Evidence for Heterophilic Adhesion of Embryonic Retinal Cells and Neuroblastoma Cells to Substratum-adsorbed NCAM

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Abstract. The adhesion of embryonic chicken retinal cells and mouse N2A neuroblastoma cells to purified embryonic chicken retinal NCAM adsorbed on a solid substratum was examined using a quantitative centrifugal adhesion assay. Both cell types adhered to NCAM and the adhesion was specifically inhibited by monovalent anti-NCAM antibody fragments. N2A cell adhesion depended on the amount of NCAM applied to the substratum, was cation independent, and was insensitive to treatment with the cytoskeletal perturbing drugs colchicine and cytochalasin D. These results indicated that the tubulin and actin cytoskeletons were not critically required for adhesion to NCAM and make it unlikely that the cell surface ligand for NCAM is an integrin. Adhesion was however temperature dependent, strengthening greatly after a brief incubation at 37°C. CHO cells transfected with NCAM cDNAs did not adhere specifically to substratum-bound NCAM and pretreatment of N2A cells and retinal cells with anti-NCAM antibodies did not inhibit adhesion to substratum-bound NCAM. These results suggest that a heterophilic interaction between substratum-adsorbed NCAM and a non-NCAM ligand on the surface of the probe cells affects adhesion in this system and support the possibility that heterophilic adhesion may be a function of NCAM in vivo.

The neural cell adhesion molecule NCAM is a vertebrate cell surface glycoprotein that is involved in cell-cell adhesion both in vivo and in vitro (Brackenbury et al., 1977; Thiery et al., 1977; Rutishauser et al., 1982, 1983, 1988; Sadoul et al., 1983; Fraser et al., 1984; Keilhauer et al., 1985; Cole et al., 1986a; Thor et al., 1987; Kadmon et al., 1990). NCAM is expressed in developmentally regulated patterns on a variety of neural and nonneural tissues (Thiery et al., 1982; Crossin et al., 1985; Pollerberg et al., 1985). A prerequisite for evaluating the physiological significance of adhesion mediated by this molecule is to understand the cellular and molecular mechanisms underlying the adhesion process.

Initial studies showed that preincubation of either adhesion partner with anti-NCAM antibody fragments could inhibit cell-monolayer or cell-substratum binding (Rutishauser et al., 1982; Cole et al., 1985) and that the aggregation of membrane vesicles containing purified NCAM could be inhibited specifically by anti-NCAM antibody fragments (Hoffman and Edelman, 1983). These studies suggested that NCAM-mediated adhesion involves homophilic interactions between two NCAM molecules on apposed cells. Other studies, however, seem less consistent with a simple homophilic mechanism for NCAM-mediated adhesion. Transfectants expressing exogenous cell surface NCAM adhere poorly to each other (Edelman et al., 1987; Pizzey et al., 1989; Reyes et al., 1990; Woo, 1990), although retinal cells (Brackenbury et al., 1977; Thiery et al., 1977) and mouse neuroblastoma N2A cells (Rathjen and Rutishauser, 1984) aggregate vigorously by NCAM-dependent mechanisms. Furthermore, no homophilic interaction of soluble NCAM to immobilized NCAM was detected in a direct binding assay (Probstmeier et al., 1989). Currently it is unclear whether NCAM functions by binding to other NCAM molecules (homophilic interactions), to non-NCAM receptors (heterophilic interactions), or both.

In addition to the extracellular binding of an adhesion molecule with its ligand, interaction of the cytoplasmic portion of the adhesion molecule with intracellular cytoskeletal elements often is required to achieve a strong and stable adhesive interaction. Cell–extracellular matrix adhesion mediated by integrins and cell–cell adhesion mediated by cadherins, for example, both require actin cytoskeleton involvement for strong adhesion (Boller et al., 1985; Volk and Geiger, 1986a,b; Hirano et al., 1987; Burridge et al., 1988; Nagafuchi and Takeichi, 1988; Albelda and Buck, 1990). The involvement, if any, of cytoskeletal systems in NCAM-mediated adhesion has not been defined.

In this study we examined the nature of adhesion to NCAM and the potential involvement of the cytoskeleton in adhesion using a centrifugal cell–substratum binding assay (McClay et al., 1981; Lotz et al., 1989) to determine the force required to dislodge adherent cells from purified NCAM adsorbed to a solid substratum (Fig. 1). This assay provides a more quantitative measure of the strength of adhesion.

1. Abbreviation used in this paper: NCAM, neural cell adhesion molecule.
than do the suspension aggregation assays used previously (Brackenbury et al., 1977; Thiery et al., 1977; Brackenbury et al., 1981). Furthermore, the centrifugation assay allows one adhesion system to be studied in isolation by immobilizing a purified adhesion molecule (in this case NCAM) on a solid substratum. In contrast, cell–cell binding experiments can be difficult to interpret because of the presence of multiple adhesion systems on the same cells (Bixby et al., 1987; Neugebauer et al., 1988; Dustin and Springer, 1991). Similar cell–substratum adhesion assays have been used to examine cell adhesion to extracellular matrix molecules (McClay et al., 1981; Lotz et al., 1989; Lalier and Bronner-Fraser, 1991) and to study cell–cell adhesion molecules involved in cellular interactions in the immune system (Springer, 1990; Dustin and Springer, 1991).

