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Binding properties of a cell adhesion molecule from neural tissue

(cell aggregation/retinal and brain cells/chicken embryo/artificial membrane vesicles/molecular interactions)

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ABSTRACT **We have previously identified and purified a cell surface glycoprotein from retina and brain, called neural cell adhesion molecule or N-CAM, that appears to be involved in neural cell–cell adhesion, the fasciculation of neurites, and the formation of normal tissue patterns in the retina. The present studies reveal that artificial vesicles containing lipid and purified N-CAM bind to different cell types with a specificity similar to that of nerve cells. The same results were obtained with soluble N-CAM that had been briefly exposed to pH 3. In both cases the binding altered the rate of aggregation of neural cells and, like cell–cell adhesion, was inhibited by antibodies against N-CAM. The results support the proposal that N-CAM is a ligand in the formation of bonds between nerve cell membranes. Moreover, results of studies of vesicle–vesicle interactions and of N-CAM binding to cells coated with anti-(N-CAM) Fab' fragments were consistent with the idea that the N-CAM molecules on different cells may interact directly to form cell–cell bonds.**

One of the major goals of developmental biology is to determine the molecular basis of cell–cell adhesion and to describe how this interaction contributes to the formation of tissues. Although an extensive literature exists on this subject (see ref. 1), a mechanism for cell–cell binding has not been established nor has any substance been directly demonstrated to be a part of such a bond.

In previous studies, we used an immunological approach (2) to identify and purify a cell adhesion molecule, called N-CAM, from nerve tissue (3). Antibodies prepared against purified N-CAM specifically inhibit the aggregation of cells from nerve tissues (4) and therefore have been used to probe the physiological consequences of that aggregation. For example, anti-(N-CAM) has been used to demonstrate that N-CAM is involved in fasciculation of nerve fibers (5) as well as in the segregation of cell bodies and neurites in cell aggregates (4) and retinal tissue (6). Anti-(N-CAM) antibodies also influence the growth of fascicles toward a source of nerve growth factor (7).

Although these investigations have been useful in analyzing developmental events in which adhesion plays a major role, they do not indicate the precise mode of action of N-CAM, particularly whether it is involved in the formation of cell–cell bonds. So far, only indirect evidence has been obtained, such as the inhibition of aggregation of cells by anti-(N-CAM) Fab' fragments and a correlation of N-CAM concentration on the plasma membrane with the rate of cell aggregation (4, 8). The present studies provide direct evidence to support the hypothesis that N-CAM may function as a ligand in cell–cell adhesion.

MATERIALS AND METHODS

Preparation of N-CAM and Anti-(N-CAM). Purified N-CAM was obtained* from a Nonidet P-40 (British Drug House, Poole, England) extract of membranes from 14-day chicken brain by affinity chromatography using an anti-(N-CAM) monoclonal antibody coupled to Sepharose CL-2B (Pharmacia). N-CAM was eluted from the Sepharose with 50 mM diethylamine/1 mM EDTA/0.5% Nonidet P-40. The detergent was removed by using Biobeads SM-2 (Bio-Rad) (9), and the protein was dialyzed against H_2O and lyophilized. This material migrated in a single zone during electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. ^{125}I-labeled N-CAM (^{125}I-N-CAM) was prepared by using the chloramine-T procedure (10). In most cases, the labeled N-CAM was subsequently dialyzed for 2 hr against 0.15 M acetate (pH 3) at 37°C and then against phosphate-buffered saline at pH 7.4 (P_i/NaCl) at 4°C.

Rabbit anti-(N-CAM) was prepared by three successive injections of 400 μg of N-CAM at monthly intervals in complete Freund's adjuvant, incomplete Freund's adjuvant, and saline, respectively. In some experiments, these antibodies were purified by affinity chromatography on N-CAM-derivatized Sepharose CL-2B. Mouse monoclonal antibodies that both bind N-CAM and inhibit nerve cell adhesion were obtained by hybridization and cloning techniques from spleens of mice immunized with N-CAM.

