The Neural Cell Adhesion Molecule N-CAM Enhances L1-dependent Cell–Cell Interactions

Guni Kadmon, Aiga Kowitz,* Peter Altevogt,* and Melitta Schachner

Department of Neurobiology, University of Heidelberg, Im Neuenheimer Feld 364; and *Institute of Immunology and Genetics, The German Centre of Cancer Research, Im Neuenheimer Feld 280, 6900 Heidelberg 1, Federal Republic of Germany

Abstract. On neural cells, the cell adhesion molecule L1 is generally found coexpressed with N-CAM. The two molecules have been suggested, but not directly shown, to affect each other's function. To investigate the possible functional relationship between the two molecules, we have characterized the adhesive interactions between the purified molecules and between cultured cells expressing them.

Latex beads were coated with purified L1 and found to aggregate slowly. N-CAM-coated beads did not aggregate, but did so after addition of heparin. Beads coated with both L1 and N-CAM aggregated better than L1-coated beads. Strongest aggregation was achieved when L1-coated beads were incubated together with beads carrying both L1 and N-CAM. In a binding assay, the complex of L1 and N-CAM bound strongly to immobilized L1, but not to the cell adhesion molecules J1 or myelin-associated glycoprotein. N-CAM alone did not bind to these glycoproteins. Cerebellar neurones adhered to and sent out processes on L1 immobilized on nitrocellulose. N-CAM was less effective as substrate. Neurones interacted most efficiently with the immobilized complex of L1 and N-CAM. They adhered to this complex even when its concentration was at least 10 times lower than the lowest concentration of L1 found to promote adhesion. The complex became adhesive for cells only when the two glycoproteins were preincubated together for ~30 min before their immobilization on nitrocellulose.

The adhesive properties between cells that express L1 only or both L1 and N-CAM were also studied. ESb-MP cells, which are L1-positive, but N-CAM negative, aggregated slowly under low Ca^{2+}. Their aggregation could be completely inhibited by antibodies to L1 and enhanced by addition of soluble N-CAM to the cells before aggregation. N2A cells, which are L1 and N-CAM positive aggregated well under low Ca^{2+}. Their aggregation was partially inhibited by either L1 or N-CAM antibodies and almost completely by the combination of both antibodies. N2A and ESb-MP cells coaggregated rapidly and their interaction was similarly inhibited by L1 and N-CAM antibodies.

These results indicate that L1 is involved in two types of binding mechanisms. In one type, L1 serves as its own receptor with slow binding kinetics. In the other, L1 is modulated in the presence of N-CAM on one cell (*cis*-binding) to form a more potent receptor complex for L1 on another cell (*trans*-binding).

THE Ca^{2+}-independent neural cell adhesion molecules N-CAM[1] (for review, see Edelman, 1985) and L1 (Rathjen and Schachner, 1984) belong to the immunoglobulin (Barthels et al., 1987; Cunningham et al., 1987; Moos et al., 1988) and the carbohydrate-based L2/HNK-1 (Kruse et al., 1984) families. L1 was first described in the central nervous system of the mouse as a 200-kD integral membrane glycoprotein (Rathjen and Schachner, 1984; Faissner et al., 1985). It is immunochemically related to NGF-inducible large external protein (NILE) in the rat (Bock et al., 1985), and to Ng-CAM (Grumet et al., 1984*a*, *b*), 8D9 (Lemmon and McLoon, 1986) and G4 (Rathjen et al., 1987) in the chicken. In the nervous system, N-CAM appears in three different major components of 180, 140, and 120 kD, respectively, which are believed to be generated by alternative splicing from one gene and to differ mainly in the extent of their cytoplasmic domains (Cunningham et al., 1987).

To date, all neural cell types in which L1 has been detected also express N-CAM. These neural cell types include subsets of postmitotic neurones, pre- and nonmyelinating Schwann cells, and certain neural tumors (Stallcup et al., 1983; Chuong and Edelman, 1984; Grumet et al., 1984*a*,*b*; Faissner et al., 1985; Nieke and Schachner, 1985; Stallcup and Beasley, 1985; Fushiki and Schachner, 1986; Lemmon and McLoon, 1986; Martini and Schachner, 1986, 1988; Mirsky et al., 1986; Chang et al., 1987; Persohn and Schachner, 1987; Miragall et al., 1988). In contrast to N-CAM, which is uniformly expressed on the cell surface of differentiated neurones, L1 is predominantly expressed on axons. Interestingly, primarily fasciculating axons or fas-

