing 1 mM NaN_3. Fractions (5 ml) 20–25 (see Fig. 7) that were enriched in neutralizing activity were pooled, dialyzed in 10 mM HEPES/0.5 M KCl/0.2 mM $CaCl_2$/0.02 mM $MnCl_2$, pH 7.4, and incubated with 15 ml of lentil lectin-Sepharose 4B (Pharmacia Inc., Piscataway, NJ) for 2 h with shaking. The gel was washed three times with 50 ml of 10 mM HEPES/0.5 M KCl/0.2 mM $CaCl_2$/0.02 mM $MnCl_2$, pH 7.4, and protein was eluted from the gel by incubation for 2 h with 100 ml of 0.2 M α-methyl-D-glucoside in the same buffer. Unless otherwise indicated all procedures were performed at 4°C.

Preparation of Monoclonal Antibodies to Ng-CAM: Mice were injected intraperitoneally at 2-wk intervals with partially purified trypsin-released extract (100 μg protein) prepared as described above and emulsified with Freund's adjuvant. 3 d after the third injection (without adjuvant), spleens were taken from the mice; the fusion, screening of hybridomas, and production of monoclonal antibodies were performed as described (21). Hybridoma culture supernatants were screened for binding to 96-well polyvinyl plates coated with 0.5 μg/well of the immunogen and select hybridomas were recloned twice by limiting dilution in 96-well dishes. Recloned hybridomas were injected into mice; ascites fluid was collected, partially purified by precipitation with 45% ammonium sulfate and dialyzed in PBS. Monoclonal antibody 10F6 obtained from culture supernatants reacted with rabbit antisera specific for subtype IgG_1.

Immunoblotting: SDS PAGE was carried out on 7.5% acrylamide gels as described (22), and samples were prepared by boiling for 3 min with sample buffer (62.5 mM Tris, pH 6.8, 5% 2-mercaptoethanol, 2% SDS, 10% sucrose, and 0.0025% bromphenol blue). Proteins were transferred to nitrocellulose paper and reacted sequentially with 50 μg of monoclonal antibody 10F6, 50 μg of rabbit anti-mouse immunoglobulin, and 1 × 10^6 cpm of ^{125}I-labeled protein A, and labeled material was detected by autoradiography (23).

RESULTS

Binding of Fluorescent Vesicles to Cells

Fluorescein-labeled chick brain membrane vesicles in suspension bound to glial cells in monolayers or in suspension during a 30-min incubation period, and little or no binding occurred with fibroblasts or cells from the meninges. When nonfluorescent membrane vesicles were incubated with glial cells, indirect immunofluorescence with anti-(N-CAM) IgG showed that the bound vesicles were brightly stained by anti-(N-CAM) but the glial cells were not stained (data not shown). Because N-CAM is a neuron-specific marker in chick brain tissue (10, 11), these results indicate that the vesicles that bound to glial cells in these experiments are mainly of neuronal origin.

When anti-(N-CAM) Fab' was absent during vesicle to cell binding, there was extensive homotypic aggregation of the neuronal membrane vesicles (Fig. 1, *A* and *B*). Earlier studies

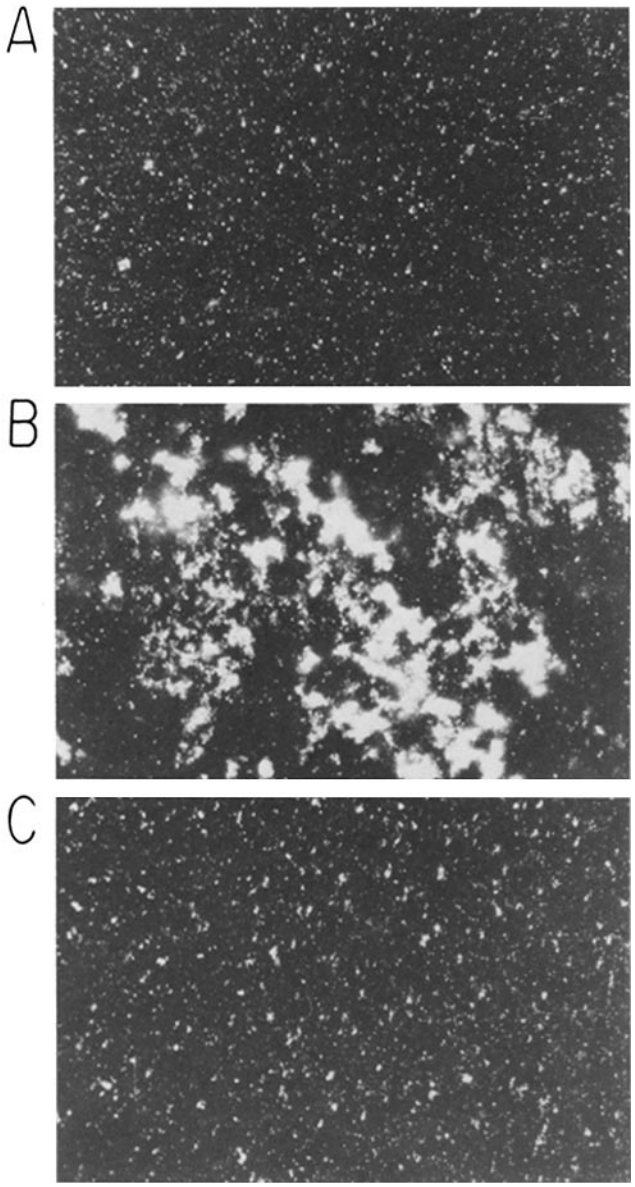

FIGURE 1 Aggregation of neuronal membrane vesicles. (*A*) Freshly prepared vesicles from 14-d chick embryo brains were prepared as described in Materials and Methods. (*B*) Vesicles incubated in 1 ml of MEM with 0.5 mg Fab′ fragments from preimmune Ig for 30 min at 37°C under the standard assay conditions as described in Materials and Methods. (*C*) Vesicles incubated as in *B* except the Fab′ fragments were from anti-(N-CAM) Ig. Fluorescence micrographs; × 470.

