Clusters of Neural Cell Adhesion Molecule at Sites of Cell-Cell Contact

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Abstract. I have examined the distribution of neural cell adhesion molecule (N-CAM) in cultured C2 myogenic cells and other cell lines to determine if N-CAM accumulates at sites of cell-cell contact. C2 cells growing in log phase display large clusters of neural cell adhesion molecule where they contact each other. These clusters are remarkably stable, do not form at cell-substrate contacts, and appear not to be enriched in a number of other cytoskeletal, membrane, or extracellular proteins. Thus, N-CAM clusters form preferentially in response to cell-cell contact and are specifically enriched in N-CAM. As C2 cultures mature and differentiate, clusters persist at contacts between aligning myoblasts and between myotubes, consistent with a role in myogenesis.

N-CAM is also enriched at cell-cell contacts in cultures of PC12, NRK, and CHO cells. These cells have significant amounts of N-CAM as detected on immunoblots. Clusters are not seen in L929 cells, which do not have detectable amounts of N-CAM. Coculture of these cells with C2 cells results in the clustering of N-CAM at heterologous contacts between C2 cells and NRK, CHO, or PC12 cells, but not between C2 cells and L929 cells. These results suggest that N-CAM specifically accumulates where N-CAM–bearing cells contact one another. Clustering of N-CAM may be an important step in strengthening intercellular adhesion.

CELL-cell adhesion plays an important role in cell migration, cell recognition, and organogenesis, and thus is essential to the development of higher eukaryotes. Several of the molecules that mediate adhesive interactions in the nervous system, including neural cell adhesion molecule (N-CAM),[1] and N-cadherin, have been isolated and extensively characterized (reviewed by Rutishauser and Jessell, 1988; Covault, 1989), but the way in which they promote cell-cell adhesion is still not completely understood.

N-CAM, the most extensively studied of these molecules, mediates homotypic adhesion (Rutishauser et al., 1982; Edelman, 1983). When N-CAM is present on the surface of two neighboring cells, the cells adhere to each other, presumably as a result of "head-to-head" interactions between N-CAM molecules (Hoffman et al., 1982; Edelman, 1983). It has generally been assumed that such interactions are sufficient for adhesion to occur, but other possibilities should be considered. In many types of cells, adhesion results in the selective redistribution of the adhesive molecules in the cell surface, so that they become concentrated at sites of intercellular contact (e.g., Ocklind et al., 1983; Schmelz et al., 1986; Hirano et al., 1987; Albelda et al., 1990; Volk et al., 1987) where they can form specialized junctional complexes (e.g., Schmelz et al., 1986; Volk and Geiger, 1986). Concentrating adhesive molecules at contacts should significantly strengthen adhesive interactions. Although some reports have suggested the possibility that N-CAM can accumulate at contact sites (Edelman, 1976; Magnai et al., 1981), and some evidence for patchy distribution of N-CAM has been reported, particularly at synapses (Rieger et al., 1985; Covault and Sanes, 1986; Persohn and Schachner, 1987; Di-Figlia et al., 1989), little is known about the distribution of N-CAM at cell-cell contacts.

I have studied the distribution of N-CAM on the surfaces of cells of the myogenic cell line, C2. C2 cells are large and flat, and grow as small islands of slightly overlapping cells before they elongate and fuse to form myotubes. Here, I show that N-CAM is concentrated at sites of contact between C2 muscle cells. These accumulations are very stable, and do not seem to be associated with other membrane, cytoskeletal, or extracellular proteins. Similar accumulations are commonly found in myoblasts shortly before fusion and on other N-CAM-bearing cells. N-CAM can also accumulate at heterologous cell-cell contacts if both types of cells bear N-CAM on their surfaces. Therefore, N-CAM aggregates at sites of homologous and heterologous cell-cell adhesion. Its aggregation may be an important step in the formation of stable intercellular contacts and, thus, in differentiation and morphogenesis.

Materials and Methods

Cell Culture

C2, a mouse myogenic cell line obtained from Mr. T. Lang (Department

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1. *Abbreviations used in this paper:* N-CAM; neural cell adhesion molecule; NRK, normal rat kidney.

of Pathology, University of Maryland School of Medicine), was grown in tissue culture plastic (Falcon, Becton Dickinson, Lincoln Park, NJ) with Dulbecco-Vogt modified Eagle's medium (DMEM) supplemented with 20% FCS (Intergen, Armour Pharmaceutical, Kankakee, IL). Subculturing utilized 0.25% trypsin-0.02% EDTA (Gibco Laboratories, Grand Island, NY), and was performed every 2-4 days, to ensure that the cell density was never more than ~70% confluent. To induce fusion of the myoblasts into myotubes, cultures were shifted into medium containing 5% horse serum (Gibco Laboratories) and incubated for several days.

L929 (mouse connective tissue) and normal rat kidney (NRK) cells were grown in DMEM containing 10% FCS. PC12 (rat pheochromocytoma) cells were grown in RPMI or DMEM supplemented with 7.5% FCS and 5% horse serum. CHO cells were grown in F12 medium with 10% FCS. These cell lines were maintained on tissue culture plastic (Falcon), but were plated onto glass coverslips (No. 1 thickness; Van Waters, Oxnard, CA) to facilitate immunofluorescence studies. All cell lines other than PC12 were passed every 3-4 d, at cell densities between 50 and 80% confluent; trypsin-EDTA was used to obtain a suspension of single cells. PC12 cells were subcultured weekly, following resuspension by mild trituration.

To mark C2 cells for some fluorescence studies, cultures were reacted with 1 mg/ml biotinyl-ε-aminocaproic acid N-hydroxysuccinimide ester (biotin-X-NHS; Calbiochem, La Jolla, CA), in Dulbecco's PBS, for 1 h at room temperature. Labeled cells were removed from the substrate with trypsin-EDTA and replated onto coverslips containing unlabeled C2, CHO, NRK, or L929 cells, in the medium appropriate for the unlabeled cell type.

For some studies of replated C2 cells, cultures at <40% confluency were treated with trypsin-EDTA, centrifuged briefly, and resuspended in fresh medium (~2 ml/60-mm dish). Samples of the cell suspension were examined to ensure that very few cells (<10%) were in pairs or larger aggregates. The suspension (0.1-0.4 ml) was spread onto fresh coverslips and replaced in the incubator. When reagents were tested for their affect on N-CAM clustering in replating C2 cells, the reagents were added to the cell suspension prior to plating.

Immunofluorescence

For immunofluorescence, cultures were first washed in PBS. They were fixed and permeabilized in three different ways: (a) 95% ethanol at −20°C for 20 min; (b) treatment for 2 min with 0.5% Triton X-100 in 2% paraformaldehyde in buffered saline (10 mM NaP, 145 mM NaCl, pH 7.2: PBS), followed by fixation for an additional 15 min in paraformaldehyde; (c) fixation in paraformaldehyde for 15 min, followed by brief (2-5 min) treatment with 0.5% Triton X-100 in PBS. Fixation in paraformaldehyde at 0° and at ambient temperature (RT) gave similar results. After fixation, but before further processing, cultures were washed briefly in PBS, or incubated in 0.1 M glycine in PBS to inactivate remaining aldehydes. In general, I used alcohol fixation for samples to be labeled with mAbs to N-CAM, and paraformaldehyde fixation for samples to be labeled with polyclonal anti-N-CAM raised in rabbits. The different protocols all yielded similar results.

Fixed and permeabilized cells were incubated for 30-60 min at RT with anti-N-CAM or other antibodies diluted in PBS supplemented with 1 mg/ml BSA (PBS-BSA). The antibodies used, with their specificities and sources, are listed in Table I. Samples were washed several times in PBS-BSA, and then incubated for 30 min at RT in fluorescent anti-antibodies, diluted to 10 μg/ml in PBS-BSA. For most experiments reported here, I used fluoresceinated goat anti-mouse IgG (FGAM) or rhodaminylated goat anti-rabbit IgG (RGAR). In double immunofluorescence experiments both anti-antibodies were used; appropriate controls showed that these secondary antibodies were species specific. For samples containing biotinylated cells (see above), fluoresceinated streptavidin (F-SAv) was included in the final labeling step. Most samples were mounted in 90% glycerol, 10% Tris HCl, pH 8.0, supplemented with 1 mg/ml p-phenylenediamine, to reduce photobleaching (Johnson et al., 1982). Some samples were mounted in buffered saline and sealed with nail polish, for studies requiring phase, Nomarski, or interference reflection optics.

Samples were observed on a Zeiss IM-35 fluorescence microscope equipped with a 63× Planeofluor objective (NA 1.25). Micrographs were taken on T-MAX P3200 film (Eastman-Kodak Co., Rochester, NY) and were developed to an ASA of 1600 using T-MAX developer (Eastman-Kodak Co.). Typical exposures were 5-20 s.

Estimates of fluorescence intensity were made with a photomultiplier attached to the fluorescence microscope, as described (Bloch, 1986). Areas of N-CAM clusters 5 μm in diameter were sampled and compared to nearby areas of the surface of the same cells that were not obviously enriched in N-CAM. Nonspecific labeling, measured similarly on cells labeled with

Table I. Antibodies Used

Antigen	Designation*	Origin	Source‡
N-CAM	anti-N-CAM	rabbit	1
	anti-N-CAM Fab	rabbit	1
	mab AG1	mouse	1
	mab 5B8	mouse	2
Intracellular/Cytoskeleton			
Vinculin	anti-vinculin	rabbit	3
	mab 11.5	mouse	4
Filamin	anti-filamin	rabbit	3
α-Actinin	anti-α-actinin	rabbit	3
Myosin	anti-myosin	rabbit	5
β-Spectrin	mab VIIF7	mouse	6
	mab IVF8	mouse	6
α-Spectrin	mab IID2	mouse	6
Erythrocyte spectrin (α+β)	antispectrin	rabbit	6
Fodrin	antifodrin	rabbit	6
Band 4.1	anti-4.1	rabbit	6
Desmin	mab DEB5	mouse	7
Vimentin	mab V9	mouse	7
Tubulin	mab 27B	mouse	8
Clathrin	mab X22	mouse	9
Membrane			
Fibronectin receptor	anti-FnR	rabbit	10
Vitronectin receptor	anti-VnR	rabbit	10
Thy-1	mab HO 13.4	mouse	11
Extracellular			
Collagen type IV	anti-TIVC	rabbit	12
Laminin	anti-Lm	rabbit	12
Heparan sulfate proteoglycan	mab B3	mouse	13
Fibronectin	anti-Fn	rabbit	10

* All rabbit antibodies except antimyosin were affinity-purified on immobilized antigen. Anti-N-CAM was used both as a crude antiserum, and after affinity purification. Both preparations gave identical results in immunofluorescence studies. Fab fragments were prepared from the total IgG fraction.
‡ Sources are: (1) Dr. J. Covault, University of Connecticut, Storrs, CN (Covault et al., 1986; DiFiglia et al., 1989); (2) Developmental Hybridoma Bank, University of Iowa, Iowa City, IA; (3) this laboratory (Bloch and Hall, 1983); (4) Dr. B. Beiger, Weizmann Institute, Rehovot, Israel; (5) Chemicon, Temecula, CA; (6) Dr. J. Morrow, Yale University School of Medicine, New Haven, CT (Yurchenco et al., 1982; Harris et al., 1986); (7) Boehringer-Mannheim, Indianapolis, IN; (8) Dr. M. Stearns, Medical College of Pennsylvania, Philadelphia, PA; (9) Dr. F. Brodsky, University of California, San Francisco, CA; (10) Telios Laboratories, San Diego, CA; (11) Dr. G. Lewis, University of Maryland School of Medicine, Baltimore, MD; (12) Dr. H. Kleinman, National Institute of Dental Research, Bethesda, MD; (13) Dr. C. Cornbrooks, University of Vermont, Burlington, VT (Eldridge et al., 1986).

nonimmune serum or mAb, used at the same final concentration as the anti-N-CAM, was subtracted to obtain the fluorescence intensities due to specific binding of anti-N-CAM.