1. *Abbreviations used in this paper*: MAG, myelin-associated glycoprotein; N-CAM, neural cell adhesion molecule.

ciculating parts of axons express L1 in vivo (Schachner et al., 1985; Martini and Schachner, 1986; Dodd et al., 1988; Bartsch et al., 1989). Antibodies to L1 and N-CAM perturb neurite fasciculation (Fischer et al., 1986; Rathjen et al., 1987), whereas only L1, but not N-CAM antibodies reduce neurite extension on other neurites (Chang et al., 1987). Likewise, neurite extension on Schwann cells (Bixby et al., 1988; Seilheimer and Schachner, 1988; Kleitman et al., 1988) and migration of granule cell neurones in the developing cerebellum can be inhibited in vitro by antibodies to L1, but less so by N-CAM antibodies (Lindner et al., 1983, 1986). Furthermore, monovalent antibodies to L1 and N-CAM, both individually or in combination, perturb aggregation among cerebellar neurones and neuroblastoma cells, with their combined inhibitory effects being stronger than expected from their individual activities (Faissner et al., 1984; Rathjen and Rutishauser, 1984). Evidence for a molecular association between the two molecules was gained by the observation that L1 and N-CAM specifically co-purify by immunoaffinity chromatography (Grumet et al., 1984*b*; Pollerberg et al., 1987; Kadmon, G., unpublished results). Moreover, antibodies to L1 induced coredistribution of the largest cytoskeleton-bound component of N-CAM, N-CAM 180 (Thor et al., 1986; Pollerberg et al., 1987), suggesting that L1 and N-CAM may interact within the surface membrane. Also, L1 and N-CAM 180 were found to accumulate at sites of cell contact between cultured cells (Pollerberg et al., 1987; Pollerberg et al., manuscript submitted for publication). These results raised the question of whether the association and selective coexpression of the two molecules has a functional significance.

In this study, we show that L1 interacts with itself, as previously shown for Ng-CAM (Grumet and Edelman, 1988; Sadoul et al., 1988) and that the avidity of this interaction is enhanced by specific association of L1 and N-CAM on the cell surface of one of the interacting partner cells.

Materials and Methods

Antibodies

IgG mAbs to mouse L1 (Rathjen and Schachner, 1984), N-CAM (BSP-2; Hirn et al., 1983), the 180-kD form of N-CAM (N-CAM 180; Pollerberg et al., 1987), myelin-associated glycoprotein (MAG; Poltorak et al., 1987) and HNK-1 (Abo and Balch, 1981) have been described. Fab fragments of polyclonal antibodies to L1 and N-CAM were produced as described (Rathjen and Schachner, 1984) and their specificity was tested by Western blot analysis (Fig. 1). For controls, Fab fragments of polyclonal antibodies to cell membranes of mouse liver (Keilhauer et al., 1985; Werz and Schachner, 1988) and ESb cells were used, because they react with all types of neural cells and ESb-MP cells.

Antigens

L1, N-CAM, and MAG were immunoaffinity purified from NP-40 detergent extracts of crude membrane preparations from adult whole mouse brains using mAb columns (Hirn et al., 1983; Rathjen and Schachner, 1984; Poltorak et al., 1987) and used in solution of 0.1% deoxycholic acid (DOC). To this end, crude membrane preparations were applied to the mAb columns in PBS, pH 7.4 containing 0.5% NP-40. The columns were then washed with 10 column volumes, each of the same buffer, PBS containing 0.1% DOC and 0.3 M NaCl, and, finally, PBS containing 0.1% DOC. The bound antigens were eluted with 0.1% DOC under alkaline conditions and immediately neutralized to pH 7.2 as described (Poltorak et al., 1987). The soluble forms of L1, N-CAM, and MAG were prepared similarly from detergent-free tissue homogenates by allowing crude membrane preparations from adult whole mouse brain to incubate for 1 h at 37°C in PBS (Sadoul et al., 1986, 1988) and purifying of the released antigens by immunoaffinity chromatography using mAb columns. The soluble forms contain the amino-terminal fragments of the molecules (unpublished observations). The soluble form of N-CAM used in this study is not equivalent to the truly secreted form of this molecule. The J1 glycoproteins (Kruse et al., 1985) were enriched by immunoaffinity chromatography from adult mouse brains by means of an L2 mAb column (Kruse et al., 1984) after removal of L1, N-CAM, and MAG (Kruse et al., 1985) from the tissue homogenate. Protein concentrations were estimated simultaneously according to Bradford (1976) and further checked by comparison of serial dilutions blotted on nitrocellulose and stained with Amido black. Purity of antigens was assessed by SDS-PAGE and Western blot analysis (Fig. 1). No evidence of contamination of antigens with glycosaminoglycans or proteoglycans could be seen by methanolysis analysis (Geyer, R., unpublished results). A contamination of ~5% of the total protein weight would have been detected.