showed that the aggregation of artificial vesicles reconstituted from lipid and N-CAM is inhibited by anti-(N-CAM) antibodies (24, 25). Therefore, to eliminate binding involving N-CAM and, in particular, any possible aggregation of neuronal membrane vesicles by the N-CAM mechanism, experiments were performed in the presence of anti-(N-CAM) Fab′ fragments. Under these conditions, the vesicles were either unaggregated or formed only small aggregates (~3 μm, Fig. 1 *C*) allowing quantitative and reproducible measurements of vesicle binding to cells.

The binding of vesicles to neuronal, glial, and fibroblastic cells that had been incubated in suspension culture for >16 h was quantified by scoring the percentage of cells that bound fluorescent vesicles after a 30-min incubation period. In previous studies (10), it was shown that neural retinal cells bound retinal membrane vesicles in the presence of preimmune rabbit Fab′ fragments and that anti-(N-CAM) Fab′ fragments inhibited neuronal vesicle binding; these results were confirmed here for cells and membrane vesicles that were prepared from 10-d chick embryo brains. In contrast, in the presence of Fab′ fragments derived from either preimmune Ig or from anti-(N-CAM) Ig, the binding of neuronal membrane vesicles to glial cells was still clearly detected and, under similar conditions, few if any vesicles bound to fibroblasts (Fig. 2 and Table I). Most important, vesicle binding to glial

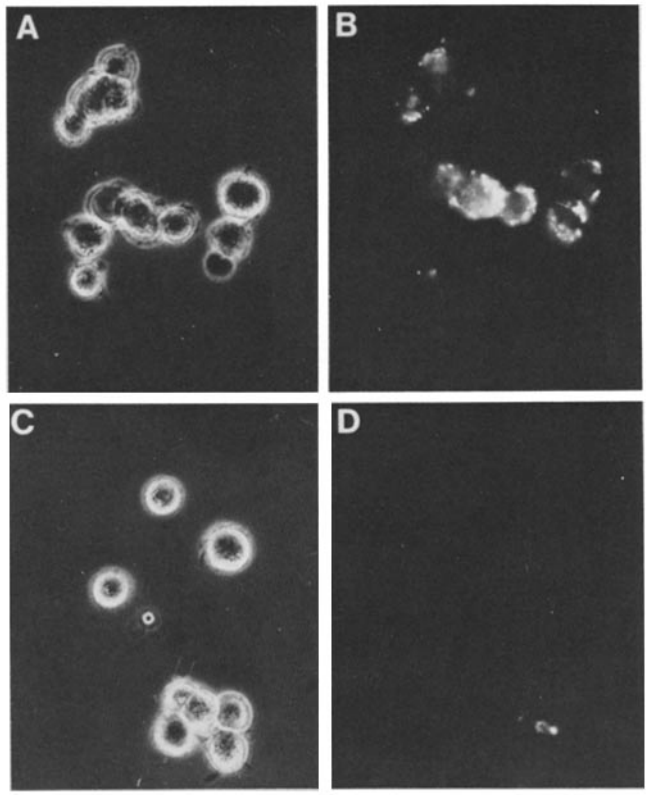

FIGURE 2 Binding of fluorescein-labeled neuronal membrane vesicles to cells. Glial cells (*A* and *B*) and fibroblasts (*C* and *D*) were each incubated with vesicles for 30 min at 37°C under the standard assay conditions as described in Materials and Methods. (*A* and *C*) Phase-contrast micrographs; (*B* and *D*) fluorescence micrographs of the same respective fields. × 510.

TABLE I

Comparison of Binding of Brain Membrane Vesicles to Cells by Two Different Methods

Cell type	Binding		
	Fab′	Fluorescence*	Radioactivity‡
		%	*cpm*
Glia	anti-(N-CAM)	47	4,251 ± 318
Glia	anti-brain membrane	9	1,308 ± 121
Fibroblasts	anti-(N-CAM)	12	1,042 ± 89
Liver cells	anti-(N-CAM)	ND	1,074 ± 108

* 200 cells were scored for fluorescence; cells containing three or more discrete points of fluorescence were scored as positive. Rabbit antibody fragments (0.5 mg) were preincubated with vesicles for 15 min on ice before addition of cells. *ND*, not done.

‡ Binding of ^{125}I-labeled vesicles to 5 × 10^6 cells was measured by gamma counting in a standard assay as described in Materials and Methods. Duplicate samples were measured; mean values of binding and the mean deviation are shown.