Our experiments used two different cell types to examine NCAM-mediated adhesion. Chicken retinal cells taken from day 10 or 11 of development were prepared by treatment with low concentrations of trypsin in the presence of EDTA (Brackenbury et al., 1981). These cells, which aggregate strongly by an NCAM-dependent mechanism, were used in the original characterization of NCAM-mediated adhesion (Brackenbury et al., 1977; Thiery et al., 1977). Mouse N2A neuroblastoma cells are tissue culture cells that aggregate by an NCAM-mediated mechanism along with other calcium-independent mechanisms involving the L1 (NgCAM) glycoprotein (Rathen and Rutishauser, 1984).

The goals of this study were to characterize quantitatively the adhesion of these two cell types to substratum-bound NCAM and to determine whether NCAM-mediated cell–substratum binding is homophilic or heterophilic. The data support a heterophilic mode of adhesion of substrate-bound NCAM with as-yet unidentified ligand on the cell surface. Adhesion to NCAM was not impaired after pharmacological perturbation of the actin or tubulin cytoskeletons, suggesting that these cytoskeletal systems are not involved in adhesion to substratum-bound NCAM.

**Materials and Methods**

**Antibodies**

Rabbit antibodies directed against purified chicken brain NCAM and mouse mAb Anti-NCAM No. 1 against chicken NCAM (Hoffman et al., 1982) were generous gifts of B. A. Cunningham and G. M. Edelman (The Rockefeller University, New York). Rabbit antibodies also were obtained from C.-M. Chuong (University of Southern California, Los Angeles, CA). To produce mAbs, hybridoma cells (2 x 10^7 cells/animal) were injected intraperitoneally into female BALB/c mice (c.6 wk old; Simonsen Laboratories, Gilroy, CA) that had been primed 3–7 d previously with a 0.5-ml intraperitoneal injection of Pristane (2, 6, 10, 14-tetramethylpentadecane; Sigma Chem. Co., St. Louis, MO). Ascites fluid was obtained 14–20 d later by pepsin digestion as described by Brackenbury et al. (1977); residual intact IgG was removed by chromatography on protein A-Sepharose (Sigma Chem. Co.). Purity and completeness of digestion was verified by SDS-PAGE in 10% polyacrylamide gels (Laemmli, 1970) followed by staining with Coomassie brilliant blue R (Sigma Chem. Co.).

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medium containing 10% FBS and supplemented with 30 μM each of glycine, hypoxanthine, and thymidine to compensate for the lack of dihydrofolate reductase.

CHO cells were cotransfected by a calcium phosphate precipitation method (Davis et al., 1986) with NCAM expression plasmids (Edelman et al., 1987) and NG4 plasmid containing functional dihydrofolate reductase minigene as a selectable marker (Gasseter et al., 1982; Johnston and Kucy, 1988). Permanent transfectants expressing dihydrofolate reductase were selected by growth in medium lacking glycine, hypoxanthine, and thymidine. Cells expressing NCAM were detected by indirect immunofluorescence using anti-NCAM polyclonal antibodies and monoclonal antibody Anti-NCAM No. 1 (Edelman et al., 1987). Levels of binding of Anti-NCAM No. 1 to the surfaces of transfected cells were determined by flow cytometry using an EPICS V Flow Cytometer (Coulter Corp., Hialeah, FL) in the Developmental Biology Center at the University of California, Irvine.

**Cell Adhesion Assay**

Our procedures were based on those of McClay et al. (1981) as modified by Lotz et al. (1989). Substrata were prepared in strips cut from 96-well polystyrene chloride flexible microtiter dishes (Falcon 3912; Fisher Scientific Co. Allied Corp., Los Angeles, CA). Substrata were coated with 5 μl of a solution of nitrocellulose in methanol (BA-85, 5 cm² in 6 ml methanol; Schleicher & Schull, Keene, NH) and were allowed to air dry (Lagenaur and Lemenon, 1987). Substrate protein solutions (20 μl) were added and were allowed to adsorb at room temperature for 30 min. Liquid was removed by aspiration and 100 μl blocking solution was added to each well to saturate unoccupied binding sites. Blocking solution contained 10 mg/ml BSA (RIA grade; Sigma Chem. Co.) dissolved in PBS; the solution was heat-inactivated by incubation at 56°C for 1 h and was then filtered through a 0.2 μm membrane filter (Fisher Scientific Co., Allied Corp.). Blocking solution was made fresh for each experiment to minimize nonspecific cell binding. After 1-2 h of blocking, the wells were washed once with PBS and once with adhesion buffer (80% Earle's balanced salts solution (Sigma Chem. Co.) + 20 μg/ml DNase I (type IV from bovine pancreas; Sigma Chem. Co.) + 20% blocking solution to give a final BSA concentration of 2 mg/ml). Wells were stored under 30 μl adhesion buffer until used in the assay. Where indicated, antibodies were included in the final buffer incubation and were left on the substrata for at least 15 min before performing the assay. N2A and CHO probe cells were labeled by incubation overnight (12-17 h) in leucine-free medium containing 10% FBS + 1 μc/ml [3H]leucine or [3H]leucine (ICN Radiochemicals, Costa Mesa, CA). After the labeling period, cultures were washed once with PBS and were incubated in complete label-free medium for 2-4 h to deplete soluble pools. Cells were released from the dish by the low trypsin with EDTA procedure described by Brackenbury et al. (1981). Briefly, cells were washed once with PBS and were incubated for 20 min in divalent cation-free medium (supernatant of 1% v/v of Sigma Chem. Co.) containing 1 mM EDTA, 20 μg/ml DNase I (type IV from bovine pancreas; Sigma Chem. Co.) and 20 μg/ml purified trypsin (Sigma Chem. Co.). Soybean trypsin inhibitor (Sigma Chem. Co.) then was added to 100 μg/ml; the cells were recovered by gentle pipetting and dispersed by repeated pipetting on ice. Cells were counted and achievement of a single cell suspension was verified microscopically using a hemocytometer. Cells were maintained on ice from this point until the assay was performed.