Preparation of Cells. Retinal and brain cells representing most of the neurons of these tissues were obtained by treatment of the tissue from 10-day chicken embryos with 0.002% trypsin. These cells display Ca^{2+}-independent aggregation (11) and have N-CAM on their surface (4, 12). Liver cells were dissociated from 11-day embryo tissue by treatment with collagenase, trypsin, and bovine serum (13). Myoblasts were obtained from leg muscles of 11-day chicken embryos by treatment with 0.1% trypsin for 30 min at 37°C. After the myoblasts were allowed to recover for 4 hr at 37°C in a spinner culture (8), they were fractionated on a Ficoll density gradient to yield a population consisting of 70% myoblasts and 30% fibroblasts (14). Fibroblasts were obtained from skin of 10-day chicken embryos by trypsinization (0.2% trypsin/0.2% EDTA, 30 min, 37°C). After the fibroblasts grew to confluence in monolayer cultures, they were harvested by trypsinization and then allowed to recover for 4 hr in a spinner culture.

Incorporation of N-CAM into Lipid Vesicles. Artificial membranes containing affinity-purified N-CAM were prepared by sonication of 30 mg of lipid in 1 ml of 0.5% Nonidet P-40/P_i/NaCl buffer, addition of 1 mg N-CAM in 5 ml of the same buffer, removal of detergent with Biobeads SM-2 (9), and isolation of the vesicles by gel filtration on Sepharose CL-2B in P_i/NaCl buffer. About 20% of the protein remained with the vesicle frac-

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Abbreviations: N-CAM, the cell adhesion molecule from neural tissue; P_i/NaCl, phosphate-buffered saline (pH 7.4); EME medium, Earle's minimal essential medium.

* The isolation and characterization of N-CAM will be described elsewhere by S. Hoffman, B. C. Sorkin, P. C. White, R. Brackenbury, R. Mailhammer, U. Rutishauser, B. A. Cunningham, and G. M. Edelman.

tion. Lipids were either synthetic dioleylphosphatidylcholine (Sigma) or total brain lipid obtained by chloroform/methanol extraction of a crude membrane preparation (15). To obtain fluorescent vesicles, 20 mM 6-carboxyfluorescein (Eastman) was included in the detergent solution. The presence of N-CAM on the exterior of the vesicles was indicated by the ability of trypsin to degrade >90% of the lipid-associated protein. The size of these vesicles ranged from submicroscopic to about 0.2 μm in diameter (see Fig. 2).

Binding Assays. Binding of soluble N-CAM to cells was measured by incubation of 10^7 cells with 2 μg of ^{125}I-labeled N-CAM (2×10^5 cpm) for 15 min at 25°C in 200 μl of Earle's minimal essential medium (EME medium) containing 3.5 mg of bovine serum albumin. The cells were then washed by centrifugation through 3.5% bovine serum albumin in EME medium and assayed in a γ-ray spectrometer.

The attachment of lipid vesicles containing N-CAM to cells was monitored by fluorescence microscopy after incubation of 100 μl of a 20% vesicle suspension with 5×10^6 cells in 2 ml of EME medium on a gyrotory shaker at 70 rpm for 15 min at 37°C. Aggregation of the vesicles themselves was observed after a similar incubation for 2 hr without cells.

The binding of N-CAM to itself was examined by incubation of 10 μg of ^{125}I-N-CAM (10^6 cpm) with 80 μl of N-CAM-Sepharose for 3 hr at 37°C in 500 μl of P_i/NaCl buffer containing 10 mg of bovine serum albumin per ml. After extensive washing, the bound radioactivity was measured in a γ-ray spectrometer.

Cell-to-cell binding was measured by using either a monolayer assay in which a suspension of fluorescein-labeled cells were incubated with a cell monolayer (8) or by measuring the decrease in particle number that accompanied the aggregation of cells in suspension (2, 16).