Bead Aggregation

Latex-beads with mean diameters of 15.8 and 11.9 μm were used (LB 16 and LB 120, respectively; Sigma Chemical Co., St. Louis, MO). Beads were coated with immunoaffinity-purified L1, N-CAM, or MAG glycoproteins (see Antigens section) during removal of the detergent from the glycoprotein solution by dialysis against PBS. Dialysis was performed at 4°C for 24 h and another 24 h under vigorous shaking. Glycoproteins were either coated individually (L1, N-CAM, MAG) or in combination (L1+N-CAM, L1+MAG, or N-CAM+MAG) at a final protein concentration of 200 μg/ml. 150 μl of a 10% bead suspension (76 cm^2 total bead surface area) were mixed with 2 ml antigen solution. Combined glycoproteins were coated at a 1:1 protein ratio with 100 μg/ml for each antigen. After coating, beads were washed by three centrifugation steps (75 *g*, 10 min, 4°C) with PBS. The amount of protein adsorbed onto the beads was ~8% of the total protein in the coating solution, yielding ~8 × 10^6 molecules per bead. Beads were kept under sterile conditions at 4°C in PBS containing 1 mg/ml BSA (BSA-PBS) for 1–7 d. During this time period, the aggregation efficiencies of the beads did not change, indicating that the protein remained attached to the beads. Control beads that had not been coated with antigens were placed directly in BSA-PBS.

For the aggregation assay, beads were diluted in BSA-PBS to 2.5 × 10^6 beads/ml in screw top glass vials. When beads coated with different proteins were allowed to coaggregate, they were mixed at bead ratio of 1:1 to the same final bead concentration as in homogeneous bead preparations. Beads were allowed to aggregate at 120 rpm for 5 h at 20°C or for 12 and 30 h at 4°C. Bead aggregation was evaluated by Coulter counter particle analysis (Coulter Electronics, Hialeah, FL) or by phase-contrast microscopy.

To measure the influence of heparin on the binding of N-CAM-coated beads, heparin (10^{-7} M, molecular mass of ~20 kD; H-3125, Sigma Chemical Co.) was added to N-CAM- or BSA-coated beads prior to bead aggregation. For control, chondroitin sulfate (10^{-7} M, molecular mass of ~50 kD; C-4384, Sigma Chemical Co.) was added instead of heparin.

In some experiments, beads were incubated before aggregation with the soluble forms of L1, N-CAM, or MAG (50 μg/ml), or with Fab fragments (0.5 mg/ml) of polyclonal antibodies to L1, N-CAM, or ESb cell membranes. Preincubation lasted 1 (for antibodies) or 4 h (for soluble antigens) at room temperature under shaking. The beads were washed three times in BSA-PBS before aggregation or left in the presence of antibodies during aggregation. The added soluble adhesion molecules were present throughout the assay.

Protein Binding Assay

Microtiter ELISA plates (Micro Test III; Falcon Labware, Oxnard, CA) were coated by overnight incubation at room temperature with 100 μl/well of serial twofold dilution steps of L1 (100 μg/ml to 780 ng/ml in 0.1 M Na_2HCO_3). J1 and MAG (200 μg/ml) and BSA (1 mg/ml) were also coated. Wells were washed with PBS, incubated for 2 h with 100 μl of 1 mg/ml BSA per well to block free protein binding sites, and washed again with PBS. To measure the binding of the combination of L1 and N-CAM to these substrates, a mixture of the two glycoproteins at a ratio of 1:1 (vol/vol) was preincubated for 4 h on ice. After dilution in BSA-PBS to 10 μg/ml (5 μg/ml for each antigen), 100 μl of this solution were added to each well and incubated overnight at room temperature. For control, soluble N-CAM (10 μg/ml in BSA-PBS; 100 μl/well) was also added. To assess maximal and background binding to wells, the proteins added for binding measurements (L1 plus N-CAM or N-CAM alone) were added to uncoated or BSA-coated