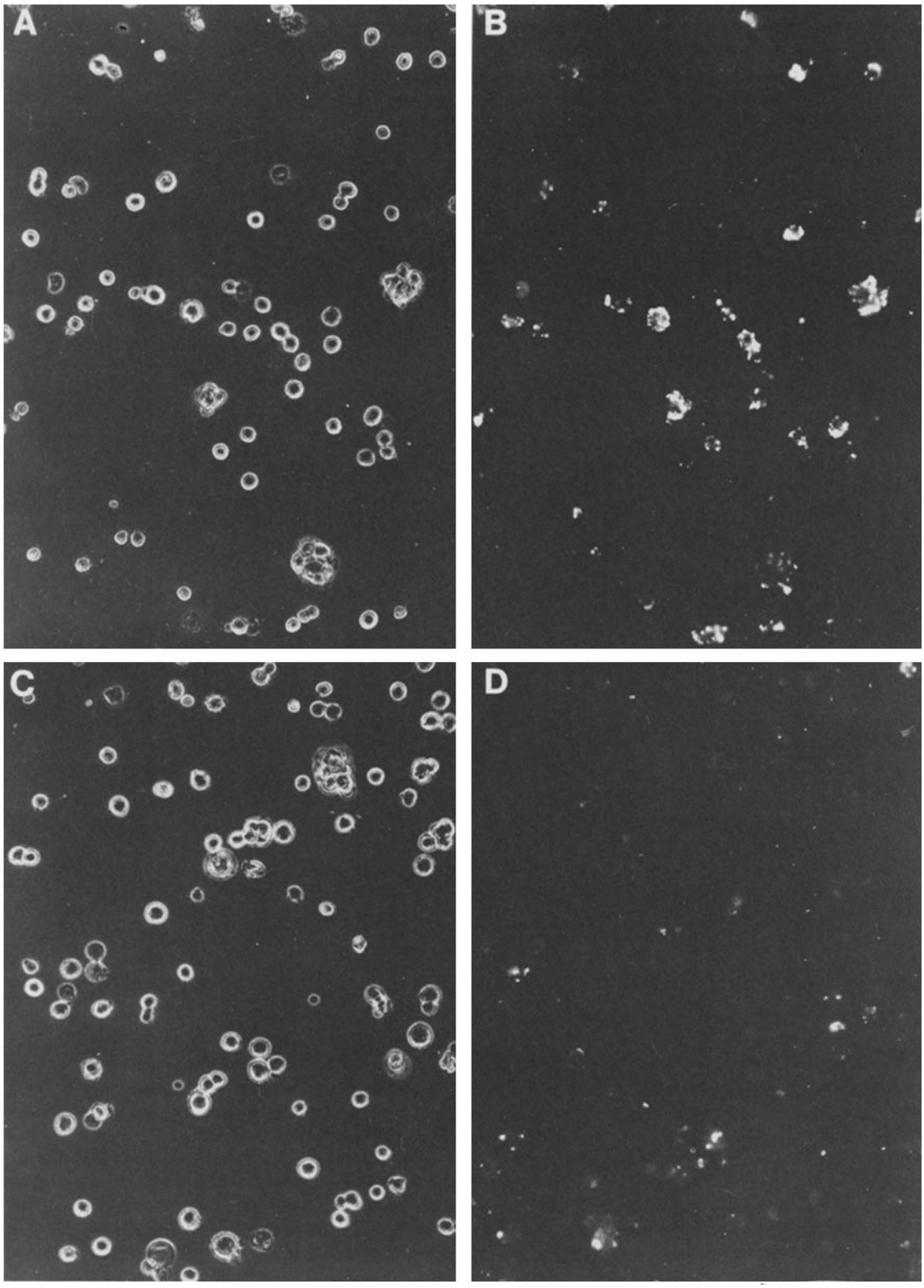

FIGURE 3 Effect of antibodies on binding of fluorescein-labeled neuronal membrane vesicles to glial cells. (*A* and *C*) Phase-contrast micrograph, (*B* and *D*) fluorescence micrographs of the same respective fields. Vesicles (100 μl) were preincubated with 0.5 mg Fab' fragments from anti-(N-CAM) Ig (*A* and *B*), and from anti-brain membrane Ig (*C* and *D*), and binding to 5×10^6 glial cells in 1.2 ml MEM for 30 min at 37°C was measured. × 270.

cells was inhibited by the Fab' fragments prepared from antibodies against embryonic chick brain membranes (Fig. 3, Table I).

These results are consistent with the interpretation that neuronal membrane vesicle aggregation is mediated primarily by N-CAM (24, 25) and that neuronal membrane vesicle binding to glial cells cells is not mediated by N-CAM but by some other mechanism. Although the binding of brain vesicles

to glial cells in suspension could be detected qualitatively with ease and measured quantitatively with these methods (Table I), scoring of the fluorescence was slow and somewhat subjective, making routine use of this form of the assay cumbersome. We therefore used radioactively labeled vesicles to facilitate the simultaneous quantification of binding in many samples.

Binding of ^{125}I-Vesicles to Cells

To accommodate many samples in a single binding experiment, the neuronal vesicles were radioactively labeled with iodinated proteins that were either incorporated in the intravesicular space or bound to the vesicle surface. To ensure that the characteristics of binding were not changed by the radioactive labeling procedure, vesicles that contained both 6-carboxyfluorescein and ^{125}I were prepared. The double label allowed independent measurements of vesicle binding by both fluorescence and radioactivity. In preliminary experiments, the vesicles were prepared from embryonic chick brains by homogenization in the presence of 6-carboxyfluorescein and ^{125}I-BSA. After purification on discontinuous sucrose gradients, vesicles were obtained that were internally labeled by both the dye and the radioactive protein. Binding of these vesicles to glial cells was then measured by both fluorescence microscopy and gamma spectroscopy. It was necessary to include anti-(N-CAM) Fab′ fragments in these experiments in order to obtain correlative quantitative binding data on individual samples by both methods; in the absence of Fab′ fragments, large aggregates of vesicles co-sedimented with the glial cells in a fashion similar to the result shown in Fig. 1 *B*.