N-CAM Clusters and Cell-Cell Contacts

N-CAM clusters, visualized by immunofluorescence, were defined as any accumulation of N-CAM that was clearly brighter than surrounding areas. This was usually easy to discern, even at clusters that were oriented along the z-axis, i.e., parallel to the direction of viewing. Any ambiguous examples were not included in the quantitations reported here.

Cell-cell contacts were considered to be any areas in which two apposing cells touched or overlapped, as visualized under phase or Nomarski optics, or as suggested by the distribution of background fluorescence. Although overlap does not necessarily imply contact, studies with mixed populations of biotinylated and control cells showed that many overlaps had structures typical of cells in contact, including lamellipodia, and cellular processes that obviously attached to the apposing cell (e.g., see Figs. 2 and 7).

SDS-PAGE and Immunoblotting

For immunoblotting, cultures at ~70% confluency in 35-mm tissue culture

dishes were washed with PBS, covered with sample buffer (Laemmli, 1970), scraped with a rubber policeman, and boiled for 3 min. Alternatively, samples were scraped into 1% NP-40, 2.5 mM NaAcetate, pH 5, and digested with neuraminidase (Type X; Sigma Chemical Co., St. Louis, MO) as described (Moore et al., 1987), before boiling in sample buffer. Samples containing 43–149 μg protein/cm gel were applied to 5–15% gradient acrylamide gels and analyzed by SDS-PAGE following the method of Laemmli (1970). Proteins were transferred electrophoretically to nitrocellulose paper (Burnette, 1981). The paper was incubated in 3% nonfat dry milk solids, 3% BSA in buffered saline, to block any remaining binding sites for protein, and then incubated further with rabbit anti-N-CAM antiserum, diluted 1:1,000 in 0.1% BSA in buffered saline. After washing, sites of antibody binding were visualized with radioiodinated protein A (Amersham Corp., Arlington Heights, IL) or with a donkey anti–rabbit IgG conjugated to HRP. The chromogenic reaction catalyzed by peroxidase was developed with 4-chloro-1-naphthol and H_2O_2.

Materials

All cell lines other than C2 were from the American Type Culture Collection (Rockville, MD) and were obtained from Dr. J. Krikorian (University of Maryland School of Medicine, Baltimore, MD). Fluorescent antibodies and streptavidin for immunofluorescence labeling, and peroxidase-conjugated anti–rabbit IgG were obtained from Jackson Immunoresearch (West Grove, PA). Dulbecco-Vogt modified Eagle's medium was prepared in our laboratory with and without $CaCl_2$. For experiments in the absence of Ca^{2+}, serum was extensively dialyzed; final concentration of Ca^{2+} in medium were <25 μM, as determined by atomic absorption spectroscopy. F12 and RPMI media were obtained from Gibco Laboratories. Unless otherwise noted, all other reagents were obtained from Sigma Chemical Co.

Results

N-CAM on C2 Myoblasts

C2 myoblasts in tissue culture contain a significant amount of N-CAM that appears in immunoblots as a broad, polydisperse band (Fig. 1, lane A). This has been shown to be a polysialylated, transmembrane form of N-CAM (Covault et al., 1986; Moore et al., 19887). In agreement with previous results (Covault et al., 1986; Moore et al., 1987), treatment of C2 extracts with neuraminidase yielded a major band of N-CAM immunoreactivity at 140 kD (Fig. 1, lane C).

When I localized this N-CAM by indirect immunofluorescence, I found that some regions displayed very intense labeling, whereas other areas labeled hardly at all (Fig. 2). Such labeling was not seen with a control antibody (Fig. 2, E and F) that failed to react with N-CAM in blots (Fig. 1, lane B), suggesting that it was specific. Consistent with this, double immunofluorescence labeling experiments (not shown) showed identical labeling by the rabbit anti-N-CAM and both of the mAbs I used (see Figs. 2 J and 5 C). Rarely, a single cell had an area that labeled brightly for N-CAM (Fig. 2 H), but the vast majority of brightly labeled areas appeared where cells overlapped with one another (Fig. 2, A–D, G, J). In many cases, brightly labeled areas were almost parallel to the substrate, revealing a distinct substructure (Fig. 2 J). There, N-CAM appeared as brightly labeled lines separated by unlabeled areas of membrane. The edges of these areas were often clearly defined. When they were not, they usually extended into the regions of cell-cell contact parallel to the z-axis (e.g., Fig. 2 I). I refer to these structures as "N-CAM clusters."

I performed several experiments to control for the possible effects of fixation on the appearance of N-CAM clusters. I obtained identical results with samples that had been fixed
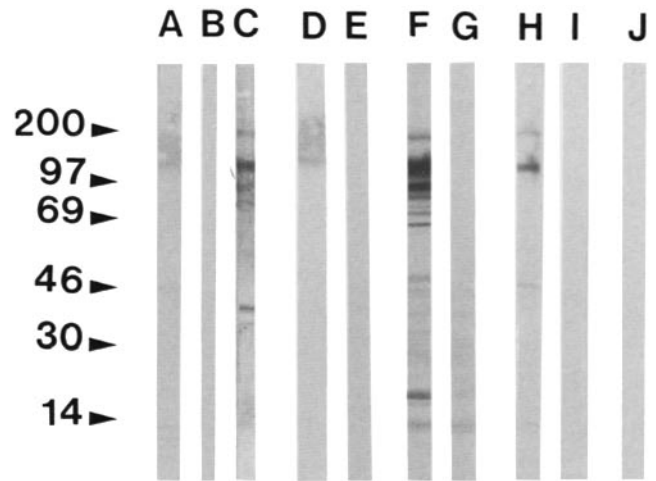
Figure 1. Immunoblotting of N-CAM in cultured cell lines. Cell cultures at ~70% confluency were scraped into sample buffer, boiled, and analyzed by SDS-PAGE in 5–15% acrylamide gels. Alternatively, cultures were scraped into a 1% NP-40 solution, pH 5, digested with neuraminidase, and then analyzed by SDS-PAGE (*C* only). Proteins were transferred electrophoretically to nitrocellulose paper and probed with a 1:1,000 dilution of rabbit anti-N-CAM antiserum (lanes *A, C, D, F, H, J*) or with normal rabbit serum (lanes *B, E, G, I*) at the same dilution, followed by a peroxidase conjugate of donkey anti–rabbit IgG. Peroxidase was visualized with 4-chloro-1-napthol and H_2O_2. (*A* and *B*) C2; (*C*) C2, extract digested with neuraminidase before boiling and SDS-PAGE; (*D* and *E*) Cho; (*F* and *G*) NRK; (*H* and *I*) PC12; (*J*) L929. Note that different samples were run on different gels and transferred to different nitrocellulose blots, so that alignment of one sample with another is only approximate. Calculated molecular weights of the bands recognized by anti-N-CAM are presented in Table II.

in ethanol (Figs. 2 *J* and 5 *C*) or acetone (not shown) at −20°C, or in paraformaldehyde in the presence of (Fig. 2, *A–F, H, I*), or followed by (Fig. 2 *G*), 0.5% Triton X-100 (see Materials and Methods). N-CAM clusters could not be labeled in cells that had not been permeabilized during or after fixation, suggesting that N-CAM epitopes in clusters were not readily accessible on the cell surface. Permeabilization with 0.2% saponin before fixation and immunolabeling

Table II. Molecular Weights of Bands Immunoblotted with anti-N-CAM

Cell	Molecular weights ($\times 10^{-3}$)*
C2	**115–200**;‡§ 52;‖ 20‖
Cs (neuraminidase-treated)	170, **140**, 115, 100,‖ 66,‖ 51, 47‖
CHO	120–225‡
NRK	190; **137**; 117; 111; 86;‖ 78; 58;‖ 23¶
PC12	215; **160**; 54‖
L929	ND**

