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Two antigenically related neuronal cell adhesion molecules of different specificities mediate neuron–neuron and neuron–glia adhesion

(cell–cell adhesion/neuron–glia interaction/neural antigens/neural development/glial cells)

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ABSTRACT **Previous studies in this laboratory have led to the identification of the neural cell adhesion molecule, N-CAM, a homophilic ligand that mediates adhesion between neurons as well as between neurons and striated muscle precursors. By means of a similar immunological approach but with different assays, we have now identified a cell adhesion molecule on neurons (Ng-CAM) that mediates the heterotypic adhesion between neuronal membranes and glial cells. In this paper, we compare certain aspects of the structure and function of Ng-CAM and embryonic N-CAM from the chicken. Ng-CAM was localized by specific antibodies on neurons but not on glia, and double-staining methods showed that individual neurons contained both Ng-CAM and N-CAM. Embryonic Ng-CAM migrates primarily as a single component of M_r 135,000; its apparent M_r shifted to 127,000 after neuraminidase treatment. In contrast, the embryonic form of N-CAM migrates on NaDodSO$_4$/polyacrylamide gels in the apparent M_r range of 200,000–250,000; after neuraminidase treatment, N-CAM migrates as two components of M_r 170,000 and M_r 140,000. Although both Ng-CAM and N-CAM have calcium-independent binding mechanisms, immunologically based cell adhesion assays suggested that they have different specificities in mediating cell adhesion. Whereas 0.25 μg of Ng-CAM partially neutralized the ability of 0.5 mg of polyspecific antineural Fab′ fragments to inhibit the heterotypic binding of neuronal membrane vesicles to glial cells and larger amounts of Ng-CAM completely neutralized this inhibition, 20 μg of N-CAM had no neutralization activity in this assay. Reciprocally, 0.25 μg of N-CAM partially neutralized the ability of 0.5 mg of the same Fab′ fragments to inhibit the direct homotypic aggregation of neuronal cells, but 20 μg of Ng-CAM had no detectable activity. Although peptide maps of the two cell adhesion molecules differed considerably and despite the differences in binding specificity of these molecules, two independently derived monoclonal antibodies were found to crossreact with both Ng-CAM and N-CAM. Therefore, these different neuronal cell adhesion molecules with distinct binding specificities share at least one antigenic determinant, raising the possibility that they arose from a common evolutionary precursor.**

Cell–cell adhesion is a key primary process in pattern formation during embryological development. The molecular bases of cell adhesion are difficult to study *in vivo*, but by means of appropriate quantitative *in vitro* assays specific cell adhesion molecules (CAMs) have been identified and purified (1). In the earliest epoch of embryological development, N-CAM, the neural cell adhesion molecule (2), is expressed on cells that develop into both neuronal and nonneuronal tissues (3, 4). L-CAM, the liver cell adhesion molecule, is also present on many cells in the earliest stages of development (4), including many which give rise to tissues other than the liver. The temporal and spatial distribution of L-CAM and N-CAM (4) suggest that they are fundamental molecules in the control of development and that they may play a specific role in embryonic induction.

Anatomical and cell biological studies (5, 6) have suggested that the development of neural tissues relies on at least two major types of cellular interactions: those occurring between neurons and those occurring between neurons and glia. Adhesion between neuronal cells can largely be attributed to N-CAM binding, which is second-order homophilic (i.e., N-CAM to N-CAM) (7, 8); the kinetics (8) and efficacy of this binding are modulated during development by changes (1, 9, 10) in the sialic acid content of N-CAM molecules [E-to-A conversion (1)] and by changes in surface density of the molecules at the cell membrane (3, 4, 8). It is an attractive hypothesis that to ensure specificity of adhesion for each cell type, the heterotypic binding of neuronal cells to glial cells is mediated by a heterophilic mechanism; this would be in accord with observations that neuron–glia interactions and neuron–neuron interactions have different morphological and physiological significance (5, 6).