wells. The binding reaction was terminated by three washes with BSA-PBS. The amount of N-CAM bound to the substrate was measured by addition of 100 μl/well of a fivefold diluted hybridoma supernatant of N-CAM mAbs, followed by an overnight incubation at 4°C, three washes in BSA-PBS, and incubation with 100 μl/well of a 1:5,000 diluted horseradish peroxidase-coupled guinea pig antibody to rat IgG for two hours at room temperature. Wells were washed again and the reaction developed with ABTS (Boehringer Mannheim, Mannheim, FRG) and H_2O_2 in acetate buffer as described (Bollensen et al., 1988) and quantitated with a Titertek Multiscan Plus ELISA analyzer (Flow Laboratories, Meckenheim, FRG).

Cell Lines and Cell Culture

Cultures of neuroblastoma C1300 clone N2A (N2A cells; Rathjen and Rutishauser, 1984; Rathjen and Schachner, 1984) and the lymphoma-derived clones Eb 288, ESb (Schirrmacher et al., 1982; Altevogt et al., 1985) and ESb-MP (Fogel et al., 1983; Lang et al., 1987) have been described. ESb-MP is an adherent variant of ESb that, in turn, is a spontaneous highly metastatic variant of Eb 288. These lymphoid cell lines are similar in cell surface markers (Lang et al., 1987; and unpublished results). In a previous study, we have shown that these cell lines are N-CAM negative and that ESb-MP, but not Eb 288 cells express L1. N2A cells express both L1 and N-CAM (Rathjen and Rutishauser, 1984). For the assays, cells were removed from the culture dish by treatment with 1 mM EDTA in PBS for ~5 min at room temperature under shaking.

Homotypic Cell Aggregation

Adherent N2A and ESb-MP cells and Eb 288 cells growing in suspension were washed three times by mild centrifugation (100 *g*, 10 min, 4°C) in Ca^{2+}-, Mg^{2+}-free HBSS (CMF-HBSS). Of each cell type, several aliquots were resuspended in 2.5 ml CMF-HBSS supplemented with 40 mM Hepes, 0.1 mM Ca^{2+}, 1 mg/ml BSA and 0.1 mg/ml DNase (Boehringer Mannheim) (low Ca^{2+} assay medium) to a concentration of 10^6 cells per ml. Ca^{2+} was added at a low concentration because in its complete absence viability of cells is strongly reduced. We ascertained that Ca^{2+}-dependent adhesion does not occur at this Ca^{2+}concentration. Aliquots were mixed with Fab fragments (0.5 mg/ml) of polyclonal antibodies to L1 or N-CAM or of polyclonal antibodies to ESb cell membranes or with no antibodies. Cells were subsequently incubated 30 min on ice to allow antibodies to react. They were then redispersed and added in 100-μl replicates to microtiter culture plates (Nunc, Roskilde, Denmark), preblocked with 10 mg/ml heat-treated (75°C, ~25 min) BSA. Cells were then allowed to aggregate in a rotatory incubator at 37°C and 85 rpm. Quadruplicates for each experimental value were taken for measurement after 0 and 30 min for N2A, and 0, 1, 3, and 5 h for Eb 288 and ESb-MP cells. Aggregation was evaluated by Coulter counter particle analysis (see below) and by phase-contrast microscopy.

In one experiment, ESb-MP cells were allowed to aggregate in low Ca^{2+} assay medium for five h after which 50 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was added with the Ca^{2+} concentration either maintained at 100 μM or increased to 1.25 mM. Measurements were taken after allowing further aggregation for 30 min at 37°C.

In a separate experiment, ESb-MP cells had been pre-incubated for 40 min on ice and an additional 20 min at 37°C and 85 rpm in low Ca^{2+} assay medium with or without soluble N-CAM (5 and 20 μg/ml) or soluble MAG (20 μg/ml). ESb-MP cells that had not been preincubated with N-CAM were then added and incubation continued for an additional 40 min.

All experiments were performed under sterile conditions.