* Results calculated for the bands seen in Fig. 1, based on molecular weight standards (206,000, 100,000, 68,000, 44,000, and 29,000) that were run simultaneously with each cell extract.
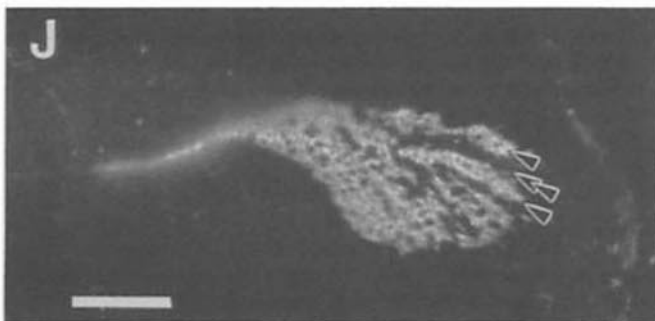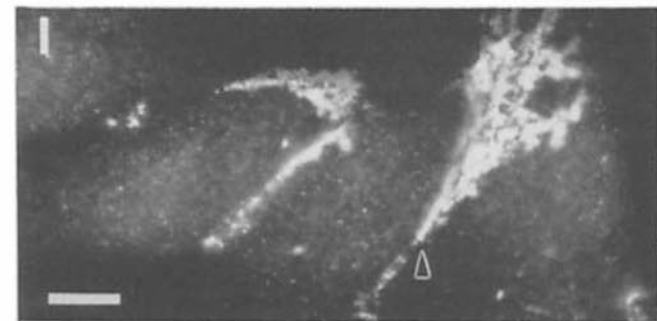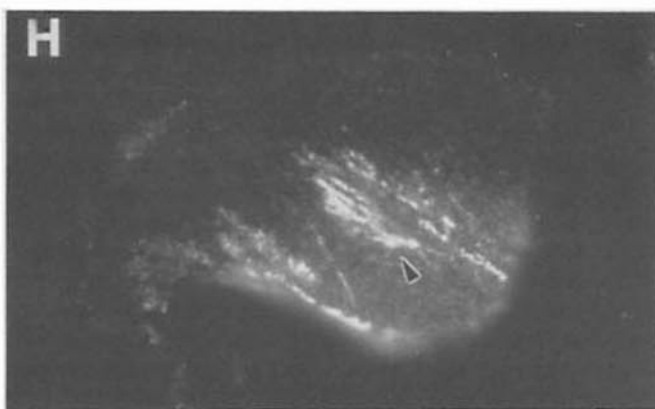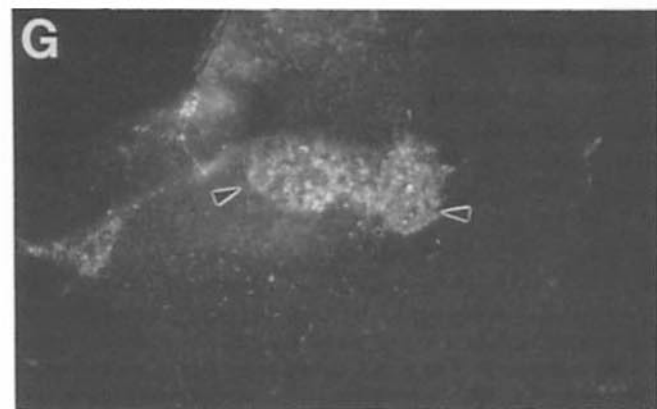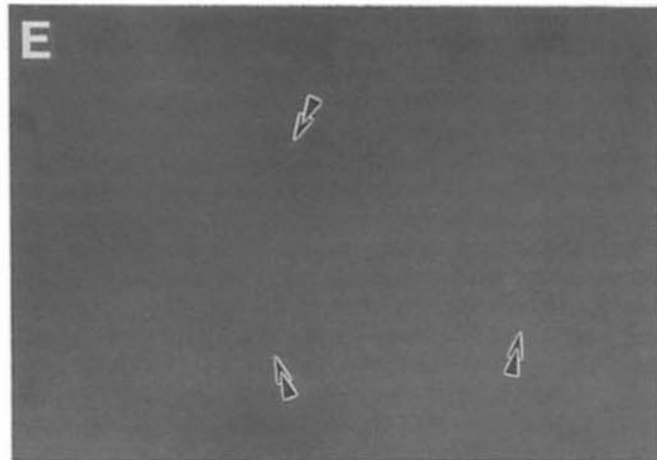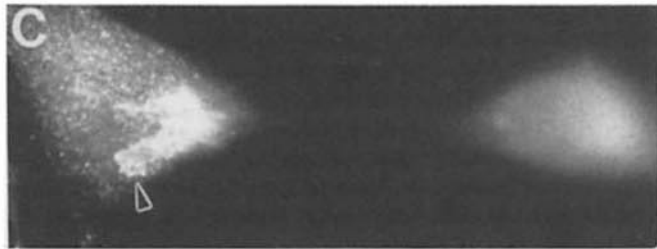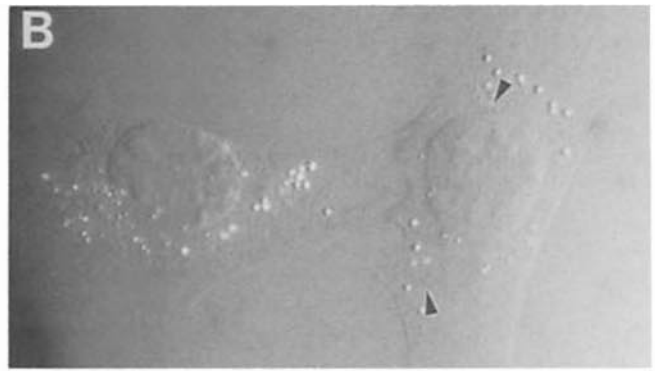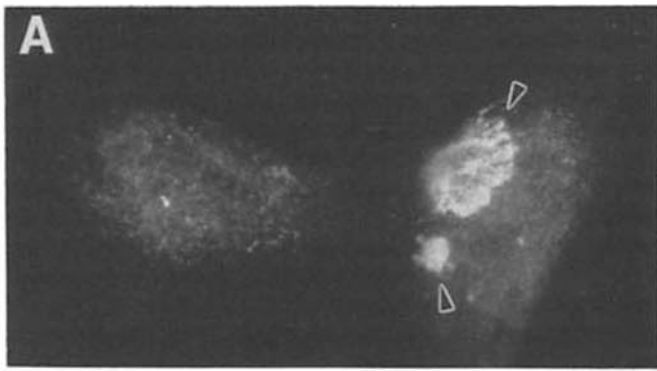‡ Range of apparent molecular weights for the broad band.
§ Values given in **bold** are those for the major band(s).
‖ Immunolabel was very faint and did not photograph well.
¶ A faint band at lower molecular weight (19,000) was also present in the blot exposed to nonimmune rabbit serum.
** ND, not detectable.
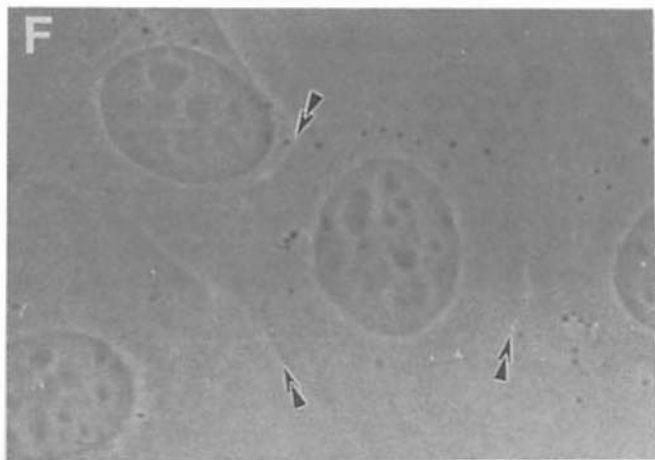
with rabbit anti-N-CAM revealed N-CAM clusters, but labeling in this case was not as bright as that obtained after permeabilization with organic solvents or Triton X-100 (not shown). Dot blot assays for N-CAM in the soluble fractions obtained after fixation with ethanol, acetone, paraformaldehyde, or paraformaldehyde plus Triton X-100, showed that N-CAM was not released in significant amounts from C2 cells by any of these fixatives (not shown). These results suggest that N-CAM clusters were not created artifactually by selective extraction of N-CAM from other regions of the cell, or by redistribution of N-CAM on the cell surface during fixation.

I did quantitative studies to characterize N-CAM clusters further. In C2 cells in log phase observed 1-2 d after replating, >85% of the contacts between C2 cells were enriched in N-CAM. I used a photometer attached to the fluorescence microscope to estimate the extent of enrichment of N-CAM at clusters. I labeled C2 cells in log phase with anti-N-CAM (polyclonal or monoclonal) followed by the appropriate fluorescent secondary antibody. I then measured the amount of fluorescence in N-CAM clusters, and compared these to nearby N-CAM–poor areas. After correcting for nonspecific labeling (see Materials and Methods), I found that N-CAM was enriched ∼4.5-fold in clusters relative to surrounding membrane (4.6 ± 0.7, mean ± SD; range, 3.7–5.3; $N = 4$ groups of 10 clusters each, in three separate experiments). Similar results were obtained for polyclonal and monoclonal anti-N-CAM antibodies. Measurements on C2 cells that overlapped without forming clusters showed no significant enrichment of labeling in the area of overlap. Thus, the enrichment of N-CAM at clusters cannot be accounted for by simple overlap of two cells bearing N-CAM on their surfaces.

Stability of N-CAM Clusters

I exposed cultures of C2 cells to several different conditions to determine if N-CAM clusters could be dispersed. I first tested the effects of metabolic inhibitors, Ca^{2+}-free conditions, colchicine, cytochalasin D, activators of protein phosphorylation, and heparin, which partially inhibits cell-cell aggregation dependent on N-CAM (Pizzey et al., 1989). Colchicine and cytochalasin D caused cells to become rounder; when this occurred, N-CAM remained concentrated in the clefts between the rounded cells (Fig. 3 A). Some cells exposed to these reagents also developed numerous smaller patches of N-CAM (Fig. 3 A), but most treated cells still had N-CAM clusters (Table III). Overnight incubation of C2 cells with inhibitors of cellular energy metabolism

(10 mM sodium azide, 10 mM 2-deoxyglucose, 1 µM oligomycin, and oligomycin or azide plus 2-deoxyglucose) also failed to disrupt N-CAM clusters (Table III). N-CAM clusters were stable for 4 h or overnight (16–23 h) in the presence of dibutyryl AMP, phorbol esters, and heparin (Table III).

The only method I have so far found to disrupt N-CAM clusters is dissociation of cultured cells with trypsin-EDTA. Before dissociation, most of the cells grew in small groups, and most had N-CAM clusters. C2 cells replated after treatment with trypsin-EDTA usually did not show clusters of N-CAM if they did not contact other cells, indicating that dissociation by trypsin-EDTA dispersed most of the clusters present in the culture before replating. However, cells rapidly recovered their clusters once they came into contact following replating (e.g., Fig. 3 C). Cells fixed and labeled 30 min after replating (i.e., shortly after they reattached to the substrate) were observed for the number of cell-cell contacts, and for those contacts that showed N-CAM clusters. Only ∼35% of the cells showed contacts, but, of these 60, 70, and 88% (in three experiments) showed N-CAM clusters. These clusters could not have been retained because of inadequate dissociation of the cells, as most (>90%) of the cells in the cell suspension before replating were single cells. These observations suggest that dissociation of cells by trypsin-EDTA disperses most N-CAM clusters, and that the reformation of N-CAM clusters occurs soon after replating of C2 cells.