To identify cell surface molecules that are involved in the heterotypic adhesion between neuronal and glial cells, a new assay was used in the present study. This assay, like that for N-CAM, was based on neutralization (2, 11) of the activity of antibodies that inhibited adhesion. Heterotypic neural–glial interactions were quantitated by measuring the binding of neuronal membrane vesicles to glial cells. Monoclonal antibodies were then obtained that inhibited adhesion in this assay and were shown specifically to recognize a neuronal protein called Ng-CAM, the neuronal–glial cell adhesion molecule.*

The existence of two binding specificities on neurons prompted us to compare the structure and function of Ng-CAM and N-CAM. Although Ng-CAM and N-CAM can be distinguished immunologically with specific monoclonal antibodies and have different specificities in the appropriate discriminatory cell adhesion assays, we found that they are both recognized by certain individual monoclonal antibodies and thus have common antigenic determinants. Moreover, we have shown that single neurons contain both Ng-CAM and N-CAM and apparently are capable of simultaneously exhibiting both binding specificities.

MATERIALS AND METHODS

Preparation of N-CAM and Anti-(N-CAM). Purified N-CAM was obtained as described (2). Monoclonal antibodies

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Abbreviations: CAM, cell adhesion molecule; N-CAM, neural cell adhesion molecule; Ng-CAM, neuronal–glial cell adhesion molecule; L-CAM, liver cell adhesion molecule; NP-40, Nonidet P-40.

*In naming Ng-CAM, we have adopted the convention that the uppercase letter refers to the cell on which the molecule appears; the lowercase letter refers to the other member of the heterotypic pair.

anti-(N-CAM) nos. 4 and 5 were prepared by immunization of mice with chicken embryo brain membranes; hybridomas were selected by the ability of their culture supernatants to recognize purified N-CAM in a radioimmune assay (2). Monoclonal antibodies were prepared by precipitation with 45% ammonium sulfate from ascites fluid of mice that were injected with hybridoma cells that had been cloned at least three times. Monoclonal antibodies anti-(N-CAM) nos. 1, 2, and 4 reacted with antisera specific for IgG1 and anti-(N-CAM) no. 5 reacted with antisera specific for IgM. The IgG fraction and monovalent Fab' fragments were prepared (11) from rabbit anti-(N-CAM) antisera (7).

Preparation of Ng-CAM and Anti-(Ng-CAM). Monoclonal antibodies that specifically recognized Ng-CAM were obtained after immunization of mice with partially purified material prepared as follows. Neuronal membranes [25 mg of protein per ml in phosphate-buffered saline (Pi/NaCl buffer) containing (per liter) 8 g of NaCl, 0.2 g of KCl, 0.2 g of KH_2PO_4, and 1.15 g of Na_2HPO_4 (pH 7.4)] were trypsinized (0.1 mg/ml, 75 min, 37°C), the trypsin was inactivated by addition of soybean trypsin inhibitor, and the supernatant fraction (100,000 × *g*, 1 hr) was passed over an anti-(N-CAM) no. 1 affinity column to remove N-CAM fragments. The unbound fraction was dialyzed into 10 mM Hepes/0.3 M KCl, pH 7.4, and passed through a column of DEAE-cellulose. The unretarded fraction was dialyzed into 10 mM Hepes/0.5 M KCl/0.2 mM $CaCl_2$/0.02 mM $MnCl_2$, pH 7.4, and incubated with lentil lectin-Sepharose 4B. This bound fraction was eluted with 0.2 M α-methyl-D-glucoside in the same buffer, dialyzed, concentrated by lyophilization, and fractionated on a column of Sephacryl S-300 in P_i/NaCl buffer; the major included peak of protein had been enriched >50-fold in neutralization activity (see below).

The culture supernatants from two of the clones, 10F6 and 16F5, inhibited binding of neuronal membrane vesicles to glial cells; their antibodies reacted with antisera specific for IgG1. Purified Ng-CAM was obtained from Nonidet P-40 (NP-40) extracts of 14-day chicken embryo brain membranes (2) by affinity chromatography using monoclonal antibody 10F6 or 16F5 coupled to Sepharose CL-2B by the CNBr method (12); Ng-CAM was eluted and the detergent was removed as described for N-CAM (2).