Although this combined procedure allowed quantitative measurement of vesicle binding in individual samples by following both fluorescence and radioactivity, labeling of vesicles with ^{125}I was subsequently found to be even more efficient when the vesicles were incubated with ^{125}I-Fab′ fragments from antibodies directed against N-CAM. This procedure served both to label the vesicles radioactively by binding of ^{125}I-(anti-(N-CAM) Fab′) to N-CAM on the surface of neuronal membrane vesicles, and simultaneously to block most of the homotypic vesicle aggregation mediated by N-CAM. The neuronal membrane vesicles that had been preincubated with ^{125}I-(anti-(N-CAM) Fab′) still aggregated to a minor extent, however, and for this reason, the assay was performed in the presence of additional unlabeled anti-(N-CAM) Fab′, which limited vesicle aggregation to the level shown in Fig. 1 *C*.

The results obtained when vesicle binding to cells was measured in identical samples by either fluorescence or radioactivity were similar. In the same experiment, anti-brain membrane Fab′ significantly inhibited vesicle binding to glial cells compared with the control level of binding in the presence of anti-(N-CAM) (Table I). Significantly less binding occurred when vesicles were incubated with an equivalent number of fibroblasts or liver cells (Table I). The data indicated that comparable results could be obtained with either the radioactive or the fluorescence method. Because radioactive tracing allowed rapid measurement of as many as 40–50 samples at the same time and was less subjective in its scoring, it was chosen for routine use.

For the development of this assay, procedures were established for isolation of both neuronal membrane vesicles and glial cells from 14-d chick embryo brains because at this time in chick neural development the glia are proliferating and interacting with neurons. Glial cells that were isolated from 10-d embryo brains showed similar binding characteristics, although fewer cells could be isolated from an equivalent mass of 10-d brain tissue. Vesicles that were isolated from 10-d embryo brains bound to glial cells, but the level of binding was 30–40% less than with 14-d vesicles. Both cells and vesicles were therefore routinely prepared from 14-d chick embryo brains.

Characteristics of Vesicle-to-Cell Binding

The extent of vesicle-to-cell binding was measured over a wide range of vesicle and cell concentrations (Fig. 4). Increasing both the number of vesicles (up to 200 μl) and the number of cells (up to approximately 7.5×10^6) in the 1.2-ml assay volume resulted in linear increases in binding (Fig. 4, *a* and *b*). At higher concentrations of either cells or vesicles, saturation of binding began. To simplify quantitative interpretation of the assay, vesicle and cell concentrations were subsequently maintained in the linear portions of the binding curves.

At a constant level of cells and vesicles, the rate of binding and the effect of temperature on binding were tested. In routine assays, binding was measured after a 30-min incubation period at 37°C following a preincubation of the vesicles with antibodies at 4°C. The extent of binding after incubation at 37°C for different intervals of time is shown in Fig. 4*c*. A significant level of binding was measured after 10 min and the level of binding increased with longer incubation periods. The level of vesicle binding to glial cells in samples that had been maintained on ice for 30 min rather than at 37°C was also measured (Fig. 4*c*, zero time point). In several experiments, this value ranged between 15–35% of the control level of binding and probably reflects the magnitude of nonspecific vesicle binding in the assay. Vesicles that were incubated without glial cells for 30 min at 37°C had a small amount of radioactivity in the final pellet (open circle, Fig. 4*c*) which ranged between 10–20% of the control level of binding to glial cells.

Previous observations (6) indicated that the binding of neuronal cells to glial cells is not dependent on the presence of calcium. In accordance with these results, the calcium-chelator EDTA (up to 3 mM) had no effect on vesicle-to-cell binding. Moreover, it was found that the metabolic inhibitor sodium azide had no significant effect on binding (<5% inhibition of control) at final concentrations ranging from 0.1 to 3.0 mM.

Specific Antibodies Inhibit Binding and Membrane Antigens Neutralize This Inhibition

Fab′ fragments of Igs against brain membranes block adhesion of neuronal cells to glial cells (6) and, as the present studies show, they also inhibit binding of neuronal membrane vesicles to glial cells (Fig. 3 and Table I). Quantitative measurements of this inhibition are shown in Fig. 5*a*. The inhibition increased with the antibody concentration and eventually reached a plateau level that in different experiments varied between 65–85% of the control level. Inhibition of >50% of the specific binding was found with 0.5 mg of Fab′ fragments that were prepared from each of eight rabbits that had been immunized with membranes from 10- or 14-d embryonic chick brains. The portion of the binding that was not inhibited by saturating amounts of Fab′ (15–35%) was similar in magnitude to the fraction of the binding that was nonspecific (compare Figs. 4*c* and 5*a*). This binding was attributed to

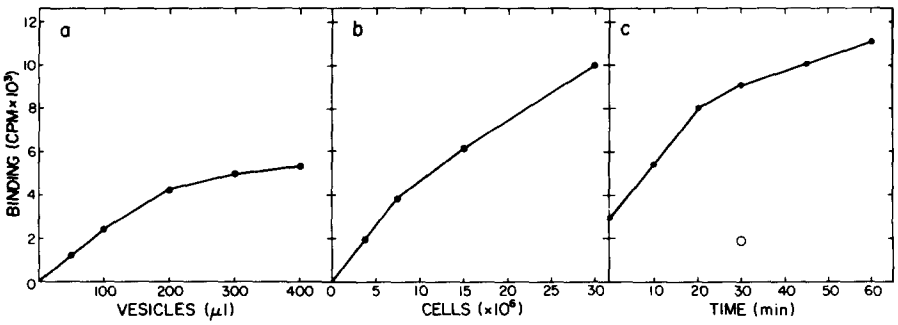

FIGURE 4 Properties of the binding of ^{125}I-neuronal membrane vesicles from 14-d chick embryos to glial cells in suspension. The level of binding was measured as a function of (*a*) the volume of radiolabeled vesicles (10% suspension) added to 5×10^6 glial cells and (*b*) the number of cells added to 0.1 ml of vesicles. (c) Binding of vesicles (0.1 ml) to 5×10^6 glial cells (●) was measured for various periods of incubation. In the absence of glial cells, the level of radioactivity found in the final pellet was also determined (○). Duplicate samples were measured as described in Materials and Methods; mean values of binding are shown in the figure and the mean deviation was <10%.