Retinal probe cells were obtained from one or two retinas dissected from embryonic day 10-12 chickens. Cells were prepared using low trypsin with EDTA as described (Brackenbury et al., 1981). Retinal cells were labeled for 4 h in the dark with 10 μc/ml [3H]leucine (McCly et al., 1981; Lotz et al., 1989) before being washed and tested in the assay. Probe cells were diluted to a final concentration of 5 x 10⁶ cells/ml (for retinal cells) or 5 x 10⁵ cells/ml (for N2A and CHO cells). Differing cell numbers were used because the retinal cells were considerably smaller than the other two cell types. 30 μl aliquots (25,000 retinal cells or 2,500 N2A or CHO cells) were applied to each well and were allowed to settle onto the substratum at unit gravity for times and at temperatures appropriate for the particular experiment. After this incubation, the sample wells were gently filled with adhesion buffer and a liquid-filled sandwich was assembled by inverting a second strip containing adhesion buffer onto the experimental strip. By inverting the sandwich was held together by a layer of double-sided carpet tape (Scotch No. 140; 3M Corp., St. Paul, MN) that had been applied to the top strip; holes were burned into the strip at the positions of the experimental wells with a heated No. 3 brass cork borer (Fisher Scientific Co. Allied Corp.).

The assembled sandwich was inverted gently and centrifuged (model CR.11, Jouan, Inc., Winchester, VA) at the indicated speeds for 8 min at 4°C. After the centrifugation, wells containing bubbles (usually not more than one per eight-well strip) were noted and later were omitted from the data analysis. The sandwich was placed gently on a dry ice-ethanol bath for 30-60 s, inverted, and allowed to freeze completely. The ends of the top and bottom wells then were cut off into 5-ml scintillation vials (Beckman Instrs., Inc., Fullerton, CA) using a modified pet nail clipper. 0.4 ml PBS + 1% (vol/vol) Triton X-100 was added to solubilize the cells and 5 ml water-compatible scintillation fluid (SafetySolve; Res. Prod. Intl. Corp., Mount Prospect, IL) was added to each vial. The vials were counted for 5 min in an LS3801 liquid scintillation counter (Beckman Instrs., Inc.) with windows adjusted for 3H/14C dual-label counting. Background radioactivity was measured using wells to which no probe cells had been added; this value was subtracted from each experimental point. Control samples contained wells into which aliquots of each labeled cell type had been centrifuged separately; these allowed calculation of the percent recovery of the added cells and of the spillover coefficients required to calculate the 3H counts for each sample. Typically 70-100% of the applied radioactivity was recovered. Percent adhesion was calculated as

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\text{cpm in substrate well} \times 100.
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Because absolute levels of adhesion to substratum-bound molecules varied somewhat from day to day, the effects of experimental perturbations were compared in all cases to control samples included in the same experiment. Each figure presents the results of a representative experiment; the number of times a particular experiment was repeated with essentially the same relative results is given in the figure legend. Each experiment was performed at least twice.

**Proteoglycans**

Crude retinal proteoglycans were prepared according to the method of Bretscher (1985) by ion exchange chromatography of Triton X-100 extracts of embryonic day 11 chick retinas (Burg and Cole, 1990) on DEAE-Trisacryl M (LKB Instruments, Inc., Gaithersburg, MD). Briefly, extracts were applied to the column in 0.15 M NaCl, 0.01 M Tris.HCl, pH 8.0, 5 mM MgSO₄, 2% (vol/vol) Triton X-100, and 1 mM PMSF. The column was washed sequentially with Triton-containing buffers containing 0.3 M NaCl, 0.3 M sodium formate, 0.5 M and 8 M urea, and 0.05 M Tris-HCl, pH 8.0. Proteoglycans were eluted with 1 M NaCl, 0.01 M Tris-HCl, pH 8.0, 0.01% (vol/vol) Triton X-100, and were dialyzed against 0.01 M Tris-HCl, pH 8.0, 0.01% (vol/vol) Triton X-100.