Heterotypic Cell Aggregation

ESb-MP and N2A cells were prepared as for the homotypic aggregation assay, but, after suspension, ESb-MP cells were stained with the fluorescent dye bisbenzimide (5 μg/ml; Calbiochem-Behring Corp., La Jolla, CA), whereas N2A cells were labeled with rhodamine isothiocyanate (RITC; 20 μg/ml; Serva Fine Biochemicals, Heidelberg, FRG). Both dyes were applied to cells for 30 min incubation at room temperature in darkness. Unbound dyes were removed by subsequent washes. The dyes did not affect aggregation. Cells were then suspended in low Ca^{2+} assay medium as described above. The two cell lines were suspended both separately or mixed together. Cell concentrations for single and mixed aggregations were 2.5×10^5/ml for ESb-MP and 1.25×10^5/ml for the larger N2A cells. Fab fragments of polyclonal antibodies to L1 or N-CAM (0.5 mg/ml) were added or omitted and the cells were allowed to aggregate as described for homotypic cell aggregation, except that replicates were incubated in a volume of 400 μl in 24-well culture plates (Costar Corp., Cambridge, MA) in darkness and aggregation allowed to proceed for only 35 min. Aggregation and coaggregation were measured by Coulter counter particle analysis (see below) and by fluorescence microscopy. It should be pointed out that an increase in mixed versus single aggregation was not observed in the highest channel numbers, probably reflecting the inability of the largest aggregates to remain intact during Coulter counter measurement (Fig. 8).

Coulter Counter Particle Analysis

Aggregation of cells or beads was measured in a Coulter counter (model T_{AII}) with Population Accessory with a 200-μm-diam nozzle. Partial volumes occupied by particles detected in channels ≥ channel no. 12 for beads and for N2A cells and no. 11 for ESb-MP cells were evaluated and yielded a sensitive measure of small changes in the extent of aggregation. Changes in partial volume measured in the channel corresponding to single cells were used for estimating coaggregation of ESb-MP and N2A cells (Fig. 8). Such measurements are possible, because N2A cells are not detectable in channel numbers < 8, whereas ESb-MP cells are detectable in channel 7, and because ESb-MP cells do not aggregate on their own in low Ca^{2+} during 1 h. To evaluate coaggregation, channel numbers were compared between cells mixed before aggregation and cells mixed after allowing them to aggregate separately for the same time (Fig. 8). A reduction in particle number in channel 7 after mixed aggregation in comparison to separate aggregation can thus be taken as indication of coaggregation of ESb-MP and N2A cells.

Cell–Cell Adhesion

ESb-MP and N2A cells (probe cells) were stained with FITC (saturated solution in DMSO diluted 1:200 in culture medium), suspended in 0.2% EDTA in CMF-HBSS, rinsed three times in CMF-HBSS, and resuspended to $\sim 2.5 \times 10^7$ cells per ml in low Ca^{2+} assay medium (See Homotypic Cell Aggregation Section). 15-mm-diam glass coverslips with confluent cultures of ESb-MP or N2A cells (target cells) were placed in duplicates into 60-mm-diam Petri dishes. Fab fragments of polyclonal antibodies to L1 or N-CAM (0.5 mg/ml) were added to probe and target cells for 25 min at 4°C to allow for antibody binding. Probe cells (1.25×10^7 in 2 ml per Petri dish) were then added to target cells and incubated in low Ca^{2+} assay medium at 37°C, 45 rpm for 1 h. Unbound cells were removed by gentle washing. Bound probe cells were fixed with 4% paraformaldehyde in PBS and counted in a fluorescence microscope (Carl Zeiss, Inc., Oberkochen, FRG). Three fields (~0.025 mm^2 each) were counted for each coverslip with aggregates of adherent probe cells scored as single particles.