I tried to prevent the reformation of N-CAM clusters in two experiments with freshly replated C2 cells. I found no significant inhibition of N-CAM clustering by either heparin or cycloheximide (Table IV). Thus, although trypsin-EDTA probably removes significant amounts of N-CAM from the cell surface, new protein synthesis is not needed in order for N-CAM clusters to reform.

The reformation of N-CAM clusters was, however, inhibited by anti-N-CAM Fab fragments but not by Fab fragments prepared from nonimmune IgG (Fig. 3, B and C; Table IV). Appropriate controls showed that anti-N-CAM Fab fragments did not block the labeling of N-CAM clusters by 5B8 after clusters had reformed. Thus, anti-N-CAM Fab fragments, which block homotypic interactions of N-CAM and N-CAM-mediated cell-cell adhesion (e.g., Thiery et al., 1977; Rutishauser et al., 1982), also inhibit de novo clustering of N-CAM.

Absence of Other Proteins at Clusters

I tested a number of antibodies (Table I) to learn if any other membrane, cytoskeletal, or extracellular proteins were en-

Figure 2. N-CAM clusters of C2 cells in log phase. C2 cells in log phase, 1-2 d after subculturing, were fixed and permeabilized, then labeled with polyclonal rabbit or monoclonal antibodies to N-CAM or normal rabbit serum, followed by RGAR or FGAM. Samples were visualized under Nomarski (*B* and *D*), phase (*F*), or fluorescence (*A, C, E, G–J*) optics. *A–F, H,* and *I* were fixed in paraformaldehyde in the presence of 0.5% Triton X-100, fixed for an additional 15 min in paraformaldehyde, and labeled with anti-N-CAM and RGAR. *A–D* show pairs of cells labeled with rabbit anti-N-CAM. Clusters are found at sites of cell-cell contact (*arrowheads*). *E* and *F* show a group of cells labeled with normal rabbit serum; no clusters appear at sites of cell-cell contact (*double arrowheads*). *H* shows a single cell with an N-CAM cluster (*arrowhead*). *I* shows a small group of cells with N-CAM clusters that extend into the cleft between cells that lies along the z-axis (e.g., *arrowhead*), parallel to the direction of observation. (*G*) Fixed for 15 min in paraformaldehyde, permeabilized for 2 min with 0.5% Triton X-100, then labeled with rabbit anti-N-CAM and RGAR. N-CAM clusters appear similar to those in *A, C,* and *I.* (*J*) A culture was fixed and permeabilized with 95% ethanol at −20°C, and labeled with mab AG1 and FGAM. A typical N-CAM cluster is shown at higher magnification. Note the fine, web-like substructure, with N-CAM -rich areas (*arrowheads*) separated by areas containing little immunofluorescence signal (*double arrowhead*). Bar in *I* refers to *A–I;* bar in *J* is for *J* only; both bars represent 10 µm.
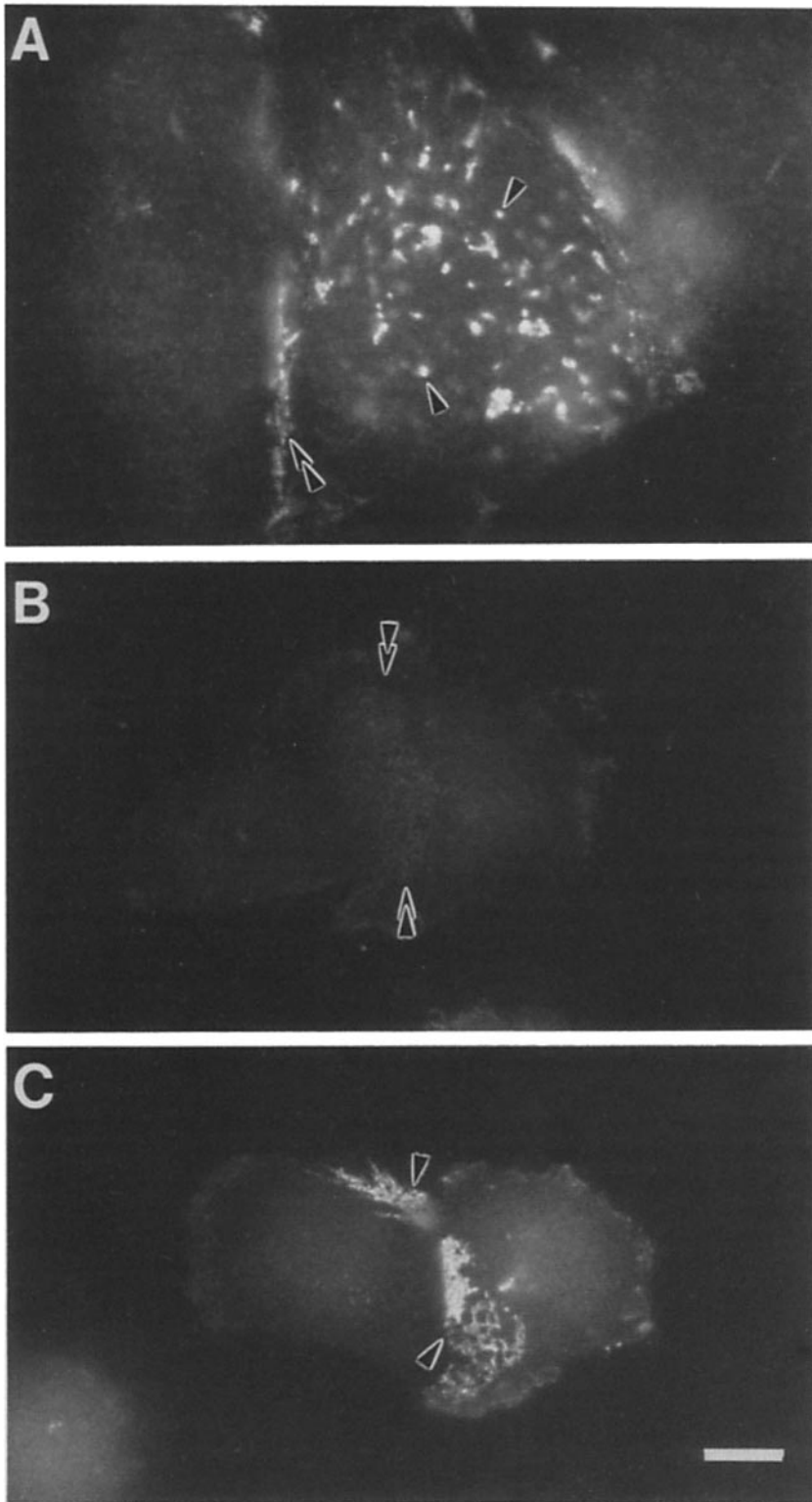
Figure 3. Stability of N-CAM clusters. (*A*) C2 cells were incubated overnight in medium containing 10 μM colchicine, washed, fixed with paraformaldehyde in the presence of 0.5% Triton X-100, fixed for 15 min more in paraformaldehyde, and labeled with anti-N-CAM and RGAR. Note the many bright spots of N-CAM label (e.g., *arrowheads*), and labeling in the cleft between cells (*double arrowhead*). (*B*) C2 cells were removed from the substrate with trypsin-EDTA, centrifuged briefly, resuspended in fresh medium, and replated onto coverslips in the presence of 0.5 mg/ml rabbit anti-N-CAM Fab. After 30 min, cultures were fixed and permeabilized as in *A*, and labeled with mab 5B8 anti-N-CAM and FGAM. Cells have attached firmly to the substrate, but N-CAM clusters have not reformed at cell-cell contacts (*double arrowheads*). Appropriate controls showed that anti-N-CAM Fab fragments did not block the labeling of N-CAM clusters by 5B8 after clusters had been allowed to reform. (*C*) As in *B*, but cells replated in the presence of 0.5 mg/ml nonimmune rabbit Fab. A pair of cells, not yet fully spread, has already formed N-CAM clusters (*arrowheads*). Bar, 10 μm.

riched at N-CAM clusters. I used double immunofluorescence to compare the labeling by each of these antibodies directly with that of N-CAM, visualized with either rabbit anti-N-CAM or with mab 5B8.

None of the antibodies to other membrane proteins reliably labeled N-CAM clusters. Anti-Thy-1 gave an almost uniform labeling of C2 cells (Fig. 4 *B*). Antiintegrins labeled C2 cells (Bloch, R. J., T. Volberg, and B. Geiger, manuscript in preparation) as described for other cells grown on a glass substrate in the presence of serum (Singer et al., 1988; Fath et al., 1989), and were not enriched at N-CAM clusters (not shown). Fluorescent lectins (concanavalin A, wheat germ agglutinin, and ricin agglutinin) also failed to label N-CAM clusters preferentially (not shown). These results suggest that

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Table III. Stability of N-CAM Clusters

Treatment or drug	Contacts with N-CAM clusters	
	4 h	Overnight
	%	%
Control (no treatment)	100	88
Ca²⁺-free medium	—*	80
Heparin, 1 mg/ml	98	98
Sodium azide, 10 mM	92	94
Oligomycin, 1 μM	92	90
2-Deoxyglucose, 10 mM	100	88
Azide + 2-deoxyglucose‡	94	86
Oligomycin + 2-deoxyglucose‡	98	100
Phorbol methylacetate (1 μg/ml)	82	94
Phorbol dibutyrate (10 μM)	90	86
Dibutyryl cAMP (1 mM)	78	94
Colchicine (10 μM)	90	86
Cytochalasin D (1 μM)	78	84
Colchicine + cytochalasin D‡	94	88

C2 cells in log phase were exposed to the conditions or drug listed for 4 h or overnight. After this time, cells were permeabilized, fixed, and labeled with anti-N-CAM, and 50 cell-cell contacts for each treatment were examined for N-CAM clusters. At most, only small effects were observed. Similar results were obtained in two other experiments. None of the effects of the treatments were judged to be significantly different from control.
* Not determined.
‡ Concentrations used are those given above in the table.

the clusters of N-CAM at sites of contact between C2 cells are not generally enriched in membrane components.