Adhesion Assays. Antisera to 14-day chicken embryo brain membranes [here designated as anti-(brain membrane) sera] were produced in rabbits (13). The amount of N-CAM activity in a sample was quantitated by its ability to neutralize the ability of anti-(brain membrane) Fab' fragments to inhibit neuronal aggregation as described (11), except that the particle counting was performed with a Coulter Counter.

In the heterotypic neuronal–glial cell adhesion assay, the amount of Ng-CAM activity in a sample was quantitated by its ability to neutralize the inhibition by anti-(brain membrane) Fab' fragments of the binding of neuronal membrane vesicles to glial cells. Neuronal membrane vesicles were labeled by incubation with ^{125}I-labeled Fab' fragments of antibodies to N-CAM (^{125}I-vesicles), fractionated on discontinuous sucrose gradients, and then washed three times with P_i/NaCl buffer. Glial cells (13) from 14-day chicken embryo brains were isolated free of neuronal cells and grown to confluence in monolayers. Cells were obtained in suspension by enzymatic treatment and incubated in medium (13) for 16 hr. Aliquots of 5×10^6 cells were tested for binding of ^{125}I-vesicles (0.1 ml of a 10% suspension) during a 30-min incubation period at 37°C in 1 ml of Eagle's minimal essential medium with spinner salts; an excess of Fab' fragments from antibodies to N-CAM was present to block all N-CAM-to-N-CAM interaction. ^{125}I-vesicle binding to glial cells was measured by scintillation spectroscopy after separation of cells from unbound vesicles by differential centrifugation (7). The protein being tested for neutralization activity was incubated with anti-(brain membrane) Fab' fragments for 15 min at 4°C; this mixture was then incubated with the ^{125}I-vesicles for 15 min at 4°C, and finally the cells were added.

Immunoblotting. Proteins were resolved by $NaDodSO_4$/polyacrylamide (7.5%) gel electrophoresis, transferred to nitrocellulose paper, reacted sequentially with monoclonal antibodies (50 μg), rabbit anti-mouse immunoglobulin (50 μg) and ^{125}I-labeled protein A (1×10^6 cpm), and detected by autoradiography (14). Although immobilized anti-(N-CAM) no. 1 could be used to purify N-CAM, it did not immunoblot N-CAM effectively and therefore anti-(N-CAM) no. 2 was used for immunoblotting. Similarly, anti-(N-CAM) no. 4 was effective in immunoaffinity purification but was ineffective in immunoblotting. On the other hand, anti-(N-CAM) no. 5 immunoblotted well but was ineffective in immunoaffinity purification.

Analytical Procedures. For peptide maps, N-CAM and Ng-CAM bands from gels were treated with *Staphylococcus aureus* V8 protease and the digestion products were resolved on $NaDodSO_4$/polyacrylamide gels (15, 16). Migration of standard proteins (myosin, M_r = 200,000; phosphorylase b, M_r = 94,000; bovine serum albumin, M_r = 68,000; ovalbumin, M_r = 43,000; α-chymotrypsinogen, M_r = 26,000; β-lactoglobulin, M_r = 18,000; and lysozyme, M_r = 14,000) is indicated in the figures next to their molecular weights × 10^{-3}. Neuraminidase treatment was performed while proteins were bound to an immunoaffinity support (17). Protein was measured by the method of Lowry *et al.* (18).

Immunofluorescence Microscopy. Cultures containing both neuronal and glial cells were prepared from 14-day chicken embryo brains (13) and were sequentially treated for 10 min with P_i/NaCl buffer containing first 3.7% formaldehyde and then 0.1 M glycine. The cultures were next incubated with specific antibodies to N-CAM (rabbit polyclonal) and Ng-CAM (monoclonal) for 1 hr in P_i/NaCl buffer containing 4% goat serum and 4% calf serum and washed four times with P_i/NaCl buffer. They were then treated for 30 min with a mixture of fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) and rhodamine-conjugated goat anti-mouse IgG (Miles) at 1:50 dilutions in P_i/NaCl buffer with 4% goat serum and 4% calf serum. After washing four times with P_i/NaCl buffer, the cultures were mounted (4) and examined with a Zeiss universal microscope equipped with a Nikon (UFX) camera.