nonspecific effects such as binding to dead cells or glass, cosedimentation of vesicles that were nonspecifically bound to cells, background radiation, and nonspecific transfer of noncovalently bound ^{125}I from Fab′ to cells. The level of binding to fibroblasts and liver cells (Table I) is approximately one-fourth the level of that to glial cells, and probably represents nonspecific binding of a similar kind.

After demonstration that specific antibodies inhibited binding of neuronal membranes to glial cells, it was possible to search for molecules containing antigenic determinants involved in the binding. The detection of such molecules depends on their ability to bind to Fab′ fragments prepared from anti-brain membrane antisera, thereby neutralizing the inhibitory activity of the Fab′ and permitting vesicle to cell binding to occur. To test initially for the presence of neutralizing material, anti-brain membrane Fab′ fragments were incubated with 14-d embryonic chick brain membranes and the mixture was centrifuged to remove the membranes; the supernatant fraction was then tested for its ability to inhibit vesicle-to-cell binding in the presence of control antibody (0.5 mg/ml anti-(N-CAM) Fab′ fragments). All of the inhibitory activity of the antibodies was depleted by incubation with the membranes. Neuronal cells in suspension effectively removed the inhibitor activity of anti-brain membrane Fab′ fragments, but glial cells in suspension were ineffective. These results suggest that one or more antigens specifically involved in neuronal cell-to-glial cell adhesion are present on neuronal and not glial cell surfaces.

The antigens were solubilized by extraction of 14-d chick brain membranes with the nonionic detergent Nonidet P-40. The detergent was removed and the fraction that remained soluble after high speed (100,000 *g*) centrifugation neutralized the inhibitory activity of anti-brain membrane Fab′ fragments in a concentration-dependent manner (Fig. 5*b*). The extract was capable of completely neutralizing the inhibition by anti-brain membrane Fab′ indicating that practically all the inhibitory antibodies were neutralized by antigenic determinants in the extract.

An important feature of the assay necessary for quantitative measurements of vesicle-to-cell binding was inhibition of vesicle self-aggregation by anti-(N-CAM) Fab′. The presence of N-CAM in the extract (20) required additional anti-(N-CAM) Fab′ to compensate for neutralization by N-CAM. Moreover, the neutralizing activity of the extract was unchanged after N-CAM antigenic determinants were removed by treatment with anti-(N-CAM) monoclonal antibody coupled to Sepharose CL-2B (20). This confirms the suggestion (6) that the CAMs on neuronal membranes mediating vesicle binding to glial cells are different from N-CAM.

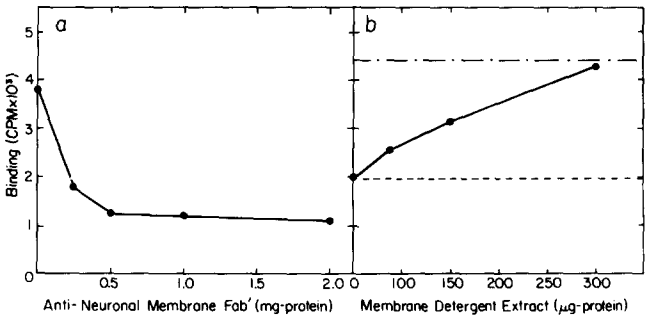

FIGURE 5 Inhibition of vesicle binding to glial cells by anti-brain membrane Fab′ fragments and neutralization of this inhibition with detergent extracts of membranes. (*a*) ^{125}I-neuronal membrane vesicles (0.1 ml) were preincubated for 15 min on ice in 0.4 ml PBS containing a mixture of Fab′ fragments from anti-(N-CAM) Ig and anti-brain membrane Ig in different ratios so that the anti-(N-CAM) titer was maintained constant while the anti-brain membrane Fab′ was increased in the 1.2-ml assay volume. The titer of anti-(N-CAM) from each antiserum was measured in an assay involving aggregation of retinal neural cells as described previously (26). In contrast to the concentration-dependent inhibition of binding found with anti-brain membrane Fab′, anti-(N-CAM) Fab′ at concentrations as high as 2 mg/ml did not affect the control level of binding or the inhibition by anti-brain membrane Fab′. (*b*) A mixture of anti-brain membrane Fab′ fragments (0.4 mg) and anti-(N-CAM) Fab′ fragments (1 mg) was preincubated with different amounts of membrane detergent extract in 0.4 ml PBS for 15 min on ice and binding of ^{125}I-neuronal membrane vesicles (0.1 ml) to 5×10^6 glial cells was measured. The mean values of binding of duplicate samples are shown, and the mean deviation was <10%. The control level of binding (- - -) and the level of binding with 0.4 mg anti-brain membrane Fab′ fragments (---) are shown in the figure.

Fractionation of Neutralizing Activity and Isolation of a Monoclonal Antibody That Inhibits Adhesion

To purify cell surface molecules involved in cell adhesion they must be solubilized. As a first step for the purification and characterization of the neutralizing activity, deoxycholate extracts of membranes were fractionated on Sephacryl S-300 (Fig. 6). Comparison with the profile of absorbance at 280 nm indicated that the neutralizing activity was enriched in the higher molecular weight fractions ($M_r \cong 150{,}000$).