Many antibodies to cytoskeletal proteins or to proteins of the extracellular matrix also failed to label N-CAM clusters (see Table I for complete list). N-CAM clusters were not enriched in microfilaments, as indicated by negative results with antibodies to actin-associated proteins (e.g., myosin, α-actinin, filamin), and with fluorescent derivatives of phalloidin. N-CAM clusters were also not associated with microtubules, intermediate filaments, peripheral membrane proteins (spectrin, band 4.1, clathrin, or fodrin: e.g., Fig. 4, C and D), or with proteins found at sites of focal cell-

Table IV. Reformation of N-CAM Clusters

Treatment or drug	Contacts with N-CAM clusters
	%
Experiment 1	
Control (no treatment)	58
Heparin, 1 mg/ml	50
Experiment 2	
Control (no treatment)	76
Cycloheximide (10 μg/ml)	84
Nonimmune Fab (0.5 mg/ml)	82
Anti-N-CAM Fab (0.5 mg/ml)	28

C2 cells were removed from the tissue culture substrate with trypsin-EDTA, centrifuged, resuspended in fresh medium containing the substance listed, and replated on fresh coverslips for 30 min at 37°C. After this time, cells were simultaneously fixed and permeabilized with paraformaldehyde plus Triton X-100 (experiment 1) and fixed further with paraformaldehyde alone, or fixed with ethanol at −20°C (experiment 2). Samples were labeled with rabbit anti-N-CAM and RGAR (experiment 1) or mab 5B8 anti-N-CAM and FGAM (experiment 2). Fifty cell-cell contacts for each treatment were examined for N-CAM clusters. Only anti-N-CAM Fab prevented the reformation of N-CAM clusters. Similar results were obtained in two other experiments.

substrate contacts (vitronectin receptor, talin, vinculin; e.g., Fig. 4, E and F). Examination of C2 cells by interference reflection microscopy, which reveals sites of close adhesion to a glass substrate, showed no correlation between sites of substrate adhesion and sites of N-CAM clustering (not shown). N-CAM clusters were not enriched in any of four extracellular proteins commonly found at or near the cell surface (Table I), including heparan sulfate proteoglycan (Fig. 4, G and H), which can bind N-CAM (Cole et al., 1986; Cole and Akeson, 1989; Probstmeier et al., 1989). The possibility remains that isoforms of these proteins that are not recognized by the antibodies I used are present at N-CAM clusters. Nevertheless, these observations are consistent with the idea that N-CAM clusters do not form in response to interactions of C2 cells with the tissue culture substrate or with other extracellular structures. They further suggest that clustering is not mediated by a common form of a cytoskeletal protein.

N-CAM Clusters in Myogenesis

All the experimental results summarized above were obtained with C2 cells in log phase growth, usually 1–2 d after subculturing. As N-CAM is believed to play a role in the fusion of myoblasts to form myotubes (Dickson et al., 1990; Knudsen et al., 1990), I studied its distribution in older, fusing cultures, and in myotubes. I found that many contacts between elongated and aligned C2 myoblasts and between pairs of C2 myotubes were also enriched in N-CAM (Fig. 5). These contacts did not appear in a single plane of focus, so I could not determine if the accumulations of N-CAM at these contacts had a fine structure similar to the clusters seen in some C2 cells in log phase growth. They did, however, resemble the accumulations of N-CAM in log phase cultures that appear to lie along the z-axis, i.e., along the axis of observation (e.g., Fig. 2 I).

## N-CAM at Other Cell-Cell Contacts

N-CAM is present in many types of cells during embryogenesis (Thiery et al., 1982). I wished to learn if N-CAM clusters also formed in other cell types in tissue culture, and so examined NRK, CHO, and PC12 cells. N-CAM in PC12 cells (Fig. 1 D, lane I) has already been described (Prentice et al., 1987). Although N-CAM has been found in developing epithelia and mesonephros (Thiery et al., 1982), N-CAM has not yet been described in NRK or CHO cells. CHO and NRK cells contained significant amounts of N-CAM, or molecules immunologically related to N-CAM, as determined by SDS-PAGE and immunoblotting (Fig. 1; Table II). The pattern seen in immunoblots of CHO cells resembled that seen with C2 cells, with a large, diffuse band in the range of 120,000–225,000 (Table II). By contrast, immunoblots of NRK cells showed distinct immunoreactive polypeptides of high molecular weights, with smaller amounts of immunoreactive polypeptides in the range of 23,000–86,000. Small amounts of these lower molecular weight polypeptides that reacted with anti-N-CAM were also seen in extracts of C2 and PC12 cells. These may be proteolytic fragments of N-CAM. Further work with NRK cells will be required to characterize these smaller molecules and to determine their relationship to the larger forms of N-CAM that predominate. As they are relatively minor components, however, they are not
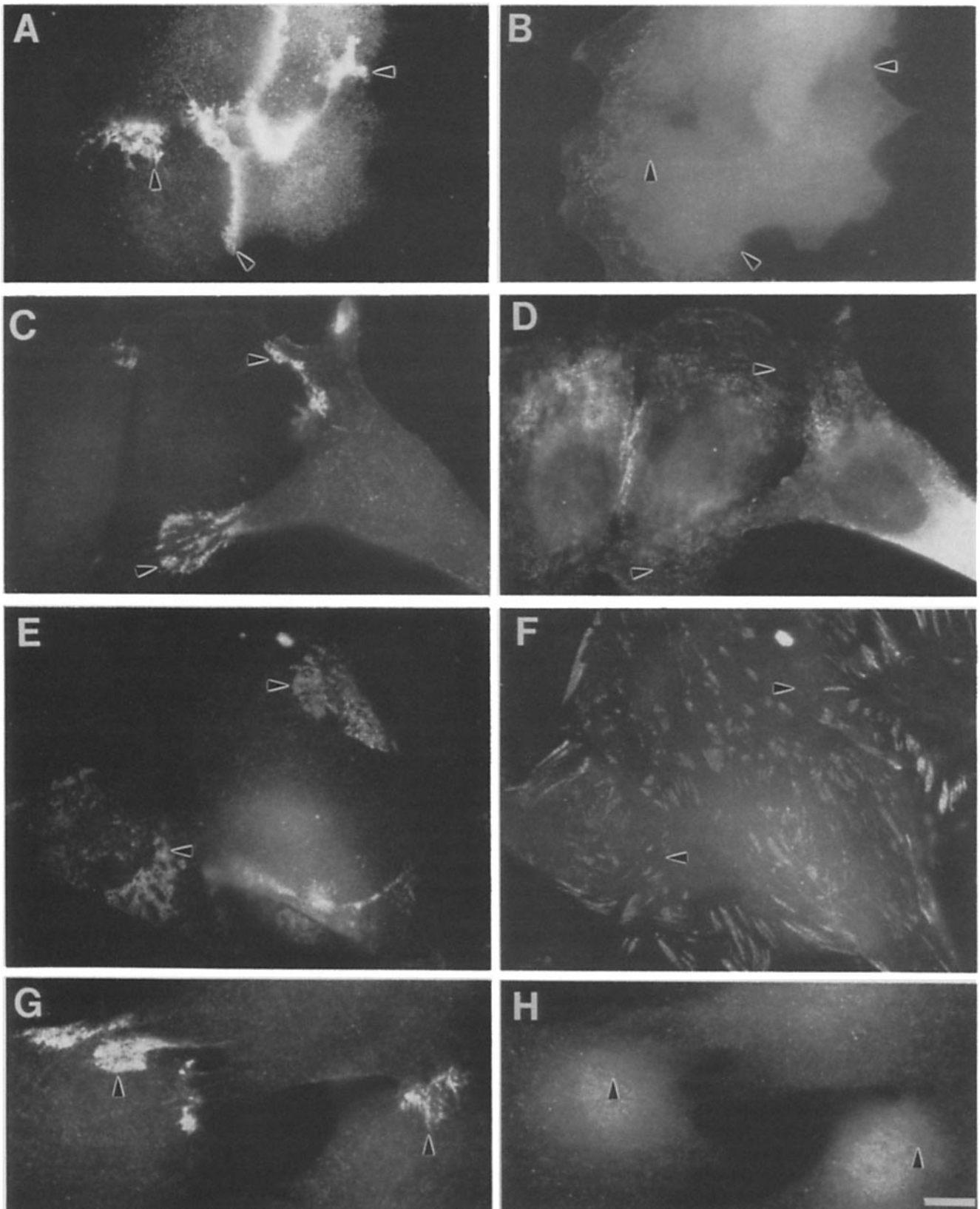
Figure 4. N-CAM clusters examined for other cytoskeletal, extracellular, and membrane markers. C2 cultures were fixed with cold ethanol (*C* and *D*), or fixed in paraformaldehyde in the presence of Triton X-100 and fixed 15 min more in paraformaldehyde alone (*A, B, E–H*; see Materials and Methods). Samples were labeled with rabbit or mouse anti-N-CAM together with antibodies to Thy-1, fodrin, vinculin, or heparan sulfate proteoglycan. Anti-N-CAM and other antibodies were visualized with appropriate fluorescent anti-antibodies. Arrowheads points to the same points in each pair of panels. All fluorescein-rhodamine pairs were photographed without changing focus. (*A* and *B*) N-CAM cluster, labeled with rabbit anti-N-CAM (*A*) is not labeled with a mAb to Thy-1 (*B*). (*C* and *D*) mAb AG1 labels an N-CAM
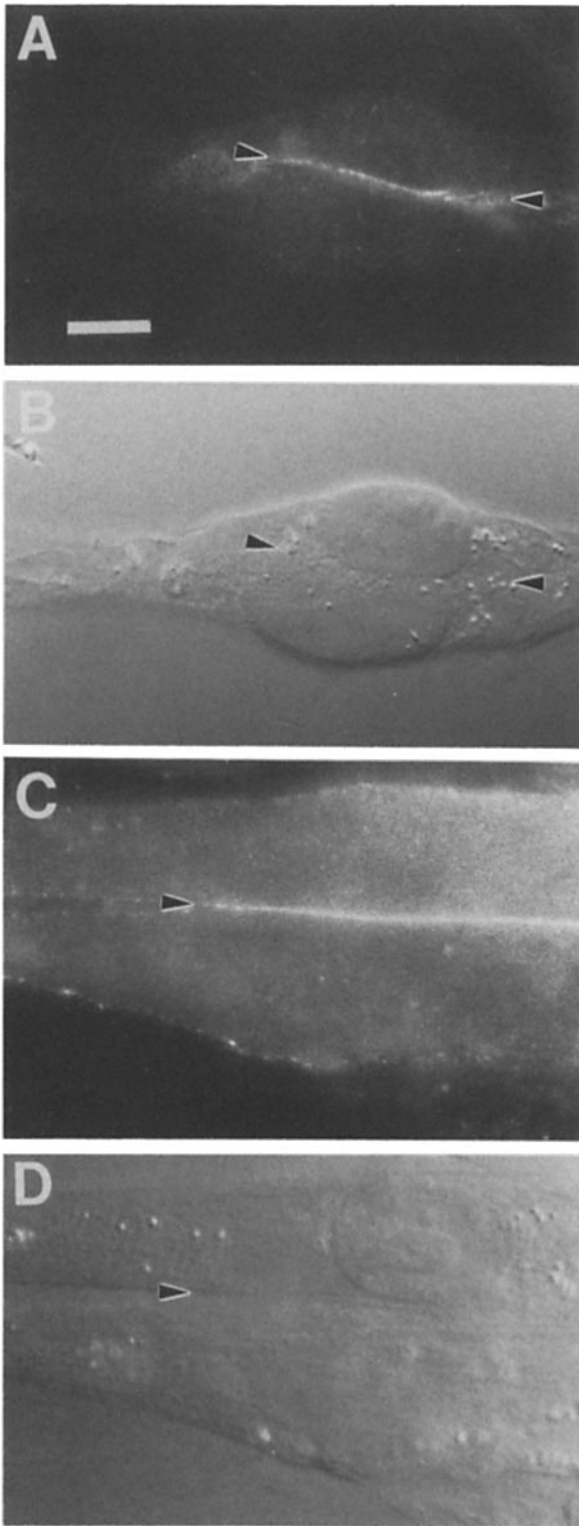
likely to contribute significantly to the immunofluorescence data presented below.