RESULTS

Immunological Characterization of Ng-CAM. The heterotypic cell adhesion assay involving the binding of neuronal membrane vesicles to glial cells allowed the identification of a neuronal molecule (Ng-CAM) that was different from N-CAM and that mediated this calcium-independent binding. The majority of neuronal vesicle binding was inhibitable by anti-(brain membrane) Fab' fragments and the inhibition could be neutralized by a partially purified fraction containing Ng-CAM. After immunization of mice with this material, monoclonal antibody-producing hybridomas were obtained and selected by the ability of their antibodies specifically to inhibit adhesion of neuronal membrane vesicles to glial cells. Culture supernatants (0.3 ml) from hybridomas 10F6 and 16F5 inhibited vesicle binding by 78% and 65%, respectively, whereas 0.5 mg of anti-(brain membrane) Fab' fragments inhibited binding by 70%; culture supernatants from control monoclonal antibodies did not inhibit binding. Furthermore, when coupled to Sepharose CL-2B, both monoclonal antibodies 10F6 and 16F5 could deplete ≈90% of the neutralization activity from NP-40 extracts of chicken neuronal membranes.

Initial Structural Comparisons of Ng-CAM and N-CAM. To examine the polypeptide chains of Ng-CAM, $NaDodSO_4$ ex-

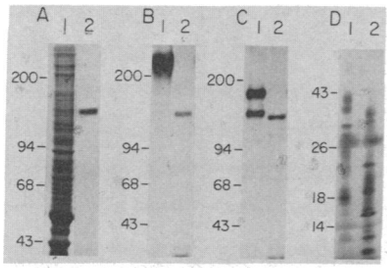

FIG. 1. Electrophoretic and structural comparisons of Ng-CAM and N-CAM. (*A*) Embryonic chicken brain membranes (100 μg of protein) were boiled in $NaDodSO_4$ sample buffer and fractionated by polyacrylamide (7.5%) gel electrophoresis and either stained with Coomassie blue (lane 1) or transferred to nitrocellulose and processed for immunoblotting. An autoradiograph of the blot using monoclonal antibody 10F6 is shown in lane 2. (*B*) N-CAM (lane 1) and Ng-CAM (lane 2) were affinity purified from NP-40 extracts of radioiodinated embryonic chicken brain membranes (2) by using anti-(N-CAM) no. 1 and 10F6 [anti-(Ng-CAM)], respectively. An aliquot of each sample was also neuraminidase-treated (*C*, lane 1, N-CAM; lane 2, Ng-CAM); the samples were then subjected to $NaDodSO_4$/gel electrophoresis and proteins were detected by autoradiography. (*D*) *S. aureus* V8 protease digests of Ng-CAM (lane 1) and N-CAM (lane 2). In this procedure, the M_r 140,000 component of neuraminidase-treated N-CAM (20 μg) and the M_r 135,000 component of Ng-CAM (20 μg) were resolved by $NaDodSO_4$/polyacrylamide gel electrophoresis, cut from the gel, and digested with 0.5 μg of *S. aureus* V8 protease (15); the digestion products were then fractionated by $NaDodSO_4$/polyacrylamide (15%) gel electrophoresis and detected by means of silver staining (19). The wide bands above the M_r 26,000 marker in both lanes represent the enzyme.

tracts of 14-day chicken embryonic brain were immunoblotted (14) by using monoclonal antibody 10F6. The major polypeptide had an apparent M_r of 135,000 (Fig. 1*A*, lane 2). To compare the molecular characteristics of Ng-CAM and N-CAM, the proteins were purified from detergent extracts of radioiodinated membranes by using monoclonal antibodies specific for each molecule. N-CAM (Fig. 1*B*, lane 1) migrated as a broad region of radioactive material from M_r = 200,000 to M_r = 250,000, the embryonic (E) form (2, 9, 10) of the molecule. In contrast, antibodies to Ng-CAM again recognized primarily a single band with a M_r of ≈135,000 (Fig. 1*B*, lane 2). In addition to the major component of M_r 135,000, Ng-CAM preparations also reproducibly contained components of M_r 200,000 and M_r 80,000 at low levels.