**Results**

**Chicken Retinal Cells and Mouse N2A Neuroblastoma Cells Specifically Adhere to Substratum-bound NCAM**

Embryonic day 10 chicken retinal cells prepared by the low trypsin with EDTA procedure adhered well in the centrifugal adhesion assay to chicken retinal NCAM adsorbed to a nitrocellulose substratum (Fig. 2 A). Monovalent Fab' fragments prepared from anti–chicken NCAM polyclonal rabbit IgG, but not Fab' fragments prepared from nonimmune IgG, completely inhibited this adhesion. Retinal cells did not adhere significantly to a BSA-adsorbed substratum in the presence or absence of antibodies. Consistent with the results of Hall et al. (1987), retinal cells adhered weakly to fibronectin. Adhesion to fibronectin was not inhibited by anti-NCAM antibodies more than with nonimmune antibodies, demonstrating the specificity of the inhibition and showing that the initiation of adhesion by anti-NCAM antibodies does not result solely from steric blockade of the cell surface NCAM on the retinal cells. Retinal cells do express NCAM, as was shown by their adhesion to the anti–chicken NCAM mAb Anti-NCAM No. 1 immobilized on the substratum; this in-
interaction also was inhibited specifically by Anti-NCAM Fab' fragments, presumably by blocking the NCAM expressed on the surface of the retinal cells.

Cultured mouse N2A neuroblastoma cells also adhered well to immobilized chicken NCAM (Fig. 2 B). This adhesion was specifically inhibited by monovalent Fab' fragments of polyclonal antibodies directed against chicken NCAM (Fig. 2 B) or by the adhesion-blocking mAb Anti-NCAM No. 1 directed against chicken NCAM (Hoffman et al., 1982) (data not shown; but see Fig. 8 B). As was the case for the retinal cells, N2A cells did not adhere significantly to BSA; they adhered to a moderate extent to fibronectin, and that adhesion was unaffected by antibodies to NCAM.

Similar results were obtained for adhesion to the extracellular matrix protein laminin (data not shown; but see Figs. 4 and 5). Mouse N2A cells did not adhere significantly to the mAb Anti-NCAM No. 1, which is specific for chicken NCAM.

The amount of force required to remove adhering N2A cells varied with the density of NCAM adsorbed to the substratum (Fig. 3 A). Increasing the concentration of NCAM applied to the substratum resulted in an increase in the force required to remove the cells from the substratum. Based on these data we used a substrate concentration of ~1 μg of NCAM applied per well and a removal force of 30 g to further characterize adhesion to NCAM.

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**Figure 2.** Cell adhesion to substratum-adsorbed molecules. E10 chicken retinal cells (A) or mouse N2A neuroblastoma cells (B) were labeled with [3H]leucine and were applied to the indicated substrata. After incubation at 37°C for 30 min, the cells were subjected to a removal force of 30 g for 8 min and percent adhesion was calculated. Substrata were NCAM, 0.5 μg (A) or 1.4 μg (B) purified chicken retinal NCAM (25 μg/ml and 70 μg/ml coating concentrations); Anti-N, 1 μg mAb Anti-NCAM No. 1 (50 μg/ml coating concentration); Fn, 0.5 μg fibronectin (25 μg/ml coating concentration); BSA, no additional substratum protein (BSA blocking solution only). Error bars indicate the range of duplicate determinations. The results of a representative experiment are shown; three additional experiments gave essentially the same results.

**Figure 3.** Adhesion of N2A cells to NCAM at different removal forces. Dilutions of a stock retinal NCAM preparation (70 μg/ml) were applied at 20 μl/well to nitrocellulose-coated dishes at final concentrations of 1.4 μg/well (solid circles; undiluted), 0.42 μg/well (open circles), 0.14 μg/well (solid squares), or no NCAM (open squares). N2A cells were labeled with [3H]leucine and were allowed to adhere for 15 min at 37°C (A) or 0°C (B) to the indicated substrata. Cells were then subjected to a removal force of 30 g for 8 min and percent adhesion was calculated. Each point represents a single well. The results of a representative experiment are shown; three similar experiments gave essentially the same results.

**Figure 4.** Effects of temperature and divalent cations on adhesion of N2A cells. Adhesion was carried out in the presence (solid bars) or absence (stippled bars) of divalent cations. N2A cells were labeled with [3H]leucine and were allowed to adhere for 15 min at 37°C (A) or 0°C (B) to the indicated substrata. Cells were then subjected to a removal force of 30 g for 8 min and percent adhesion was calculated. Substrata were NCAM, 0.64 μg purified chicken retinal NCAM (32 μg/ml coating concentration); BSA, no additional substratum protein (BSA blocking solution only); Lm, 0.5 μg laminin (25 μg/ml coating concentration). Error bars indicate the range of duplicate determinations. The results of a representative experiment are shown; two similar experiments gave essentially the same results.
Adhesion to NCAM Is Cation Independent but Temperature Dependent

Many cell adhesion processes occur in two phases. The initial phase involves a weak interaction, thought to result from the binding of the adhesion molecules themselves; this is followed by a dramatic strengthening of the adhesion that in many cases involves interactions with the cytoskeleton (Umbreit and Roseman, 1975; McClay et al., 1981). This strengthening can occur in as little as 5 min; it requires incubation at warm temperatures and the presence of metabolic energy. To see if a similar phenomenon occurs during adhesion to NCAM, we tested the effect of temperature on N2A cell adhesion in the centrifugation assay. Like adhesion to laminin, NCAM-mediated adhesion was reduced dramatically at 0°C compared with 37°C (Figs. 3 B and 4).