Cell–Substrate Adhesion

60-mm-diam bacteriologic Petri dishes were coated with nitrocellulose (BA 85, Schleicher & Schuell, Keene, NH) dissolved in methanol according to Lagenaur and Lemmon (1987). Cell adhesion molecules (1 μl of a 200 μg/ml 0.1% DOC-containing solution per spot) were spotted in a Petri dish (three spots for each adhesion molecule) and three Petri dishes were taken for an experimental value. When adhesion molecules were tested in combination, samples were mixed at a ratio of 1:1 (vol/vol) to a final protein concentration of 200 μg/ml (100 μg/ml for each molecule) and incubated for 4 h on ice before spotting. The single proteins were preincubated in parallel. To determine the protein concentrations critical for cell attachment and neurite extension, L1, N-CAM, and their combination were serially diluted in 0.1% DOC-containing CMF-HBSS in threefold steps from 200 to 2.5 μg/ml. A concentration of 0.4 μg/ml was also used. For controls, MAG and J1 (200 μg/ml) and the combinations of each, MAG, or J1 with L1 or N-CAM (100 μg/ml for each molecule) were also used. To evaluate whether the efficacy of the combination of L1 and N-CAM depended on the preincubation time of the mixture, L1 and N-CAM (each 10 μl, 200 μg/ml 0.1% DOC-containing solution) were added to 60 μl of detergent-free CMF-HBSS, mixed and allowed to stand for 0, 1, 10, 30, and 60 min, or 4 h on ice before spotting. The spots were not allowed to dry and, after spotting, excess spotting solution was removed and Petri dishes were flooded with a blocking solution containing 50 mg/ml bovine hemoglobin and 10 mg/ml BSA for 30–60 min before addition of single cell suspensions.

Single cell suspensions of cerebella from 8-d-old NMRI mice were dissociated with 2 U/ml Dispase II (Boehringer Mannheim) according to Faissner et al. (1984) and seeded onto the adhesion molecule-spotted Petri dishes

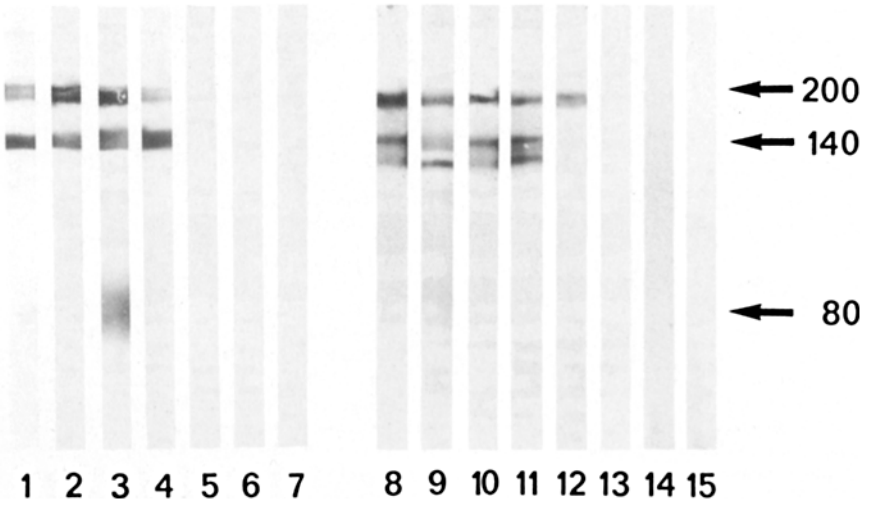


Figure 1. Documentation of the purity and specificity of adhesion molecule preparations and antibodies. L1 (lanes *1–7*) and N-CAM (lanes *8–15*), immunoaffinity purified from adult mouse brain, were separated by SDS-PAGE on 7% minislab gels and visualized by silver nitrate staining (lanes *1* and *8*) or Western blot (lanes *2–7* and *9–15*) with the following antibodies: immunoaffinity purified polyclonal L1 (lane *2*) and N-CAM (lane *9*) antibodies; Fab fragments of polyclonal antibodies (0.02 mg/ml) to L1 (lanes *3* and *13*) and N-CAM (lanes *5* and *10*); HNK-1 mAb (lanes *4* and *11*); mAb to N-CAM 180 (lane *12*); polyclonal antiserum to laminin (lanes *6* and *14*). Primary antibodies were omitted in lanes *7* and *15* and only alkaline phosphatase-conjugated anti-rat IgG (1:7,500) was used.

in CMF-HBSS. Treatment with Dispase II leaves L1- and N-CAM-dependent aggregation mechanisms intact (Faissner et al., 1984). In some cases, cells were first preincubated with Fab fragments of polyclonal antibodies to L1 or liver cell membranes (0.5 mg/ml). 2 h after seeding, the medium was changed to basal Eagle's medium containing 10% horse serum. To assure cell survival, fresh medium was mixed one to one with medium preconditioned by primary cerebellar cultures. Cultures were examined by phase-contrast microscopy 20 h after seeding the cells. All procedures were carried out under sterile conditions.