Attempts to further purify the neutralizing activity from detergent extracts of membranes were unsuccessful. We therefore attempted to release a soluble form of the neutralizing activity from neuronal membrane vesicles with proteolytic enzymes. Most of the neutralizing activity on neuronal mem-

brane vesicles was depleted from the membranes after treatment with trypsin and was recovered in a high speed supernatant (100,000 *g*) fraction. The trypsin-released material remained soluble in the absence of detergent, and survived several purification steps (Table II). The trypsin-released extract also contained N-CAM antigenic determinants which were detected with a neutralization assay for N-CAM (26); the extract was therefore passed over a column of anti-(N-CAM) monoclonal antibody coupled to Sepharose CL-2B to remove most of these determinants (20). Nucleic acids and some protein were then removed by passing the extract over a column of DEAE-cellulose in 0.3 M KCl. The unretarded fraction, containing most of the neutralizing activity (Table II), was then filtered on a column of Sephacryl S-300 (Fig. 7). High molecular weight fractions ($M_r \cong 100{,}000$) from the column neutralized inhibition by anti-brain membrane Fab' of binding of neuronal vesicles to glial cells (Ng-CAM neutralizing activity). Residual N-CAM neutralizing activity (26) eluted in the lower molecular weight fractions, and was separated from the peak of Ng-CAM neutralizing activity. The

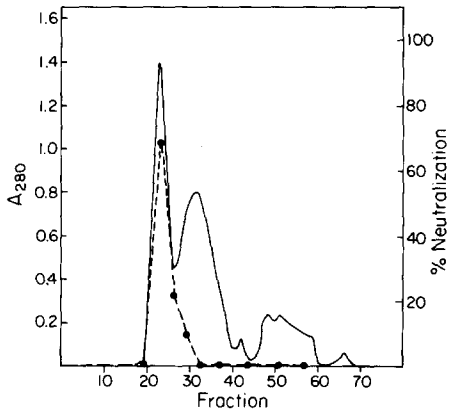

FIGURE 6 Fractionation of a detergent extract of membranes by gel filtration and Ng-CAM neutralizing activity of fractions. A soluble extract of membranes (5 ml) was applied to a Sephacryl S-300 column and the fractions that were eluted were assayed for absorbance at 280 mm (——). Fractions (0.5 ml) were dialyzed and were tested for neutralization activity (●---●) as described in the legend to Fig. 5*b*.

TABLE II

Purification of Ng-CAM Neutralizing Activity From 14-d Embryonic Chick Brain Membranes

Fraction	Protein	Activity units*	Specific activity	Yield	Purification
	mg		*U/mg*	*%*	*-fold*
Trypsin-released extract	55	2,200	40	100	1
Anti-(N-CAM)-Sepharose unbound	53	2,120	40	96	1
DEAE-Cellulose unbound	40	2,000	50	91	1.25
Sephacryl S-300 (fractions 20–25)	4.8	1,440	300	65	7.5
Lentil lectin-Sepharose eluate	0.5	1,000	2000	45	50

* One unit of Ng-CAM neutralizing activity is defined as the amount required to reduce by 50% the inhibition of binding of neuronal membrane vesicles to glial cells in the standard assay (see Materials and Methods).

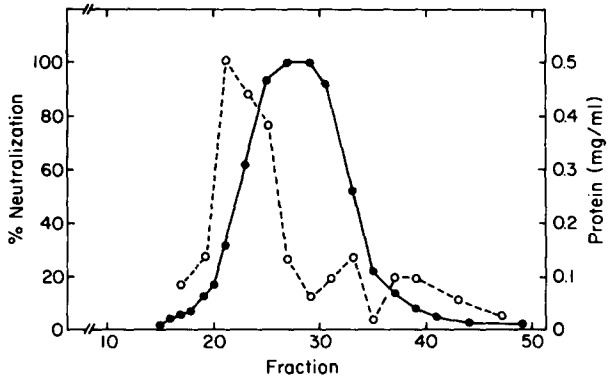

FIGURE 7 Fractionation of a trypsin-released extract of membranes and determination of Ng-CAM activity of fractions. The fraction that passed through a DEAE-cellulose column (Table II) was concentrated and 2.6 ml was applied to a Sephacryl S-300 column that was equilibrated and eluted in PBS. Fractions (50 μl) were tested for protein (19) (●——●) and for Ng-CAM neutralizing activity in the presence of 1 mg/ml anti-(N-CAM) Fab' fragments (O---O).

neutralizing activity from the Sephacryl S-300 fractions was purified further by affinity chromatography on lentil lectin-Sepharose CL-4B, (Table II). This material was enriched approximately 50-fold in neutralizing activity, but it still contained many polypeptides as estimated by SDS PAGE (data not shown). To obviate this difficulty, this material was injected into mice to produce monoclonal antibodies which were then used to specifically identify the neutralizing molecules.

Monoclonal antibodies that bound to the partially purified trypsin-released material were tested for their ability to inhibit binding of neuronal membrane vesicles to glial cells. One clone, 10F6, was found to secrete an antibody that inhibited binding of neuronal membrane vesicles to glial cells to the same extent as polyclonal anti-brain membrane Fab' (Table III). Detergent extracts of embryonic chick brain membranes were immunoblotted using monoclonal antibody 10F6; a polypeptide of $M_r = 135{,}000$ was specifically recognized (Fig. 8) and relatively lower levels of reactivity were detected at $M_r = 200{,}000$ (12). In addition to these bands, SDS PAGE of immunoprecipitates from detergent extracts of membranes using this antibody showed small amounts of a diffuse band of $M_r = 80{,}000$ (12). Monoclonal antibody 10F6 did not recognize N-CAM in immunoblots of both detergent extracts of membranes (Fig. 8) and purified N-CAM (12). Furthermore, a monoclonal antibody to N-CAM that inhibits the aggregation of vesicles reconstituted with N-CAM (25) did not inhibit binding (Table III). These results suggest that the antigens specifically recognized by monoclonal antibody 10F6 are involved in neuron to glial cell adhesion and that these antigens are immunologically distinguishable from N-CAM.