Neuroblastoma cells adhered well to NCAM in the presence or absence of divalent cations (Fig. 4), confirming that NCAM is a calcium-independent adhesion molecule (Brackenbury et al., 1977; Thiery et al., 1977; Brackenbury et al., 1981). Adhesion to laminin, on the other hand, was inhibited in the absence of divalent cations. Although the receptors responsible for N2A cell adhesion to laminin have not been defined, such adhesion is most likely to be mediated by members of the integrin family of cell surface proteins, whose adhesive activities are cation dependent (Fujimura and Phillips, 1983; Gailit and Ruoslahti, 1988; Albelda and Buck, 1990).

Adhesion to NCAM Is Insensitive to Treatment With Colchicine or Cytochalasin D

The strengthening of adhesion to NCAM by incubation at 37°C suggested that adhesion to NCAM, like adhesion to laminin, might involve the cytoskeleton. To test this, we treated N2A cells with the microtubule inhibitor colchicine (Fig. 5 B) or the actin microfilament inhibitor cytochalasin D (Fig. 5 A). Neither of these drugs significantly inhibited adhesion of N2A cells to purified NCAM. In fact, adhesion to NCAM appeared to be moderately increased in the presence of cytochalasin D. In contrast, and consistent with the properties of integrin-mediated adhesion, adhesion of N2A cells to laminin was significantly inhibited by treatment with cytochalasin D (Fig. 5 A) but not by treatment with colchicine (data not shown). These experiments make it unlikely that adhesion to NCAM critically requires attachment to actin microfilaments or to colchicine-sensitive microtubules.

Cells Expressing Transfected NCAM Do Not Attach to Substratum-bound NCAM

To examine further the mechanism of NCAM-mediated adhesion, we transfected CHO cells with full-length cDNA constructions encoding different NCAM protein isoforms (Edelman et al., 1987). Because earlier experiments suggested that NCAM-mediated adhesion is homophilic, we anticipated that these cells would adhere specifically to sub-

Figure 5. Effects of cytoskeleton-disrupting drugs on N2A adhesion to NCAM substrata. Mouse N2A neuroblastoma cells were labeled with [3H]-leucine and were applied to the indicated substrata in the presence of the indicated concentrations (0, 1, or 10 μM) cytochalasin D (A) or colchicine (B). After incubation at 37°C for 30 min, the cells were subjected to a removal force of 30 g for 8 min, and percent adhesion was calculated. Substrata were NCAM, 1.4 μg purified chicken retinal NCAM (70 μg/ml coating concentration); Laminin, 0.5 μg laminin (25 μg/ml coating concentration); BSA, no additional substratum protein (BSA blocking solution only). Error bars indicate the range of duplicate determinations. The results of a representative experiment are shown; three additional experiments with cytochalasin D and one additional experiment with colchicine gave essentially the same results.

Figure 6. Adhesion of transfected CHO cells to substratum-adsorbed NCAM. 3H-labeled CHO transfectant IA6 (1) and 14C-labeled transfectant IIC6 (II) were allowed to adhere in the same wells to the indicated substrata for 15 min at 37°C and were then subjected to a removal force of 30 g for 8 min. Substrata were NCAM, 1.4 μg purified chicken retinal NCAM (70 μg/ml coating concentration); Anti-N, 1 μg mAb Anti-NCAM No. 1 (50 μg/ml coating concentration); Fn, 0.5 μg fibronectin (25 μg/ml coating concentration); BSA, no additional substratum protein (BSA blocking solution only). Error bars indicate the range of duplicate determinations. The results of a representative experiment are shown; one additional experiment gave essentially the same results.
stratum-bound NCAM. Surprisingly, no specific adhesion of these transfectants to adsorbed NCAM was observed (Fig. 6). A few transfected lines adhered to some degree to purified NCAM (for example, line f in Fig. 6), but this adhesion was completely insensitive to treatment with anti-NCAM antibody fragments, and we therefore concluded that it was not specific. Flow cytometric analysis using mAb Anti-NCAM No. 1 showed that the levels of NCAM expression at the surface of the transfected cells were comparable to those on chicken retinal cells, which bind well to these same substrata (data not shown). These results are in agreement with our previous data (Edelman et al., 1987) and those of others (Pizzey et al., 1989) indicating that cell–cell adhesion of transfected cells is at best very weak and are inconsistent with a simple homophilic adhesion mechanism. In contrast, previous experiments have shown that neural retinal cells exhibit strong adhesion (Brackenbury et al., 1977; Thiery et al., 1977) and suggest that NCAM-mediated adhesion is homophilic (Rutishauser et al., 1982; Hoffman and Edelman, 1983). Because the transfected cells express NCAM in an unnatural context, we decided therefore to test the homophilic or heterophilic nature of NCAM adhesion in the cell substratum assay using cells that are known to aggregate using an NCAM-dependent mechanism.