PC12 cells grow as small groups of cells that are usually only loosely attached to the substrate. In PC12 cells, enrichment of N-CAM resembled that seen between fusing C2 myoblasts and myotubes (Fig. 6 *D*), as it was clearly enriched in areas of cell-cell contact oriented along the z-axis, parallel to the direction of observation. Such accumulations tended to be more common between cells at the center of the group and less common between cells near the periphery.

NRK cells, and especially CHO cells, formed N-CAM clusters that more closely resembled those seen in C2 cells in log phase. NRK cells grew as small islands of closely associated cells. N-CAM accumulated almost uniformly around the edges of these cells where they contacted one another (Fig. 6 *B*) but not at edges that were free (not shown). These N-CAM clusters were not as broad as those of C2 cells, perhaps because the extent of cellular overlap is less in NRK cultures. CHO cells formed larger, broader N-CAM clusters, like those seen in C2 cells (Fig. 6 *A*). These results suggest that if a particular type of cell makes significant amounts of N-CAM, N-CAM clusters will form at sites of cell-cell contact (Table V).

I wanted to determine how labeling by anti-N-CAM would appear in a culture of cells that did not have significant amounts of N-CAM, and so examined L929 cells. L929 cells contain no N-CAM by detectable immunoblotting (Fig. 1, lane *J*; Table II). When labeled for immunofluorescence observations, these cells did not display clusters (Fig. 6 *C*; Table V).

The immunofluorescence results summarized in Fig. 6 were all obtained with polyclonal rabbit anti-N-CAM. I obtained identical results when I examined CHO, NRK, and L929 cells that had been fixed in ethanol at −20°C and labeled with the mAb anti-N-CAM antibodies, 5B8 and AG-1 (not shown).

N-CAM at Heterologous Cell-Cell Contacts

I next examined the distribution of N-CAM in co-cultures of C2 cells and either NRK, CHO, PC12, or L929 cells. In mixed cultures containing C2 cells and either NRK, CHO, or L929 cells, the C2 cells were labeled with biotin before plating. Subsequent incubation with fluoresceinated streptavidin readily distinguished C2 cells from the other cell types in the culture. PC12 cells and C2 cells could be easily distinguished from one another by their different morphologies.

Figure 5. N-CAM accumulates at contacts between aligning myoblasts, and between myotubes. C2 cells were cultured for several days in medium to induce myogenesis, fixed in the presence of Tri-ton X-100, and labeled with anti-N-CAM followed by RGAR. Cultures were viewed under rhodamine (*A* and *C*) and Nomarski (*B* and *D*) optics. (*A* and *B*) N-CAM accumulates in the cleft between aligned myoblasts (*arrowheads*). (*C* and *D*) N-CAM accumulates at contacts between myotubes (*arrowheads*). Bar, 10 μm.

cluster (*C*) that is not significantly labeled by rabbit antifodrin (*D*). (*E* and *F*) Rabbit anti-N-CAM labels N-CAM clusters (*E*) that are not significantly labeled by mouse mAb 11.5 antivinculin (*F*). Antibodies to talin and to the vitronectin receptor gave results similar to those with antivinculin (not shown). (*G* and *H*) Rabbit anti-N-CAM labels N-CAM clusters (*G*) that are not labeled by a mouse mAb against heparan sulfate proteoglycan (*H*). Bar, 10 μm.
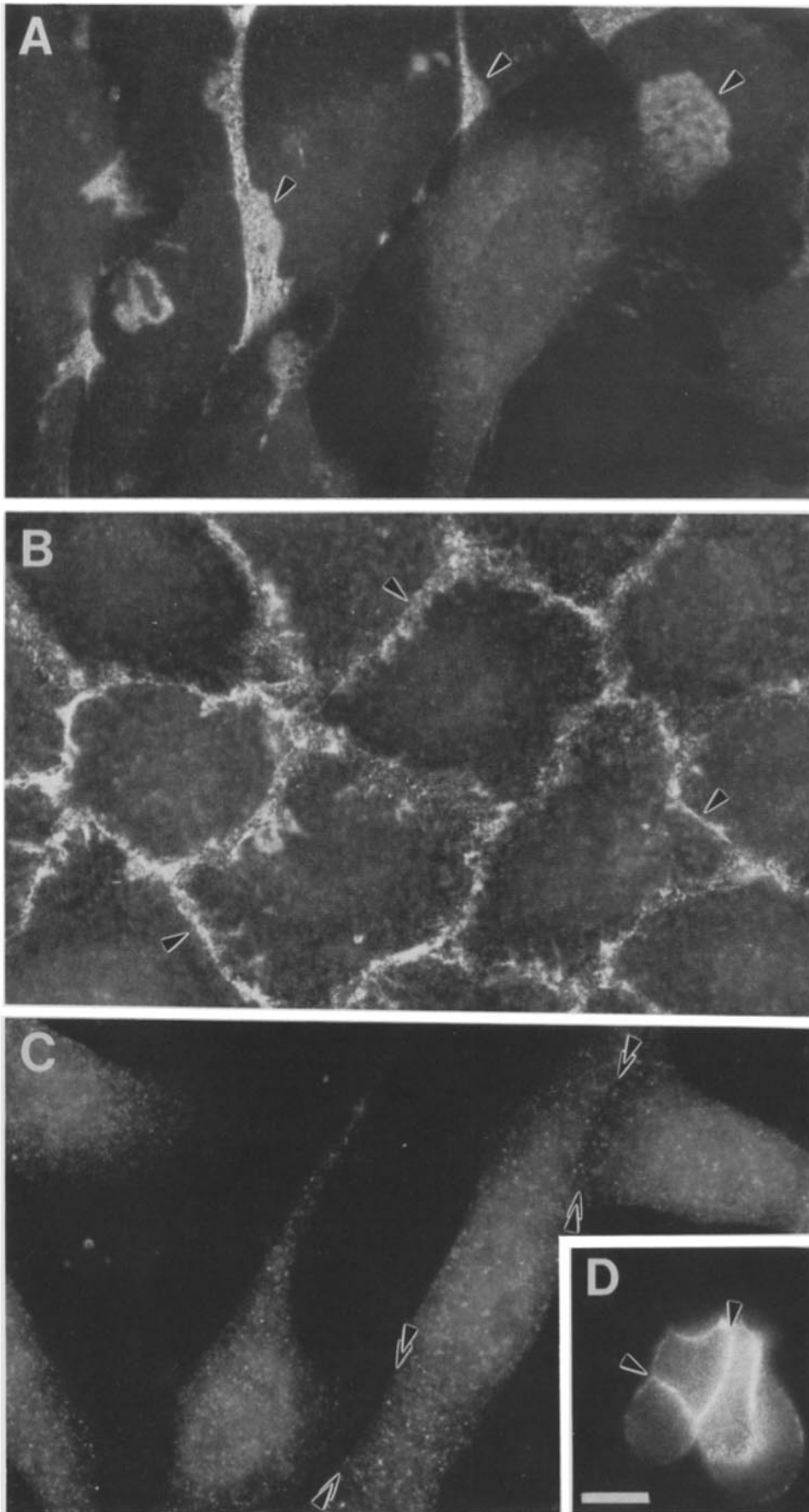
Figure 6. N-CAM clusters form at cell-cell contacts made by other types of cells. Cultures of CHO, NRK, PC12, and L929 cells were fixed in the presence of Triton X-100, fixed further with paraformaldehyde, and labeled with anti-N-CAM followed by RGAR. (*A*) CHO cells have N-CAM clusters resembling those of C2 cells (*arrowheads*). (*B*) NRK cells grow closely packed, and assume polygonal shapes. N-CAM accumulates where the edges of two cells appear to come together (*arrowheads*). (*C*) L929 cells do not form N-CAM clusters at sites of cell-cell contact (*double arrowheads*). (*D*) PC12 cells accumulate N-CAM at clefts between rounded cells (*arrowheads*). Bar, 10 μm.

Table V. N-CAM Clusters at Homologous and Heterologous Cell-Cell Contacts

Type of cell–cell contact	Cell–cell contacts with N-CAM clusters
	%
C2/C2	88
C2/bC2	78
CHO/CHO	98
CHO/bC2	64
NRK/NRK	88
NRK/bC2	52
L929/L929	0
L929/bC2	10

C2 cells in log phase were biotinylated, removed from the substrate with trypsin-EDTA, centrifuged briefly, and resuspended in DMEM containing 10% FCS. One or two drops of the suspension were applied to coverslips containing cultures of NRK, CHO, L929, or C2 cells, in ~0.4 ml of medium. Other coverslips with cultures of these cells were left unchanged. Cultures were incubated overnight, and then were permeabilized, fixed, and labeled with anti-N-CAM, followed by RGAR, and F-SAv when appropriate. Cell–cell contacts in control cultures, and between biotinylated C2 cells (bC2) and the other cell type in mixed cultures, were examined for N-CAM clusters. In most cases, 50 such contacts were examined per culture to obtain the results given here. Similar results were obtained in 2 other experiments.