DISCUSSION

The results presented here indicate that Ng-CAM is present on neuronal cells of the chick embryo and that it is responsible for the interaction of neuronal cells with glial cells. This molecule is distinct from the previously described N-CAM. Moreover, since Ng-CAM is not detected on glial cells, neuron-glial interaction, unlike neuron-neuron and neuron-myotube adhesion, appears to be between different molecules, one of which is Ng-CAM and the other remains to be identified.

TABLE III

Binding of Brain Membrane Vesicles to Glial Cells

Rabbit Fab'	Monoclonal antibody*	Binding†	Inhibition
		cpm	*%*
anti-(N-CAM)	—	5,064 ± 307	0
anti-brain membrane	—	2,105 ± 171	58
anti-(N-CAM)	Anti-(N-CAM) No. 1	5,004 ± 271	1
anti-(N-CAM)	Antibody 10F6	2,157 ± 194	57

* 20 μg of ammonium sulfate precipitate of ascites fluid was used. Anti-(N-CAM) No. 1 was obtained as described (20).

† Binding was measured as described in Table I.

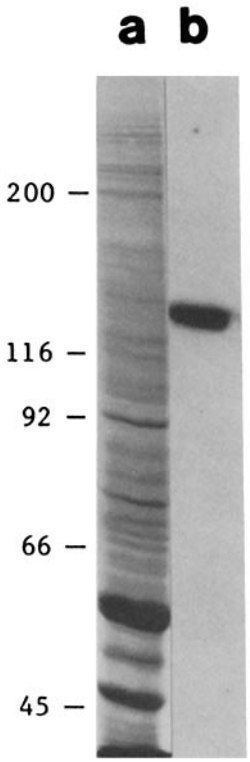

FIGURE 8 Immunoblot of chick brain membrane extract with monoclonal antibody 10F6. Embryonic chicken (14 d) brain membranes (100 μg of protein) were boiled in SDS sample buffer, fractionated by gel electrophoresis, and either stained with Coomassie Blue (lane *a*) or transferred to nitrocellulose and processed for immunoblotting. An autoradiograph of the blot using monoclonal antibody 10F6 is shown in lane *b*. Molecular weight standards were from Bio-Rad Laboratories and their migration positions in the gel are indicated ($M_r \times 10^{-3}$).

In examining cell-cell adhesions, both the cellular mode of binding, e.g., homotypic vs. heterotypic, and the molecular nature of the binding that mediates the adhesion, e.g., homophilic vs. heterophilic, must be distinguished. These mechanisms are schematically represented in Fig. 9, and for simplicity they are considered only in terms of binding between two partners. Homotypic adhesion (cell type A to cell type A) can occur in two different molecular modes; either one CAM binds to itself (Fig. 9, panel *I*) or one CAM binds to a complementary and different CAM (Fig. 9, panel *II*). Previous studies on the molecular bases of homotypic cell-cell adhesion employed cell aggregation assays (4, 27, 28). These assays were based on the inhibition of the aggregation of a particular cell type by monovalent antibody fragments raised against cell surface determinants and on the reversal of this inhibition by neutralization of the antibody with cell surface molecules. The use of such neutralization assays facilitated the identification and purification of molecules from the cell surface of vertebrate neuronal cells (20, 26, 29), liver cells (15, 30), and embryonal carcinoma cells (31, 32); the involvement of these molecules in homotypic cell-cell adhesion has been amply demonstrated in vitro (reviewed in reference 4).

Heterotypic adhesion (cell type A to cell type B) can occur in the same two molecular modes (Fig. 9, panels *III* and *IV*). The adhesion between neurons and myotubes, for example, appears to be mediated by a homophilic mechanism in which N-CAM on the neuron binds to N-CAM on the myotube (13, 16, 24), whereas the interaction between neurons and glial cells described here appears to be heterophilic. However, use of the neutralization approach with specific heterotypic adhesion presented several problems.

The major problem posed by heterotypic adhesion is that each of two different cell types can potentially bind homotypically, i.e., to itself, as well as to the other cell type. This required that a number of conditions be met in order to detect the molecules responsible for the heterotypic interaction. First, it was necessary to unequivocally identify cells or membrane vesicles derived from particular tissue types. In this study, the identity of the different cell types was established by a combination of behavior in culture, morphology, and surface determinants. A major (but not the only) criterion was the difference between the two chicken cell types in their ability to bind two different specific monoclonal antibodies, the first against N-CAM (for neurons) and the other against an as yet unidentified glial surface antigen (reference 6 and J.-P. Thiery, M. Grumet, and G. M. Edelman, unpublished observations).