RESULTS

Artificial Vesicles Containing N-CAM Bind Specifically to the Surface of Neural Cells. Vesicles prepared with N-CAM and either total brain lipid or dioleylphosphatidylcholine contained about 0.6% protein, most of which was displayed at the external surface. The binding of these vesicles to retinal cells during a 15-min incubation was easily visualized (Fig. 1 *a* and *b*) by fluorescence microscopy using intravesicular carboxyfluorescein as a label. Binding was observed only with vesicles that contained N-CAM freshly eluted from the immunoaffinity column and directly incorporated from the detergent-containing eluate into vesicles. Although not obvious in the photographs, there was little if any fusion of vesicles with the cells. Both the binding of vesicles and the aggregation of cells were completely inhibited by affinity-purified anti-(N-CAM) Fab' (Fig. 1 *c* and *d*) and, in contrast to at least two other mechanisms of cell aggregation (11–13), neither required the presence of Ca^{2+}. Antibodies against whole brain cells, which react with many surface antigens including N-CAM, also blocked vesicle-cell binding. When anti-(N-CAM) was specifically removed from this antiserum by affinity chromatography on N-CAM-derivatized Sepharose, the remaining antibodies were found to have no effect on binding.

Neural Retina Cells and N-CAM Vesicles Exhibit Similar Binding Specificities. If N-CAM is a ligand in neural cell–cell adhesion, then its binding affinity for cells should resemble that of intact neural cells. In comparing the adhesion of N-CAM vesicles and retinal neurons to various cell types (Table 1), we observed that both vesicles and neurons bound to cells having N-CAM on their surfaces. These observations include muscle cells, which recently have been shown to express N-CAM and to bind specifically to nerve cells (see Table 1). With one exception, neither vesicles nor nerve cells bound to cells lacking N-CAM. The exception, binding of retinal cells to hepatocytes, is particularly interesting because this adhesion was not inhibited by anti-(N-CAM) Fab' and required Ca^{2+}; both of these findings suggest that this adhesion mechanism is distinct from that involving N-CAM (12).

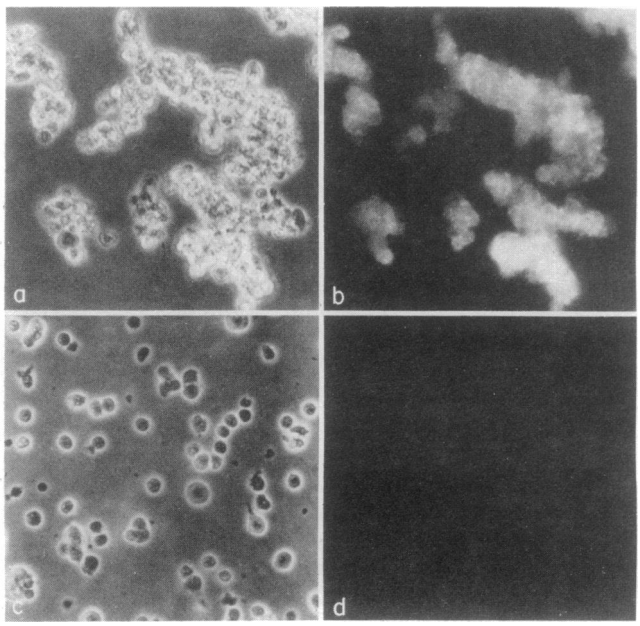

FIG. 1. Binding of fluorescent N-CAM vesicles to retinal cells. (*a*) Phase-contrast micrograph. (× 330.) (*b*) Fluorescence micrograph of the same field. (*c* and *d*) Same experiment carried out in the presence of anti-(N-CAM) Fab'.

N-CAM Vesicles Inhibit Retinal Cell Aggregation. Also shown in Table 1 is the effect of anti-(N-CAM) Fab' and N-CAM vesicles on the aggregation of retinal and liver cells. The antibody and vesicles had similar effects: a marked decrease in the rate of retinal cell aggregation but no effect on hepatocytes; vesicles that did not contain protein had no effect on aggregation. The retinal cell aggregates that did form in the presence of N-CAM vesicles often contained vesicles between cells and within the aggregate. Because the vesicles themselves can aggregate (see below), it is likely that the partial inhibition obtained with the N-CAM vesicles reflects a combination of inhibition and agglutination.