The heterogeneity of the electrophoretic migration of the E form of N-CAM has been attributed to the presence of varying amounts of sialic acid that can be enzymatically removed by treatment with neuraminidase (2, 17). A comparison of immunoprecipitates of N-CAM and Ng-CAM that were both treated with neuraminidase is shown in Fig. 1*C*. This treatment converts N-CAM to discrete bands on $NaDodSO_4$/polyacrylamide gel electrophoresis; Ng-CAM and N-CAM still showed different patterns and the M_r 140,000 component of neuraminidase-treated N-CAM differed in migration from the major M_r 127,000 component in similarly treated Ng-CAM. These results suggest that the two cell adhesion molecules that are both present on neurons have major differences in both their protein and carbohydrate structures.

Chemical differences between the two CAMs were further revealed by comparisons of the fragments produced after digestion of each protein with *S. aureus* V8 protease. After electrophoresis (Fig. 1*D*), the patterns of their peptide fragments differed considerably, confirming that Ng-CAM and N-CAM are dissimilar in structure. This finding does not preclude the possibility of structural similarities between certain fragments of Ng-CAM and N-CAM.

Ng-CAM and N-CAM Have Different Roles in Neuronal Adhesion. In previous studies (2), it was shown that purified N-CAM specifically neutralized the inhibition of neural cell adhesion by polyspecific antineural antisera. Experiments were done to compare the binding specificities of Ng-CAM and N-CAM. By using appropriate polyspecific anti-(brain membrane) Fab' fragments (13), 0.25 μg of N-CAM had detectable neutralization in the N-CAM assay testing neuron–neuron adhesion and 2.0 μg gave 90% neutralization. On the other hand, the inhibition of binding of ^{125}I-vesicles to glial cells by the same Fab' fragments was specifically neutralized by purified Ng-CAM protein (Ng-CAM assay); 0.25 μg of Ng-CAM had detectable neutralization activity and 4.0 μg completely neutralized the inhibition by antibodies (Table 1). Ng-CAM (20 μg) had no neutralization activity in the N-CAM assay and N-CAM (20 μg) had no effect in the Ng-CAM assay (Table 1).

A few preparations of Ng-CAM were found to have neutralizing activity in the N-CAM assay; they contained ≈5% by mass of the activity of purified N-CAM. However, careful analyses of these Ng-CAM preparations showed that they actually contained N-CAM, as indicated by immunoblotting with a specific anti-(N-CAM) monoclonal antibody (no. 2). After removal of N-CAM from these fractions by immunoaffinity chromatography, their activity in a N-CAM assay was completely depleted and N-CAM could not be detected by immunoblotting. This result suggests either that N-CAM was bound weakly to the anti-(Ng-CAM) immunoaffinity column or that a small amount of N-CAM was bound weakly to Ng-CAM itself. The possibility that these two molecules might interact on the same cell requires further exploration.

Two Different Monoclonal Antibodies Each Specifically Recognized Both Ng-CAM and N-CAM. The data presented above strongly suggest that Ng-CAM and N-CAM are different and largely unrelated molecules. Moreover, as shown in Fig. 1, monoclonal antibody 10F6 recognized only Ng-CAM and anti-(N-CAM) no. 1 recognized only N-CAM. Nevertheless, it was found that two anti-(N-CAM) monoclonal antibodies (nos. 4 and 5) that were originally selected by their ability to recognize N-CAM also recognized Ng-CAM. This was demonstrated for anti-(N-CAM) no. 4 by using this antibody to immunoaffinity purify proteins that simultaneously comigrated with both N-CAM and Ng-CAM on $NaDodSO_4$/polyacrylamide gel electrophoresis; as indicated by Coomassie blue staining, no other polypeptides were copurified (data not shown).