Adhesion Appears to Involve a Heterophilic Interaction of Substrate-bound NCAM with a Non-NCAM Molecule on the Cell Surface

There are at least two possible explanations that could account for the failure of transfected cells to adhere to substratum-adsorbed NCAM. Some essential cofactor for adhesion may be supplied normally to NCAM in neural cells but not in CHO cells, or NCAM-mediated adhesion could in fact be heterophilic, i.e., involve binding to a ligand different from NCAM (Fig. 7). Under the latter hypothesis, transfected CHO cells would lack the NCAM ligand and thus would not aggregate. If this were so, then the adhesion of retinal cells and N2A cells to NCAM substrata would involve the postulated NCAM ligand, not cell surface NCAM, and antibody-mediated inhibition of cell surface (as opposed to substratum-bound) NCAM would have little or no effect on adhesion.

To test this possibility, we treated N2A cells and NCAM-coated substrata separately with monovalent anti-NCAM Fab' fragments and washed away the unbound antibodies before performing the centrifugation assay (Fig. 8). Treatment of the substrata with anti-NCAM antibodies abolished adhesion of cells to NCAM but not to fibronectin, confirming that the substratum-adsorbed NCAM molecules were participating in adhesion. Both polyclonal (Fig. 8 A) and monoclonal (Fig. 8 B) antibody fragments against NCAM were effective. In contrast, treatment of the cells with polyclonal anti-NCAM antibody fragments had no specific effect on adhesion to purified NCAM (Fig. 8 A). Similar results were obtained for adhesion of retinal cells to substratum-bound NCAM and fibronectin (Fig. 9 A). These results are most easily explained by postulating that the cell surface ligand for substratum-bound NCAM is not itself NCAM. As expected, treatment of N2A cells with anti-NCAM No. 1 did not affect adhesion; this antibody does not react with mouse NCAM.

It is possible that the lack of inhibition of adhesion of antibody-coated cells was the result of antibody internalization or insertion of new NCAM onto the cell surface during the brief incubation at 37°C. To test this, adhesion experiments were performed at 4°C, where these processes should not be occurring but where retinal adhesion to substratum-bound NCAM, although weak, is still detectable. Similar experiments could not be performed with N2A cells because their adhesion is so weak at 4°C (Fig. 3 B). Treatment of the

Figure 7. Models of NCAM-mediated adhesion. (A) Schematic diagram of cell–cell adhesion mediated by homophilic (top) or heterophilic (bottom) interactions with NCAM. (B) Corresponding model of cell adhesion to substratum-bound NCAM by a homophilic (left) or heterophilic (right) mechanism. (Solid figure) NCAM; (gray figure) NCAM ligand.

Figure 8. Inhibition of N2A adhesion by separate antibody treatment of cells and substrata. (A) Polyclonal anti-NCAM Fab' fragments or nonimmune Fab' fragments were used as indicated to treat the indicated substrata alone, cells alone, or both cells and substrata together. (B) mAb anti-NCAM No. 1 was used to treat the indicated substrata alone or the cells alone. Cells were allowed to adhere to the substrata for 20 min at 37°C before being subjected to a removal force of 30 g for 8 min. Substrata were NCAM, 0.5 μg purified chicken retinal NCAM (25 μg/ml coating concentration); Fn, 0.5 μg fibronectin (25 μg/ml coating concentration); BSA, no additional substratum protein (BSA blocking solution only). Error bars indicate the range of duplicate determinations. The results of a representative experiment are shown; one additional experiment gave essentially the same results.
Effect on adhesion to NCAM substrata, whereas treatment of the retinal cells. As before, incubation of the substratum dramatically inhibited adhesion (Fig. 9 B). To characterize this adhesion further, similar experiments of others suggest that a heparan sulfate ligand on the retinal cell surface.

Retinal cells with anti-NCAM antibodies had no specific effect on adhesion to NCAM substrata, whereas treatment of the substratum dramatically inhibited adhesion (Fig. 9 B). To characterize this adhesion further, similar experiments were carried out using a range of removal forces (Fig. 10). Preincubating the retinal cells with anti-NCAM antibody fragments did significantly inhibit adhesion to NCAM (Fig. 10, A and B) but did not significantly inhibit adhesion to substratum-adsorbed Anti-NCAM No. 1 mAb (Fig. 10 C). The latter result showed that the antibody incubation procedure was effective in covering up the NCAM on the surface of the retinal cells. As before, incubation of the substratum with anti-NCAM antibody fragments significantly inhibited the adhesion of both antibody-treated and untreated retinal cells (compare Fig. 10 B with A). Antibody treatment had no effect on adhesion to the nonadhesive BSA-adsorbed control substratum (Fig. 10 D). These results suggest that the simple homophilic model of NCAM-mediated adhesion is inadequate. Rather, they are consistent with the possibility that adhesion to substratum-bound NCAM involves a non-NCAM ligand on the retinal cell surface.

The heterophilic ligand for NCAM is not known, although experiments of others suggest that a heparan sulfate proteoglycan may play this role (Cole et al., 1985, 1986a,b). In preliminary experiments, crude proteoglycans prepared from embryonic chicken retinal membranes did not support N2A cell or retinal cell adhesion when adsorbed to a nitrocellulose substrate, and incubation of these proteoglycans with an NCAM substrate inhibited cell adhesion (Fig. 11). This is consistent with previous work showing that heparin inhibits NCAM-mediated adhesion (Cole et al., 1985, 1986b; Reyes et al., 1990) but does not support the hypothesis that adhesion to NCAM is mediated by a heparan sulfate ligand. However, it is possible that the crude proteoglycan preparations used for these experiments might contain both adhesive and inhibitory components, so further experimentation will be required to resolve this point definitively.