I tested the effect of biotinylation on the ability of C2 cells to form N-CAM clusters by culturing biotinylated and untreated C2 cells together. The mixed cultures formed N-CAM clusters almost as well as did controls alone (Fig. 7, *A* and *B*; Table V). These samples also permitted direct comparison of the extent of N-CAM clustering with the extent of overlap between neighboring C2 cells. N-CAM clusters usually filled most of the area of cell-cell overlap (Fig. 7, *A* and *B*). When the edges of the biotinylated cell could be clearly discerned in the vicinity of an N-CAM cluster, they were often lamellipodial in shape (e.g., Fig. 7 *B*, *arrowheads*). The outline of the biotinylated cells at N-CAM clusters was not always clearly visible, however, suggesting that some contacts occurred at thin extensions of one C2 cell over or under another.

When I prepared mixed cultures of biotinylated C2 cells with NRK or CHO cells, N-CAM accumulated at many, but not all, sites of heterologous cell-cell contact (Fig. 7, *C–F*; Table V). Mixed cultures of C2 myoblasts and PC12 cells also formed N-CAM clusters (Fig. 7, *I* and *J*), but, because PC12 cells usually grow as large aggregates, results with mixed PC12-C2 cultures were hard to quantitate. When I cultured C2 cells with L929 cells, N-CAM was not significantly enriched at sites of intercellular contact (Fig. 7, *G* and *H*; Table V).

Discussion

I began the experiments described above with the aim of learning if N-CAM accumulates at sites of cell-cell contact. N-CAM is, perhaps, the best characterized of the cell adhesion molecules, and some evidence suggests that it can accumulate at adherent surfaces of cells in suspension (e.g., Pizzey et al., 1989). In the nervous system, the distribution of N-CAM can be patchy, with high concentrations of the molecule found at some synapses (Rieger et al., 1985; Covault and Sanes, 1986; Persohn and Schachner, 1987; DiFiglia et al., 1989). Clustering of N-CAM at cell-cell con-

tacts, if it occurred, could therefore be an important stage in cell-cell recognition and synaptogenesis.

My results strongly suggest that N-CAM is significantly enriched at sites of cell-cell contact. In cultures of C2, NRK, and CHO cells, clusters of N-CAM are seen at almost all sites where pairs of cells overlap or come into close proximity, and rarely form on single cells or on regions of paired cells that are not in close proximity. In addition, where the outline of one cell against the other is clear (e.g., Fig. 7), N-CAM clusters are contained within the overlapping area. Labeling of clusters with anti-N-CAM antibodies is only possible after permeabilization with detergents or organic solvents, suggesting that extracellular epitopes are normally not accessible. This is consistent with the idea that N-CAM clusters form at sites of close cell-cell contact. Although ultrastructural evidence will be needed to prove this, the indirect evidence that this is the case seems compelling.

Quantitatively, N-CAM clusters appear to be enriched about 4.5-fold in N-CAM relative to surrounding membrane areas, suggesting that N-CAM does not appear clustered simply because two cells overlap. Simple overlap would be expected at most to double the level of fluorescence, but overlapping regions of C2 cells that do not form N-CAM clusters show levels of specific fluorescence due to N-CAM similar in intensity to nonoverlapping regions. A 4.5-fold enrichment of N-CAM at clusters is less than one might expect from many of the immunofluorescence micrographs. One possible reason for this is that N-CAM clusters contain significant areas of membrane that are not enriched in N-CAM (e.g., Fig. 2 *J*). The 5 μm diameter area sampled by the photomultiplier tube would include such N-CAM-poor areas. The photographic procedures we normally use would also tend to enhance the contrast between N-CAM-rich and N-CAM-poor areas. Still another possibility, that N-CAM remains selectively enriched in clusters because these are more stable during detergent extraction and fixation, is highly unlikely, for several reasons. First, it would not explain the apparent discrepancy between the quantitative measurements and the micrographs, which were made on samples that were processed similarly. Second, quantitations with mAb anti-N-CAM, on samples that were fixed and permeabilized with ethanol, and with polyclonal anti-N-CAM, on samples that were fixed with paraformaldehyde and permeabilized with detergent, gave similar results. These different fixation and permeabilization protocols might be expected to create different artifacts, but they yield qualitatively and quantitatively similar results. Finally, I could detect no N-CAM in any of the extracts generated by the different fixation and permeabilization I followed. Thus, N-CAM clusters are real structures; they are not created by selective loss or redistribution of N-CAM from other areas of the cell surface.

Clusters of N-CAM are not enriched in other membrane markers. This suggests that N-CAM clusters cannot be due to nonspecific trapping of membrane proteins or of antibodies. N-CAM clustering does not occur in response to substrate adhesion, or to adhesion to cells that do not bear N-CAM. N-CAM clustering must therefore involve the specific recruitment of N-CAM in response to a specific stimulus. As discussed below, this stimulus is probably the proximity of a second N-CAM-bearing cell.

The unusual structure of N-CAM clusters is consistent
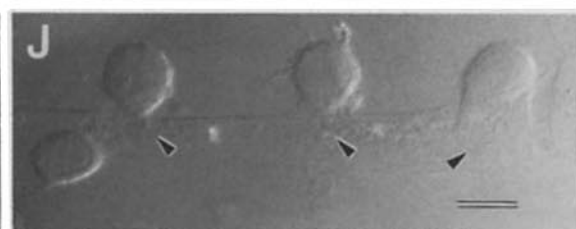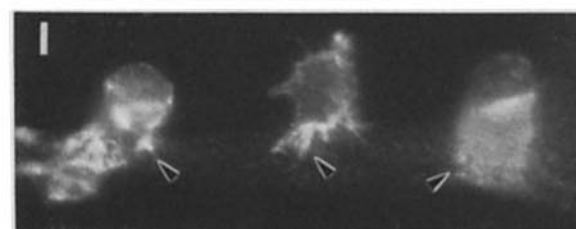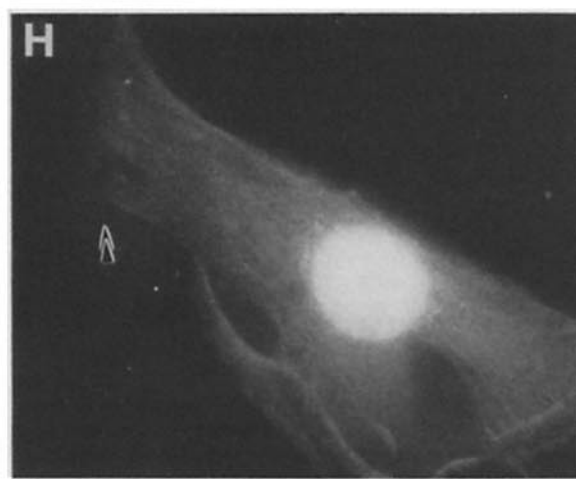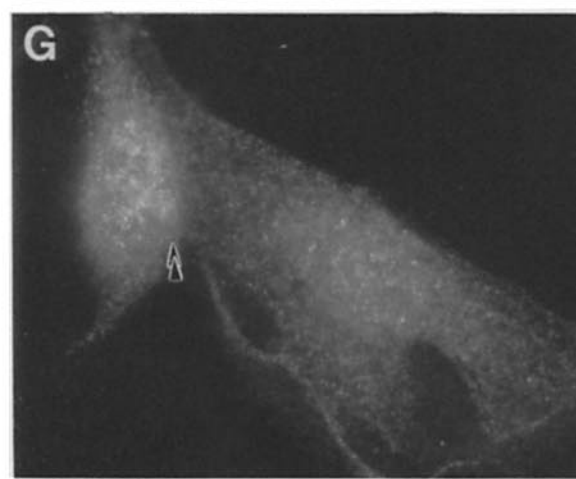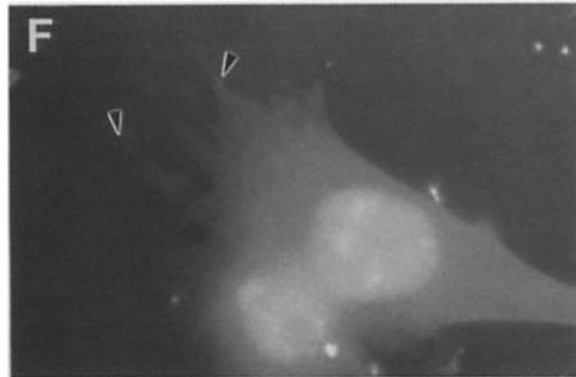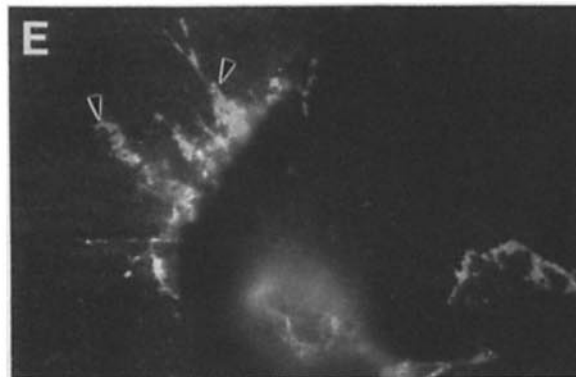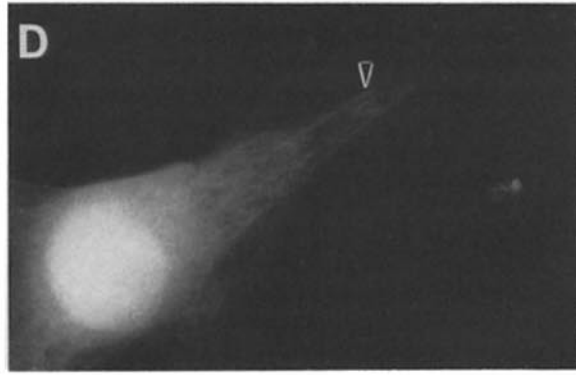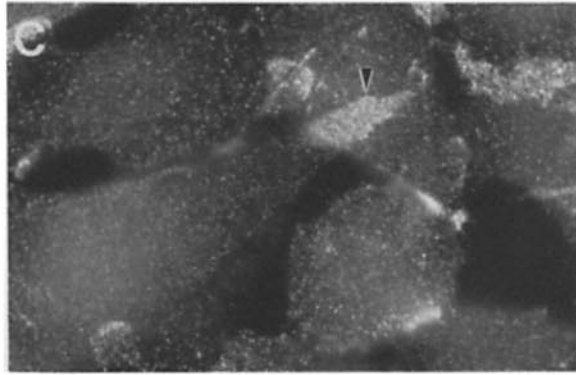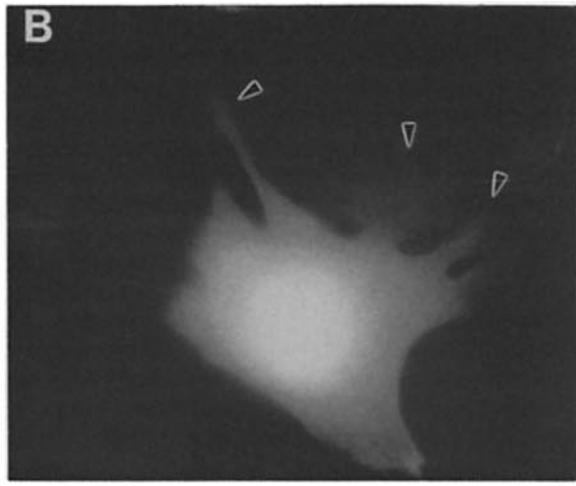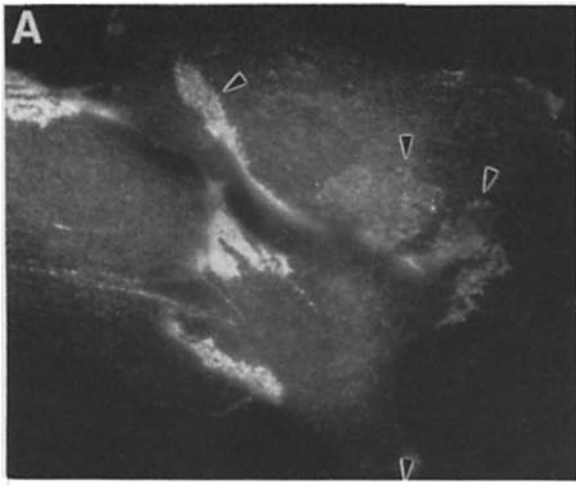
with their forming in a distinctive way. When they appear almost parallel to the substrate, N-CAM clusters usually resemble an irregular meshwork. There is no a priori reason to suppose that N-CAM clusters would contain relatively large areas of N-CAM-free membrane. Such an organization of N-CAM clusters may be due to the influence of other proteins. N-CAM can bind with brain spectrin or fodrin (Pollerberg et al., 1986, 1987), and with heparan sulfate proteoglycan and collagen (Cole et al., 1986; Cole and Akeson, 1989; Probstmeier et al., 1989), but these do not appear to accumulate at N-CAM clusters (Fig. 5). None of the many other peripheral membrane proteins and proteins of the cytoskeleton and extracellular matrix that I tested appear enriched at N-CAM clusters. Such proteins may, however, be present at N-CAM clusters but remain undetected by the antibodies I used.