A comparison among Ng-CAM, N-CAM, and the proteins purified with anti-(N-CAM) no. 4 was made by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by immunoblot-

Table 1. Specificity of N-CAM and Ng-CAM in different neuronal cell adhesion assays

Added protein, μg	Neutralization, %			
	N-CAM assay		Ng-CAM assay	
	N-CAM	Ng-CAM	N-CAM	Ng-CAM
0.25	15	<5	<5	14
0.5	29	<5	<5	30
1.0	61	<5	<5	46
2.0	90	<5	<5	71
4.0	ND	ND	<5	100
20	90	<5	<5	100

In both the N-CAM assay and the Ng-CAM assay, adhesion was inhibited with 0.5 mg of polyspecific Fab' fragments derived from anti-(brain membrane) antisera. The Fab' fragments were preincubated with the indicated amounts of protein and the resultant neutralization of inhibition of adhesion was quantitated as described (11). Bovine serum albumin (2 mg) had no detectable activity in either assay. ND, not done.

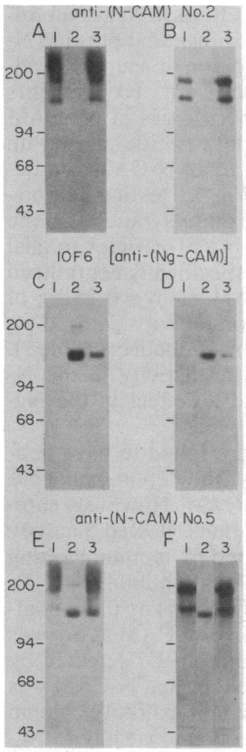

FIG. 2. Antigenic crossreactivity between N-CAM and Ng-CAM. N-CAM (lanes 1) and Ng-CAM (lanes 2) were purified from NP-40 extracts of embryonic chicken brain membranes by affinity chromatography by using anti-(N-CAM) no. 1 and anti-(Ng-CAM) (10F6), respectively. Material was similarly purified by using the crossreactive monoclonal antibody anti-(N-CAM) no. 4 and appeared to be a mixture of N-CAM and Ng-CAM (lanes 3). The purified proteins, either untreated (*A*, *C*, and *E*) or neuraminidase-treated (*B*, *D*, and *F*), were resolved by electrophoresis and immunoblotted with the indicated monoclonal antibodies against N-CAM (*A* and *B*), Ng-CAM (*C* and *D*), or a second crossreactive monoclonal antibody (*E* and *F*). Direct comparison of the immunoblots showed that the M_r 140,000 component of N-CAM differed in migration from the major component of Ng-CAM; (for example, see *E*, lanes 1 and 2). The material in *E* (lane 3) and *F* (lane 3) that migrates in this molecular weight range appears to include both Ng-CAM and the M_r 140,000 component of N-CAM.

ting of the gels by using monoclonal antibodies that distinguished between the two proteins. As shown in Fig. 2*A*, a monoclonal antibody specific for N-CAM recognized authentic N-CAM (lane 1) as well as material purified by anti-(N-CAM) no. 4 (see lane 3); Ng-CAM (lane 2) was not recognized. The same results were obtained after treatment with neuraminidase (Fig. 2*B*; compare corresponding lanes). When the gels were immunoblotted using specific anti-(Ng-CAM) (10F6), a component (Fig. 2*C*, lane 3) that was copurified with N-CAM by anti-(N-CAM) no. 4 and that corresponded to Ng-CAM (Fig. 2*C*, lane 2) was visualized. Comparisons after neuraminidase treatment gave the same results (Fig. 2*D*, lanes 2 and 3).

When the crossreacting anti-(N-CAM) monoclonal antibody (no. 5) was used to immunoblot the gels of native (Fig. 2*E*) and neuraminidase-treated (Fig. 2*F*) material, patterns consistent with these findings were obtained. Lanes marked 1 showed patterns for N-CAM, those marked 2 showed Ng-CAM, and those marked 3 showed a pattern revealing a mixture of both proteins. Thus, the proteins that were purified by anti-(N-CAM) no. 4 appeared to be a mixture of Ng-CAM and N-CAM and anti-(N-CAM) no. 5 immunoblotted components of both of these molecules. As an independent control and by using the same immunological criteria, L-CAM, another cell adhesion molecule that has a different binding specificity (20), did not share antigenicity with either Ng-CAM or N-CAM (data not shown).