It was also necessary to develop a quantitative system for evaluating the adhesiveness between vesicles and cells, each of different origin. Previous studies (13, 16, 24, 25, 33, 34) support the idea that the adhesive specificity is maintained when vesicles are substituted for cells in adhesion assays. The large difference in size between the vesicles and the cells allowed distinct recognition of the specific adhesion between neuronal and glial membranes. In the present assay, the interaction was followed quantitatively by using doubly labeled neuronal vesicles. Microscopic observation indicated that the fluorescent vesicles only bound to the surface of ~50% of the glial cells. This result may indicate heterogeneity in the glial cell population and more detailed cell identification and fractionation are necessary to resolve this issue. ^{125}I-Fab' fragments from anti-(N-CAM) antibodies were particularly useful as a label for vesicles because the abundance of N-CAM on the surface of neuronal membrane vesicles allows sufficient radioactive labeling. In addition, the antibody

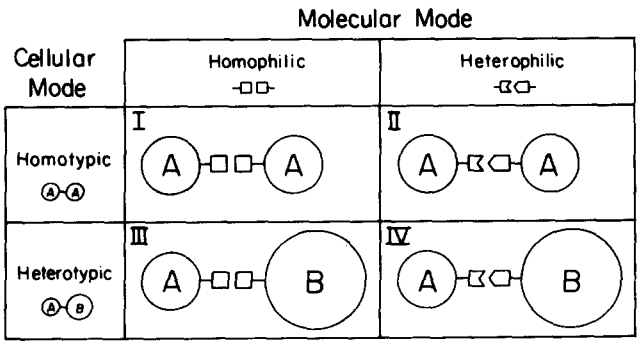

FIGURE 9 Schematic representation of different modes of cell-cell adhesion. The circles represent cells; similar cells are represented by circles (panels *I* and *II*) of the same size, different cells are represented by circles of different sizes (panels *III* and *IV*). Cell adhesion molecules are represented by the other geometric shapes; similar molecules are represented but squares (panels *I* and *II*) and complementary molecules by the five-sided figures (panels *II* and *IV*).

blocks the bulk of homotypic vesicle aggregation (a necessary condition for the quantitation of heterotypic binding), and acts as a convenient marker for neuronal membranes in this system. It should be stressed that this unique label for neuronal vesicles does not limit the generality of the assay inasmuch as both native vesicles from a variety of cell types and reconstituted vesicles can be internally labeled with ^{125}I-BSA and fluorescent dyes. Furthermore, neuronal membrane vesicles have been directly labeled with ^{125}I by the lactoperoxidase method (described in detail in reference 16) and specific binding of such ^{125}I–neuronal membrane vesicles to glial cells has been measured (unpublished observations). Nevertheless, the use of a specific and abundant surface label such as N-CAM is to be preferred when available.

With these conditions met, the immunological approach to cell-cell adhesion previously developed for homotypic adhesion (4) could be applied in a compound assay. Under the conditions used, the adhesion of neuronal membranes was specific for glia. Because only the vesicles were labeled, their adhesion to cells could be unequivocally determined after the cells were separated from the unbound vesicles by differential centrifugation. Fab′ fragments prepared from antisera raised against neuronal membranes inhibited the binding of neuronal membrane vesicles to glial cells. The finding that extracts of brain membranes neutralized the inhibition by these Fab′ fragments completed the requirements necessary for a quantitative assay to measure neutralizing activity during purification.

Use of this assay allowed isolation of a monoclonal antibody that strongly inhibited the binding of neuronal vesicles to glial cells. The CAM (Ng-CAM) that was specifically recognized by the antibody is different both chemically and functionally from N-CAM. Whereas embryonic N-CAM migrates on SDS PAGE in a broad region, M_r = 200,000–250,000 (20), the major component of Ng-CAM migrated as a discrete band at M_r = 135,000. Furthermore, after gel filtration of a deoxycholate extract of membranes, a peak of Ng-CAM neutralizing activity was detected at $M_r \cong$ 150,000, implying that Ng-CAM is not aggregated in this detergent. Cell adhesion assays indicated that monoclonal antibodies to Ng-CAM (10F6) and N-CAM (anti-(N-CAM) No. 1) specifically inhibit neuron-glia adhesion and neuron-neuron adhesion, respectively (Table III and reference 25). Other monoclonal antibodies to neuronal antigens, however, have recently been obtained that recognize both Ng-CAM and N-CAM; this cross-reactivity is considered in detail elsewhere (12).

Several results suggested that the adhesion between neurons and glial cells is heterophilic. The finding that neurons, but not glia, could deplete the inhibiting antibodies suggested that Ng-CAM is present on neurons but not on glia. In indirect immunofluorescence experiments, monoclonal antibody 10F6 has been found to stain neurons but not glial cells (12); these results further indicated that the adhesion between neuronal cells and glial cells is heterophilic. The behavior of Ng-CAM neutralizing activity on gel filtration columns suggested that it was not self-aggregated, consistent with the idea that Ng-CAM binds to a complementary heterophilic CAM on glial cells and not to itself (Fig. 9, panel *IV*). With an appropriate anti–glial cell antibody, the present assay can be applied to test for CAMs present on glial cells that are involved in binding to Ng-CAM on neurons.

As with any in vitro phenomenon, the direct relevance of heterotypic vesicle adhesion to physiological mechanisms in vivo must be established by separate criteria. The new assay developed in the present studies was used mainly as a tool to help identify Ng-CAM by means of a specific monoclonal antibody. It is now possible to test additional effects of specific antibodies to Ng-CAM on adhesion of neurons to glial cells (6) as well as on more complex in vitro and in vivo systems. We are also in a position to search for protein components in addition to the major neuronal protein of M_r = 135,000 that may take part in making up the heterophilic system; until all such components are identified and the role of the molecules of M_r = 200,000 and 80,000 is fully determined (12), the characterization of Ng-CAM must be considered incomplete. Further pursuit of such studies may reveal the role of both Ng-CAM and its putative ligand on glial cells in forming connections between migrating neurons and glial fibers during development, and in the organization and the maintenance of mature neural tissues.

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