Experiments with Soluble N-CAM. The studies described above were also carried out with ^{125}I-N-CAM in detergent-free solution. Neither significant binding nor consistent inhibition of retinal cell aggregation was observed. Other studies* suggested that such preparations contain N-CAM in an aggregated form, and various treatments were attempted in order to restore binding activity. One of these, brief dialysis against pH 3 buffer at 37°C, was successful in that after neutralization a significant amount of N-CAM bound to cells (Table 2). The effect of low pH on N-CAM structure and function is not yet explained, but it is possible that this treatment exposes binding sites by altering the size or conformation of N-CAM aggregates.

The binding to cells obtained with pH 3-treated N-CAM was inhibited by affinity-purified or monoclonal anti-(N-CAM) Fab' and competitively decreased by the addition of similarly treated but unlabeled N-CAM. This binding required undenatured protein: it disappeared after exposure of the molecule to deoxycholate, high temperature, or trypsin (Table 2). Binding was not affected by 10 mM EDTA or 10 mM azide, indicating that Ca^{2+} was not required and suggesting that the N-CAM was not being ingested by phagocytosis. As with N-CAM vesicles, the

Table 1. Comparison of retina cells and N-CAM vesicles for amount and effect of binding to different cell types

Cell type	Cell surface N-CAM*	Binding of retina cells† Amount	Binding of retina cells† % Inhibition by anti-(N-CAM)¶	Binding of N-CAM vesicles‡	Rate of cell aggregation§ Control	Rate of cell aggregation§ With N-CAM vesicles	Rate of cell aggregation§ With anti-(N-CAM)¶
Retina	+	450 ± 32	85	+	51 ± 2	23 ± 1	8 ± 2
Brain	+	420 ± 25	90	+			
Myoblasts‖	+	208 ± 35	60	+			
Hepatocytes	−	414 ± 41	2	−	62 ± 4	59 ± 2	60 ± 4
Retina (trypsin)**	−	25 ± 8		−			
Fibroblasts	−	10 ± 6		−			

Values are mean ± SD of three or four experiments.
* Determined by immunofluorescence staining with anti-(N-CAM).
† Binding between the indicated cell type and 1 mm^2 of retinal cell monolayer.
‡ Vesicles containing carboxyfluorescein were visualized by fluorescence microscopy (see Fig. 1).
§ Percentage decrease in particle number during a 30-min incubation at 37°C and rotation at 90 rpm.
¶ Affinity-purified anti-(N-CAM) was present at 20 μg/ml during binding.
‖ The myoblast data were provided by M. Grumet in our laboratory.
** Cells were treated with 0.1% trypsin for 20 min at 37°C and washed.

specificity of soluble N-CAM binding to different cell types was similar to that of intact cells from neural retinas (Tables 1 and 2). Although the pH 3-treated soluble N-CAM did have detectable effects on the rate of retinal cell adhesion in the rotation-suspension assay, they were less dramatic and more complex than those obtained with N-CAM vesicles. With gyrotory agitation at 200 rpm, addition of N-CAM at 100 μg/ml caused a 40% inhibition of aggregation; at 90 rpm, however, the rate was enhanced by about 30%. As suggested for vesicles, the enhancement observed with less agitation may reflect cell agglutination caused by interactions among soluble N-CAM molecules.

Coating of Cells with Anti-(N-CAM) Fab' Inhibits the Binding of Cells, Vesicles, and Soluble N-CAM. The antibody inhibition studies described above were carried out with Fab' fragments present in solution throughout the binding assay. In a second protocol, designed to test the hypothesis that binding involves the interaction of one N-CAM molecule with another, cells were preincubated with Fab' fragments for 15 min at 4°C, washed thoroughly, and then assayed for their binding properties. When retinal cells were coated with affinity-purified or monoclonal anti-(N-CAM) Fab', their ability to bind uncoated retinal cells or soluble N-CAM was reduced by 72% or 93%, respectively; the binding of vesicles was diminished by a similar proportion (Table 3). In each case, this level of inhibition represented 80–90% of the effect obtained when Fab' was present in solution throughout the assay.