Inasmuch as two separately derived monoclonal antibodies crossreacted with purified Ng-CAM and N-CAM fractions that were completely distinguishable by chemical, independent immunological, and functional criteria, we conclude that these two neuronal CAMs share at least one antigenic determinant. The common site recognized by the crossreactive antibody anti-(N-CAM) no. 4 is not a N-linked oligosaccharide for this antibody was found to react specifically with N-CAM synthesized in the presence of tunicamycin (data not shown).

Antibodies to Ng-CAM and N-CAM Simultaneously Bind to Single Neurons. Within the chicken brain, N-CAM is found specifically on neurons (1, 3, 4). To determine the cell types

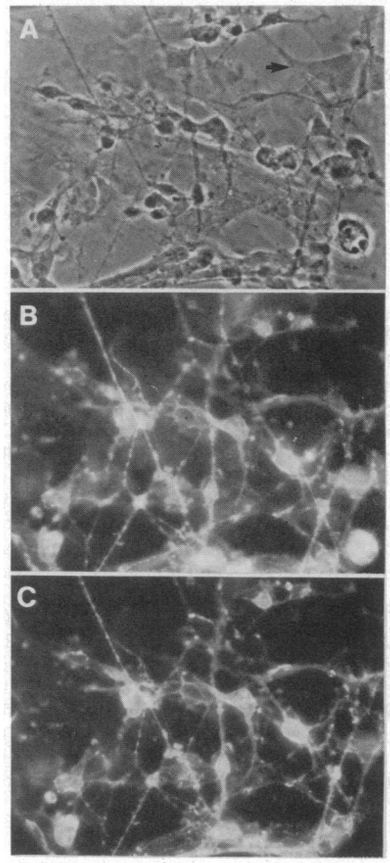

FIG. 3. Localization of both N-CAM and Ng-CAM on single neuronal cells. Cultures that contained neuronal and glial cells were indirectly stained simultaneously with 0.1 mg of rabbit anti-(N-CAM) IgG and monoclonal anti-(Ng-CAM) 16F5 (1:25 dilution of ascites fluid) per ml. Photographs were taken with Tri-X film (Kodak) under phase-contrast (*A*) and epifluorescence for fluorescein-labeled goat anti-rabbit IgG known to react with the anti-(N-CAM) (*B*) and rhodamine-labeled goat anti-mouse IgG known to react with the anti-(Ng-CAM) (*C*). (×270.) Treatment with preimmune sera (0.1 mg of IgG per ml) gave no neuronal or glial staining, but the flat nonneuronal cells (see arrow in *A* for an example) that were not recognized by anti-(N-CAM) or anti-(Ng-CAM) antibodies were found to be specifically stained (data not shown) with a monoclonal antibody specific for glial cells.

that contain Ng-CAM and to determine whether Ng-CAM and N-CAM are expressed on the same cell, immunofluorescence experiments were performed on cultures of embryonic brain cells with differently labeled specific antibodies to Ng-CAM and N-CAM. By indirect immunofluorescence, monoclonal antibody 16F5 [anti-(Ng-CAM)] and specific rabbit anti-(N-CAM) IgG stained neuronal cells but not the flat nonneuronal glial cells (Fig. 3). The nearly complete coincidence of staining on the same cells by anti-(N-CAM) (Fig. 3*B*) and anti-(Ng-CAM) (Fig. 3*C*) antibodies demonstrated that individual neurons expressed the two different CAMs simultaneously; only a few cells showed divergence in the staining patterns.

DISCUSSION

The findings revealed by this investigation are: (*i*) neurons show a previously unrecognized CAM, Ng-CAM, at their surfaces; (*ii*) this molecule has binding specificity for glia but not for neurons and is not found at glial surfaces; (*iii*) it can coexist with N-CAM on the neuronal surface. This is the first unequivocal evidence that single cells can display CAMs of two different specificities simultaneously at their

surfaces, although circumstantial evidence suggests that N-CAM and L-CAM may appear together on very early embryonic cells (4); and (*iv*) Ng-CAM shares at least one antigenic determinant with N-CAM, although the two molecules are structurally and functionally different.