**Figure 9.** Inhibition of chick retinal cell adhesion by separate antibody treatment of cells and substrata. NCAM-adsorbed substrata (0.4 μg/well, 20 μg/ml coating concentration) were treated with nonimmune Fab' fragments (I) or polyclonal anti-NCAM Fab' fragments (2), after which the unbound antibodies were removed by washing. Control wells (3) contained nonimmune (solid bars) or polyclonal anti-NCAM Fab' fragments (stippled bars) throughout the assay. Other wells contained fibronectin (4) or BSA (5) substrata without added antibodies. Probe cells were treated in solution with nonimmune (solid bars) or anti-NCAM (stippled bars) Fab' fragments, washed (except for 3), and brought into contact with the indicated substrata. Cells were allowed to adhere to the substrata for 20 min at 37°C (A) or 0°C (B) before being subjected to a removal force of 30 g for 8 min. Error bars indicate the range of duplicate determinations. The results of a representative experiment are shown. Two additional experiments at 37°C gave essentially the same results seen in A; further experiments at 0°C are described in the legend to Fig. 10.

**Figure 10.** Effect of removal force on chick retinal cell adhesion to NCAM at 4°C. NCAM-adsorbed substrata (0.7 μg/well, 35 μg/ml coating concentration) were treated with nonimmune Fab' fragments (A) or polyclonal anti-NCAM Fab' fragments (B), after which the unbound antibodies were removed by washing. Other wells contained Anti-NCAM No. 1 mAb (1 μg/well, 50 μg/ml coating concentration) (C) or BSA (D) substrata without added antibodies. Embryonic day 12 retinal cells were treated in solution with nonimmune (closed circles) or anti-NCAM(open circles) Fab' fragments, washed, and brought into contact with the indicated substrata by centrifugation at 17 g for 8 min at 4°C. Cells were allowed to adhere to the substrata for 20 min at 0°C before being subjected to the indicated removal forces for 8 min. Each symbol represents a single well. The results of a representative experiment are shown; two additional experiments gave essentially the same results.
and extend the binding results to N2A neuroblastoma cells.

Interactions between neurons, glia, and muscle cells (Bixby et al., 1977). These results imply that different mechanisms underlie adhesion to laminin and to NCAM. In particular, the insensitivity to divalent cation deprivation and to cytochalasin D, which disrupts the actin cytoskeleton, suggests that the NCAM ligand is not an integrin. This is of interest because there are several examples in the immune system in which integrins act as cell surface adhesion receptors for immunoglobulin superfamily members with structural similarities to NCAM (Springer, 1990; Dustin and Springer, 1991).

Although interactions with the actin cytoskeleton appeared not to be involved in adhesion to NCAM, the adhesion did strengthen upon incubation at 37°C. This might reflect strengthening by diffusion of cell surface NCAM ligands in the plane of the membrane into the region of cell–substratum contact and consequent interaction with substrate bound NCAM or possibly an interaction with cytoskeletal elements not sensitive to the inhibitors used in this study. Adhesive strengthening by membrane diffusion of adhesion molecules has been suggested to occur for calcium-dependent retinal cell adhesion in the presence of metabolic poisons (McClay et al., 1981) and for the adhesion of lymphoid cell lines to substratum-adsorbed LFA-3 through cell surface CD2 molecules (Chan et al., 1991). The latter workers reported a moderate strengthening of cell–substratum adhesion in the presence of cytochalasin, similar to that seen in the experiments reported here.

It is difficult to distinguish homophilic from heterophilic adhesion in cell–cell adhesion assays in suspension. Both homophilic and heterophilic cell aggregation should be inhibited by anti-NCAM antibodies because NCAM is a critical component of every adhesive bond under both models (Fig. 7). The use of a cell–substratum adhesion assay, on the other hand, allows one to separate experimentally the two halves of the adhesive interaction and thus to determine whether the partner for substratum-adsorbed NCAM is another NCAM molecule on the adhering cell (Fig. 7). The use of the centrifugal adhesion assay thus allowed us to test the homophilic or heterophilic nature of NCAM binding in a more direct fashion than has been possible in the past.

Our antibody blocking experiments supported a heterophilic binding mechanism for adhesion to substratum-bound NCAM. Coating of cell surface NCAM molecules with anti-NCAM antibodies failed to inhibit the adhesion of the cells to substratum-bound NCAM, although it did inhibit their adhesion to substratum-bound mAb's that are known to recognize cell surface NCAM. These results are difficult to interpret in the context of a simple homophilic interaction between substratum-bound and cell surface NCAM molecules; rather they support a heterophilic adhesion model in which substratum-adsorbed NCAM binds to a non-NCAM ligand on the surface of the adhering cell.

Earlier experiments showed that separate incubation of anti-NCAM antibodies with cells in suspension or with substrate-attached cells could significantly inhibit adhesion (Rutishauser et al., 1982). These results were interpreted to support the homophilic adhesion model. However, since each adhesion partner presumably would carry both NCAM and its ligand, such a result also could be explained under the heterophilic model by weakening only of the adhesive bonds using the NCAM molecules on the targeted cells. This might suffice to allow the shear forces in the cell monolayer binding assays to inhibit retention of the probe cells on the monolayers.