Table 2. Binding of soluble N-CAM to cells

Cell type	Treatment of N-CAM*	Inhibitor or competitor	Bound N-CAM, cpm†
Retina	pH3	—	23,665 ± 1,414
Retina	None	—	2,449 ± 526
Retina	pH 3	Anti-(N-CAM)‡	720 ± 286
Retina	pH 3	N-CAM §	7,154 ± 1,010
Retina	pH 3, DOC	—	1,124 ± 215
Retina	pH 3, 100°	—	1,599 ± 94
Retina	pH 3, trypsin	—	2,326 ± 255
Retina (trypsin)¶	pH 3	—	2,506 ± 456
Myoblasts	pH 3	—	11,212 ± 1,103
Myoblasts	pH 3	Anti-(N-CAM)‡	2,525 ± 520
Fibroblasts	pH 3	—	3,156 ± 212
Hepatocytes	pH 3	—	2,324 ± 250

* All experiments denoted "pH 3" used ^{125}I-N-CAM that had been dialyzed at 37°C against 0.15 M acetate (pH 3) for 2 hr and then against P_i/NaCl buffer, (pH 7.4). DOC, 0.5% sodium deoxycholate.
† After incubation of 10^7 cells for 15 min at 25°C in 200 μl of medium containing 2 μg of ^{125}I-N-CAM (2 × 10^5 cpm). Values are the mean ± SD of three experiments.
‡ Presence of 20 μg of affinity-purified anti-(N-CAM) Fab'. Monoclonal anti-(N-CAM) gave the same result.
§ 100 μg of unlabeled N-CAM.
¶ Cells were treated with 0.1% trypsin for 20 min at 37°C and washed.

N-CAM in Solution or on Vesicles Binds to N-CAM. The decreased binding of N-CAM vesicles and soluble N-CAM to cells coated with anti-(N-CAM) Fab' is consistent with the idea that there is an interaction between N-CAM molecules. Direct evidence for such an interaction came from the observations that pH 3-treated soluble ^{125}I-N-CAM adhered to N-CAM immobilized on Sepharose (Table 4), that vesicles containing only synthetic lipid and purified N-CAM spontaneously formed large aggregates (Fig. 2), and that N-CAM appears to exist in solution as a heterodisperse oligomer. The binding of N-CAM to N-CAM-Sepharose was inhibited by anti-(N-CAM) Fab' and was subject to competition by unlabeled N-CAM but not by serum albumin. No binding of N-CAM was observed with un-derivatized Sepharose or with serum albumin coupled to Sepharose (Table 4). Similarly, the aggregation of N-CAM vesicles was completely blocked by the presence of anti-(N-CAM) Fab' fragments (Fig. 2); trypsinized N-CAM vesicles or vesicles consisting only of lipid did not aggregate.

The aggregation of N-CAM molecules in solution (or onto cells) probably accounts for the absence of a saturation plateau in the binding of soluble N-CAM to retinal cells (Fig. 3), the highest point shown representing more than 2 × 10^7 molecules bound per cell. Although this phenomenon could reflect nonspecific adsorption of N-CAM to the cell surface, the absence

Table 3. Binding properties of retinal cells coated with Fab'

Fab' used to coat cells*	Binding of: Retinal cells†	N-CAM vesicles‡	Soluble N-CAM, cpm§
Nonimmune	423 ± 23	+++	30,124 ± 3,067
Anti-(N-CAM)	120 ± 15	+/−	2,013 ± 164

* Monoclonal Fab' was used at 20 μg/ml, and unbound Fab' was removed by washing. The same result was obtained with affinity-purified Fab'.
† Binding of untreated cells to a monolayer of Fab'-coated cells (see Table 1). Values are the mean ± SD of three experiments.
‡ Visual estimation of bound fluorescent vesicles.
§ See Table 2 for details. Values are the mean ± SD of three experiments.