What is the relationship of Ng-CAM to other CAMs? Previous work from this laboratory (1, 4) has shown that following gastrulation, N-CAM and L-CAM have divergent tissue distributions. In addition, they have different specificities and ion dependencies (20) of binding. Ng-CAM, like N-CAM and unlike L-CAM, has a Ca^{2+}-independent binding mechanism. N-CAM and L-CAM (20) have been classified as members of a primary set of CAMs that are expressed in the earliest embryonic epoch of embryological development, and they have been proposed to play a role in embryonic induction (4). They are also expressed and used subsequently in later epochs of histogenesis. In contrast, Ng-CAM is not detectable in the earliest epoch of chicken development and it does not appear until a time just prior to the first appearance of glial cells (unpublished observations). Therefore, it apparently is a member of a secondary set of CAMs postulated to appear in later epochs of organogenesis, presumably to meet specific histogenetic requirements, particularly those related to heterotypic cellular interactions during cytodifferentiation.

The finding that the two neuronal molecules Ng-CAM and N-CAM share one or more antigenic determinants raises the possibility that they both arose from a common evolutionary precursor. Alternatively, secondary-set CAMs such as Ng-CAM may have evolved after duplication of genes for primary-set CAMs. However, the possibility still exists that anti-(N-CAM) nos. 4 and 5 recognize small regions of Ng-CAM and N-CAM containing antigenic determinants that are fortuitously similar; such regions, if they exist, are likely to be on the polypeptide chains of the molecules, for we have found that N-CAM synthesized in the presence of tunicamycin (17) is still recognized by anti-(N-CAM) no. 4. Primary structural comparisons between the two molecules are required to assess the significance of their antigenic crossreactivity.

That neural cells can contain the two CAMs, N-CAM and Ng-CAM, is an important observation in attempting to understand the specificity of cell adhesion. It provides an opportunity to study the ability of a single cell to adhere simultaneously to two different types of cells by means of different adhesive mechanisms. Homotypic aggregation among neurons is mediated by N-CAM via a straightforward homophilic mechanism (ref. 8; see ref. 1 for discussion of binding mechanisms). The present studies on the heterotypic adhesion between neural membranes and glial cells raise the possibility that additional cell adhesion mechanisms, particularly those utilized during histogenesis, may be somewhat more sophisticated. It appears that Ng-CAM is not present on glial cells (see Fig. 3) and that its ligand on glial cells is therefore different from Ng-CAM. Inasmuch as heterotypic cell adhesion must occur between neurons and glia in the presence of homotypic adhesion between neurons, it is reasonable to expect that the glial interaction would be mediated by a heterophilic mechanism to ensure discrimination between the different types of cells during morphogenesis. To produce this specificity, it would be necessary that Ng-CAM and its corresponding ligand on glial cells are heterophilic—i.e., they bind to each other but not to themselves. As we have shown here, Ng-CAM, although found on neural cells, makes no direct contribution to the adhesion between neural cells and thus does not bind to itself, at least in the form present on neural membranes. This result is in accord with the hypothesis that Ng-CAM does not interact with Ng-CAM on other cell membranes and therefore that adhesion mediated by Ng-CAM is not homophilic.

So far, we have no direct evidence concerning the nature of the ligand for Ng-CAM present on glial cells. The fact, however, that minor amounts of M_r 200,000 and M_r 80,000 proteins copurified with Ng-CAM is consistent with the possibility that one or both of these components may comprise the ligand. The alternative that these molecules represent additional components of Ng-CAM at the neuronal cell surface must be considered; decision between the alternatives awaits more refined cytochemical and immunological explorations. It also remains to be determined whether, in addition to the mechanism for neuron–glia interaction, yet another adhesive mechanism exists to mediate binding between glial cells of different kinds. Deeper insight into these binding mechanisms may come from explorations of the role of Ng-CAM in the mouse mutants *reeler* and *weaver*, in which Bergmann glial fibers are implicated in the cerebellar connectional disorders that ensue from the mutations (21–23). Such studies may reveal a role for Ng-CAM both in establishing neural connections related to guide glial fibers (6) and in determining early cytoarchitecture.

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