Nevertheless, the current experiments do not rule out an additional, weaker NCAM to NCAM adhesion, although inhibited NCAM-mediated adhesion in suspension adhesion assays. These experiments confirm and extend the work of Cole and co-workers (Cole and Glaser, 1984; Cole et al., 1985; Reyes et al., 1990), who used a different assay to show that retinal cells can bind to substratum-adsorbed NCAM, and extend the binding results to N2A neuroblastoma cells.

The use of purified NCAM in a cell–substratum binding assay had the advantage of isolating the analysis of NCAM-mediated adhesion from other adhesive systems present on the same cells. This is particularly significant because cell–cell binding studies have demonstrated that multiple adhesion systems, including those involving NCAM, contribute in an apparently redundant fashion to the adhesive interactions between neurons, glia, and muscle cells (Bixby et al., 1987; Neugebauer et al., 1988). The presence of such multiple adhesion systems may have contributed to the difficulty of identifying NCAM-mediated adhesion in earlier cell monolayer adhesion experiments using retinal cells and transfected fibroblasts (Woo, 1990).

The adhesion of N2A cells and retinal cells to laminin and to NCAM both were greatly strengthened by incubation of the cells with the substratum at 37°C. Both adhesion systems were insensitive to colchicine treatment, suggesting that microtubules are not required for either. However, unlike the adhesion of N2A cells to laminin, adhesion to NCAM did not require divalent cations and was unaffected by treatment with cytochalasin D, which disrupts the actin cytoskeleton. The cation-independent nature of NCAM-mediated adhesion in this assay system is consistent with the original definition of NCAM as a calcium-independent adhesion molecule (Rutishauser et al., 1976; Brackenbury et al., 1977; Thiery et al., 1977). These results imply that different mechanisms underlie adhesion to laminin and to NCAM. In particular, the insensitivity to divalent cation deprivation and to cytochalasin treatment suggests that the NCAM ligand is not an integrin. This is of interest because there are several examples in the immune system in which integrins act as cell surface adhesion receptors for immunoglobulin superfamily members with structural similarities to NCAM (Springer, 1990; Dustin and Springer, 1991).
such adhesion was not detected in our transfectant studies or in direct binding experiments of others (Probstmeier et al., 1989). Vesicle aggregation experiments using purified NCAM and lipids supported the hypothesis of homophilic NCAM binding (Hoffman and Edelman, 1983; Hall et al., 1990) and weak cell–cell adhesion was observed with some NCAM transfectants (Edelman et al., 1987; Pizzey et al., 1989); these results might reflect a weak homophilic interaction in the absence of a stronger heterophilic ligand. Thus it is possible that NCAM might exhibit multiple binding specificities, as do a number of other cell surface and extracellular matrix adhesive molecules. For example, the Ig superfamily member ICAM-1 (Rothlein et al., 1986) binds to different ligands (LFA-1 and Mac-1) through Ig-like domains 1 and 3, respectively (Diamond et al., 1991).

What might the heterophilic NCAM ligand or ligands be? The results presented here suggest that the NCAM ligand is not an integrin. Probstmeier et al. (1989) found that purified NCAM bound to several species of collagen, suggesting that collagen might act as an NCAM ligand in cell adhesion; however, in preliminary experiments we were not able to detect specific adhesion of transfected CHO cells to purified collagens I, II, IV, V, or VI (data not shown).

Our results also failed to support a role for heparan sulfate proteoglycans in adhesion to NCAM, although several previous studies suggest that a heparan sulfate proteoglycan is an attractive candidate for an NCAM ligand. Heparin and heparan sulfate are known to bind to NCAM (Cole et al., 1986a) and to inhibit NCAM-mediated adhesion (Cole et al., 1985; Cole et al., 1986b) and a putative heparin binding domain of NCAM is required for adhesion of retinal cells to substratum-bound NCAM (Reyers et al., 1990). However, it has not been shown directly that a heparan sulfate proteoglycan serves as an NCAM adhesive ligand, and no such adhesion was detected in the current study. On the other hand, proteoglycans isolated from neural tissues, including the embryonic chicken retinal proteoglycans used in the current study, comprise a complex mixture including several different heparan sulfate–bearing and chondroitin sulfate–bearing protein cores (Cole and Burg, 1989; Burg and Cole, 1990). This raises the possibility that inhibitory proteoglycan species present in this mixture might act as inhibitors, promoting activities of an NCAM proteoglycan ligand. Further studies of separated proteoglycan species isolated from retinal tissue will be required to resolve this point.

The physiological ligand or ligands for NCAM thus cannot be identified yet with any degree of certainty. It should be possible to use the centrifugal adhesion assay along with the transfected cells expressing NCAM polypeptides both to test purified potential ligands for their ability to mediate attachment through cell surface NCAM and to purify additional ligand candidates from cell membrane extracts. It will be necessary to identify and characterize the NCAM ligand or ligands to define the physiological role of NCAM in vivo and to determine whether control of expression of the ligand, as well as of NCAM itself, is significant for the control of cellular adhesive activities in the intact organism.

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