Table 4. Binding of ^{125}I-N-CAM to N-CAM-Sepharose

Protein coupled to Sepharose*	Inhibitor or competitor	Bound cpm†
N-CAM	None	228,791
N-CAM	Anti-(N-CAM)	46,889
N-CAM	Unlabeled N-CAM (20 μg)	137,136
N-CAM	Unlabeled N-CAM (100 μg)	44,252
Un-derivatized	None	9,625
Serum albumin	None	12,321

* Protein (160 μg) was coupled to 80 μl of beads.
† Sepharose was incubated with 10 μg of N-CAM (10^6 cpm) in 500 μl of P_i/NaCl buffer containing bovine serum albumin at 10 mg/ml.

of binding to anti-(N-CAM)-coated cells (Table 2) suggests that the initial attachment of N-CAM to the cells was specific.

DISCUSSION

The results reported here establish that purified N-CAM in certain states can bind specifically to the surface of neural cells and suggest that this binding is correlated with adhesion between cells that express N-CAM on their surface. Binding of N-CAM or N-CAM vesicles to cells appears to be specific in that it occurred only with cell types known to have N-CAM on their surface and was inhibited by anti-(N-CAM) Fab' but not by other antibodies against the neural cell surface. This activity was destroyed by protease, heat, and ionic detergents and was stimulated by brief exposure to low pH or by incorporation of N-CAM into or onto a lipid bilayer. It is therefore dependent upon both molecular conformation and environment.

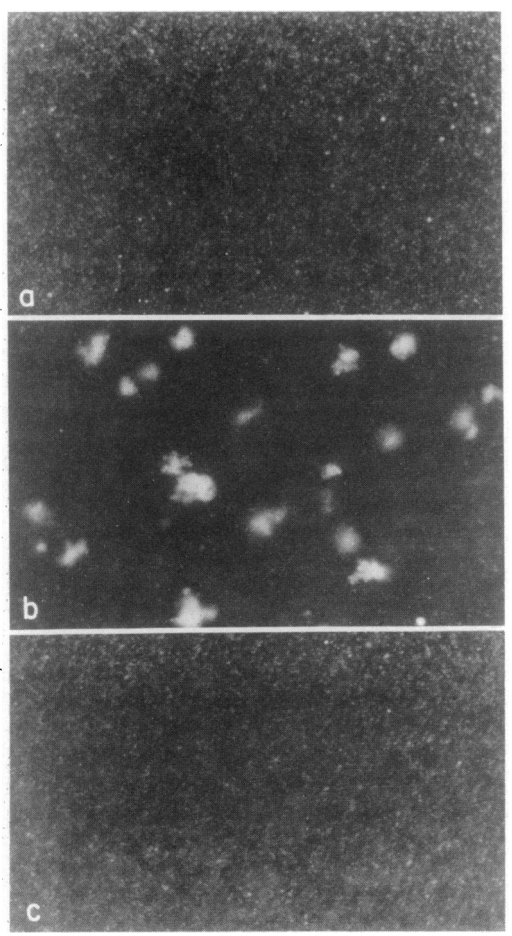

FIG. 2. Aggregation of N-CAM vesicles as observed by fluorescence microscopy (× 460.) (*a*) Freshly prepared vesicles. (*b*) Vesicles incubated with shaking for 2 hr at 37°C. (*c*) Vesicles incubated as in *b* in the presence of affinity-purified anti-(N-CAM) Fab' at 20 μg/ml.

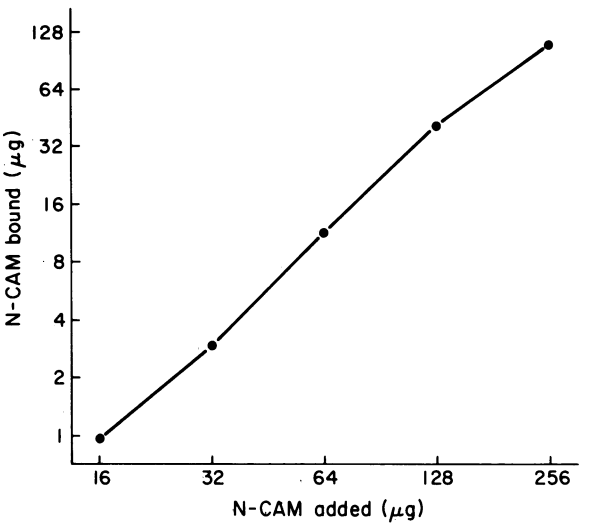

FIG. 3. Binding of soluble ^{125}I-N-CAM to retinal cells. The N-CAM was treated at pH 3 after iodination and then incubated with 10^7 cells.