Once they form, N-CAM clusters are unusually stable. N-CAM clusters in cultures of C2 cells are not disrupted by a variety of treatments that break up accumulations of other integral membrane proteins (Raff and DePetris, 1973; Yahara and Edelman, 1973; Karnovsky and Unanue, 1973; Ukena et al., 1974; Schreiner and Unanue, 1976; Bourguignon and Kerrick, 1983), or by heparin, which binds to N-CAM (Cole et al., 1986; Cole and Akeson, 1989; Probstmeier et al., 1989). Heparin does not interfere with homotypic interactions between N-CAM molecules (Moran and Bock, 1988), but it does reduce N-CAM-dependent aggregation of cells (Cole et al., 1986; Pizzey et al., 1989). N-CAM clusters are much stabler than the acetylcholine receptor (AChR) clusters of cultured rat myotubes. The latter are disrupted by several treatments which leave N-CAM clusters intact, including inhibition of energy metabolism (Bloch, 1979), Ca^{2+}-free conditions (Bloch, 1983; Bursztajn et al., 1983), and phorbol esters (Ross et al., 1988). Clustered AChRs are closely associated with cytoskeletal and extracellular matrix proteins (reviewed by Bloch and Pumplin, 1988) which appear to be absent from N-CAM clusters. This is consistent with the idea that N-CAM clusters form by a mechanism different from that responsible for clustering AChR.

The factor most likely to be responsible for the formation, and, perhaps, for the stability of N-CAM clusters, is cell-cell contact. N-CAM clusters form at most homotypic cell-cell contacts, and at many heterotypic contacts (Table V). They do not form at sites of adhesion to the substrate or to cells poor in N-CAM. This suggests that, for clusters to form, N-CAM present on the surface of one cell must interact with N-CAM on the opposing cell surface. This is the type of interaction expected of a ligand, like N-CAM, that is involved

in homotypic adhesion (Hoffman and Edelman, 1983; Hoffman et al., 1982). If the amounts of N-CAM on opposing cell surfaces are not limiting, and if N-CAM is mobile, homotypic interactions should result in extensive concentration of N-CAM at sites of cell-cell contact, which would serve as "diffusion traps" (e.g., Edwards and Frisch, 1976; Axelrod, 1983). The large, stable clusters of N-CAM at sites of cell-cell contact, described here for the first time, confirm this prediction. The observation that anti-N-CAM Fab fragments, which inhibit homotypic interactions and cell-cell adhesion mediated by N-CAM (e.g., Thiery et al., 1977; Rutishauser et al., 1982), block the reformation of N-CAM clusters is also consistent with this prediction.

The notion that homotypic interactions are necessary for N-CAM clustering can be challenged because occasionally a single C2 cell has an N-CAM cluster. These rare examples may have been pairs of cells that were difficult to distinguish from a single cell, single cells interacting with small membrane fragments containing N-CAM, or cells that had recently interacted with another cell but separated, leaving the cluster temporarily intact. If, however, N-CAM clustering can occur in response to a signal other than contact with another cell, it may be difficult to learn what this signal might be.

Although homotypic interactions may be required for clustering, some contacts between N-CAM-bearing cells are not enriched in N-CAM. One factor that may influence the extent and frequency of N-CAM clustering is the presence on cell surfaces of different isoforms of N-CAM. Many different forms of N-CAM, produced by alternative splicing of a single transcript, have so far been identified (e.g., Murray et al., 1986; Barthels et al., 1988; Kreig et al., 1989; Santoni et al., 1989; Thomson et al., 1989; Small and Akeson, 1990). In addition, polysialylation of N-CAM influences its ability to undergo homotypic interactions (Rothbard et al., 1982). I have not yet studied the ability of these different forms of N-CAM to cluster, but some of the data reported here are relevant to this question.

In C2 cells, both transmembrane forms and lipid-linked forms seem able to cluster at cell-cell contacts. In myoblasts, mAbs 5B8 and AG1 label N-CAM clusters. Both antibodies recognize only the 140- and 180-kD forms of N-CAM, i.e., those with transmembrane domains (DiFiglia et al., 1989; J. Covault, University of Connecticut, Storrs, CT, personal communication). This result, coupled with the size of the N-CAM in C2 cells, as determined by SDS-PAGE and immunoblotting (Fig. 1; Covault et al., 1986; Moore et al., 1987), suggests that most of the N-CAM in clusters spans the

Figure 7. N-CAM clusters at heterologous cell-cell contacts. C2 cells were biotinylated and plated down onto cultures of CHO, NRK, or L929 cells (*C–H*), or onto unlabeled cultures of C2 cells (*A* and *B*). Alternatively, PC12 cells were triturated and plated onto cultures of C2 cells (*I* and *J*). One (*A–H*) or several (*I* and *J*) days later, cultures were fixed in the presence of Triton X-100, fixed further with paraformaldehyde alone, and labeled with anti-N-CAM followed by RGAR (*A, C, E, G, I*) and with fluoresceinated streptavidin (*B, D, F, H*). Cocultures of PC12 and C2 cells were observed under Nomarski optics (*J*). (*A* and *B*) A biotinylated C2 cell (*B*) contacts unmarked C2 cells at several places. N-CAM accumulates at each (e.g., *A* and *B*, *arrowheads*). One of these contacts occurs at a large, flat, lamellipodial structure (*B, middle arrowhead*). (*C* and *D*) A biotinylated C2 cell (*D*) contacts an unmarked CHO cell. An N-CAM cluster is present at the site of overlap (*C* and *D, arrowheads*). (*E* and *F*) A biotinylated C2 cell (*F*) contacts an unmarked NRK cell. An N-CAM cluster is present at the sites of contact (*E* and *F, arrowheads*). (*G* and *H*) A biotinylated C2 cell (*H*) contacts an unmarked L929 cell. N-CAM is almost uniformly labeled in the C2 cell (*G*), and no N-CAM cluster is present at the sites of contact (*G* and *H, double arrowheads*). (*I* and *J*) PC12 cells contact an elongated C2 cell. N-CAM accumulates at the sites of contact (*I* and *J, arrowheads*). Bar, 10 μm.

membrane of C2 myoblasts. In contrast, the lipid-linked form of N-CAM predominates in C2 myotubes (Covault et al., 1986; Moore et al., 1987), and presumably accounts for much of the N-CAM that accumulates at myotube-myotube contacts. Homologous cell-cell contacts may therefore accumulate N-CAM, regardless of the isoform. Although some heterologous contacts are less able to accumulate N-CAM, this may be due to factors other than differences in the isoforms of N-CAM they express on their surfaces.

The accumulation of N-CAM at cell-cell contacts is likely to be important in many biological processes, but it has been clearly documented in vivo in only a few instances in the adult nervous system (Persohn and Schachner, 1987; DiFiglia et al., 1989) and in adult and developing muscle (Rieger et al., 1985; Covault and Sanes, 1986). The possible role of N-CAM clusters in myogenesis is consistent with the observation that N-CAM is enriched at contacts between fusing C2 muscle cells (Fig. 5). Myogenesis is inhibited by anti-N-CAM antibodies (Knudsen et al., 1990). However, the binding of antibodies to N-CAM may block not only homotypic interactions between N-CAM molecules, but also the formation of close contacts between cells. It will be of interest to determine if clustering of N-CAM also occurs during myogenesis in vivo, and in primary muscle cells in vitro. If so, further experiments will be required to distinguish between the myogenic roles of the homotypic binding of N-CAM and N-CAM clustering.

N-CAM is found in many tissues during the early stages of embryogenesis (Thiery et al., 1982). The ability of some types of cells to form structures enriched in N-CAM, and the relative inability of some heterologous cell pairs to do so, has obvious implications for the developmental processes of cell recognition and sorting, cell migration, and organogenesis. The fact that N-CAM clusters can be examined in a variety of cell lines suggests that their role in cell adhesion and sorting may be amenable to further experimentation.

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