Although a molecule involved in cell–cell adhesion would be expected to have a binding affinity for surface membranes, no unequivocal evidence exists that the adhesion and binding phenomena are related. Removal of N-CAM from its natural environment could create a binding activity that is not involved in cell adhesion. This interpretation could account for the failure of the N-CAM–cell binding curve (Fig. 3) to reach a saturation plateau. Nevertheless, Fab' fragments that were bound to cells (which express N-CAM in a "native" state) blocked binding of both N-CAM vesicles and soluble N-CAM. Therefore, an adventitious binding of purified N-CAM would require the unlikely possibility that such an "altered" molecule has a specific affinity for a native site on cell surface N-CAM and, moreover, that this site is covered by the same monoclonal Fab' fragments that inhibit cell–cell adhesion.

The alternative proposal that the binding of N-CAM to cells is related to neural cell adhesion is based on the following strong circumstantial evidence. (*i*) Both the cell adhesion and N-CAM binding phenomena were inhibited by anti-(N-CAM). (*ii*) Neither type of binding required calcium, in contrast to at least two other adhesion mechanisms (11–13). (*iii*) Binding of N-CAM to neural cells altered their rate of aggregation. (*iv*) Previous studies have indicated that the rate of aggregation of neural cells is correlated with the amount of N-CAM on their surfaces (4). (*v*) The specificity of binding of neural cells, N-CAM vesicles, and soluble N-CAM to various cell types was identical except for nerve–hepatocyte adhesion, which appears to be unrelated to interactions involving N-CAM. (*vi*) To date, anti-(N-CAM) is the only specific antibody against a neural cell surface component that inhibits adhesion among the neural cells used in our studies; moreover, the adhesion-blocking activity of polyspecific antisera against these cells was lost upon specific removal or neutralization of anti-(N-CAM) antibody.

Unequivocal evidence that N-CAM is a ligand in cell adhesion can be obtained only by characterizing a cell–cell bond in molecular detail. Little is known about the molecular interactions involved in cell adhesion, but the rapid and specific aggregation of N-CAM vesicles raises the possibility that N-CAM molecules can bind directly to each other to form a mem-

brane–membrane link. Aggregation was observed with vesicles made with either crude brain lipids or pure dioleylphosphatidylcholine and therefore is unlikely to involve an interaction of N-CAM with a nonglycoprotein component of cellular origin. The binding of N-CAM to N-CAM-Sepharose and the aggregated state of N-CAM in solution may represent the same phenomenon although, in these cases, one must also consider the possibility of nonspecific aggregation, particularly via hydrophobic interactions.

The suggestion that interactions between N-CAM molecules are important in cell–cell adhesion was supported by the observation that the binding, by a nerve cell, of N-CAM, N-CAM vesicles, or other cells expressing N-CAM was strongly inhibited by the prior coating of that cell with monovalent Fab' fragments prepared from monoclonal anti-(N-CAM). These results do not exclude the possibility that, in cell–cell binding, N-CAM may bind to another kind of molecule as well as to itself. It is striking, however, that adhesion between two cells that was inhibitable by anti-(N-CAM) only occurred if both cells had N-CAM on their surface.

Taken together, the present experiments provide evidence in support of the idea that N-CAM is an important part of the ligating apparatus for the specific calcium-independent adhesion that occurs among nerve cells and their fibers. Nevertheless, a precise description of the binding process, in which each molecular component is identified and the interactions are defined chemically, will require considerably more information, particularly the elucidation of the detailed molecular structure of N-CAM.

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