Statistical Methods

Nested analysis of variance (ANOVA), the *t* test, and multiple comparisons among means using the Student-Newman-Keuls (SNK) test were performed according to Sokal and Rohlf (1969). Differences between means that could be marginally accepted as significant with $\alpha \leq 0.05$ (range between means only slightly larger than the least significant range at $P \leq 0.05$) were considered to indicate tendencies.

Results

Bead Aggregation

To test how L1 and N-CAM could interact with each other, the purified glycoproteins were coated on Latex beads and their interactions measured by following bead aggregation.

Microscopic examination after 5 h of rotary incubation at room temperature (Fig. 2) revealed that L1-coated beads (L1 beads) formed few small aggregates (Fig. 2 *a*). N-CAM-coated beads (N-CAM beads) did not aggregate (Fig. 2 *b*) and only few aggregates were formed when L1 beads were coincubated with N-CAM beads (Fig. 2 *c*). Beads coated with both L1 and N-CAM (L1+N-CAM beads) also formed small aggregates with each other (Fig. 2 *d*). When L1 beads were incubated together with L1+N-CAM beads, very large aggregates were observed (Fig. 2 *e*). Conversely, incubation of N-CAM beads together with L1+N-CAM beads did not result in strong aggregation (Fig. 2 *f*).

Aggregation was much more pronounced after an additional 7 h of incubation at 4°C, such that very large aggregates were formed homotypically by L1 beads and L1+N-CAM beads. N-CAM beads did not aggregate. However, N-CAM beads aggregated in the presence of heparin (10^{-7} M), but not of chondroitin sulfate (10^{-7} M). The heterotypic mixture of L1 and L1+N-CAM beads produced giant aggregates.

Bead aggregation was also measured by Coulter counter particle analysis (Fig. 3). Although 19.2 ± 2.8% of all L1 beads formed large aggregates, N-CAM beads did not aggregate (3.1 ± 1.38%) (Fig. 3). Coincubation of L1 and N-CAM beads produced low aggregation (6.9 ± 0.49%), suggesting that heterotypic aggregation did not occur. When L1+N-CAM beads were incubated homotypically, 29.5 ± 0.68% of all beads were measured as large aggregates. The highest value (42.2 ± 3.87%) was measured when L1 beads were incubated together with L1+N-CAM beads. This heterotypic aggregation of L1 beads with L1+N-CAM beads was not inhibited by the soluble form of MAG nor by Fab fragments of polyclonal antibodies to ESb cell membranes (Table I *A*). However, incubation of the beads with the soluble forms of L1 or N-CAM resulted in 60% inhibition. Inhibition was also achieved by preincubation of the beads with Fab fragments of polyclonal antibodies to L1 or N-CAM. Complete inhibition of aggregation was observed after preincubation with Fab fragments of polyclonal antibodies to both L1 and N-CAM or when Fab fragments to L1 were present during the incubation (Table I *A*). Weaker or no aggregation was also measured in the following heterotypic bead incubations: N-CAM with L1+N-CAM (9.1 ± 0.17%), N-CAM with L1+N-CAM after pre-incubation with Fab fragments of polyclonal antibodies to N-CAM (0.5 mg/ml; 0% ± 0%), L1 with MAG (9.3 ± 0.59%), and N-CAM with MAG (8.2 ± 0.39%). Stronger aggregation was observed for the combinations L1 with L1+MAG (15.7 ± 1.33%) and L1 with N-CAM+MAG (16.9 ± 0.38%).

Because of the striking absence of homophilic binding between N-CAM beads under the conditions of this study, the influence of heparin on the aggregation of N-CAM beads was studied. N-CAM beads aggregated in the presence of 10^{-7} M heparin, but did not aggregate in the presence of 10^{-7} M chondroitin sulfate (Fig. 4). N-CAM beads also did not aggregate in the presence of 10^{-5} or 10^{-9} M chondroitin sulfate (not shown). Aggregation of N-CAM beads in the presence of 10^{-7} M heparin was fully inhibited by Fab fragments (0.5 mg/ml) of polyclonal N-CAM antibodies, but not by Fab fragments (0.5 mg/ml) of polyclonal MAG antibodies (Fig. 4). BSA-coated beads did not aggregate in the presence of 10^{-7} M heparin (Fig. 4).

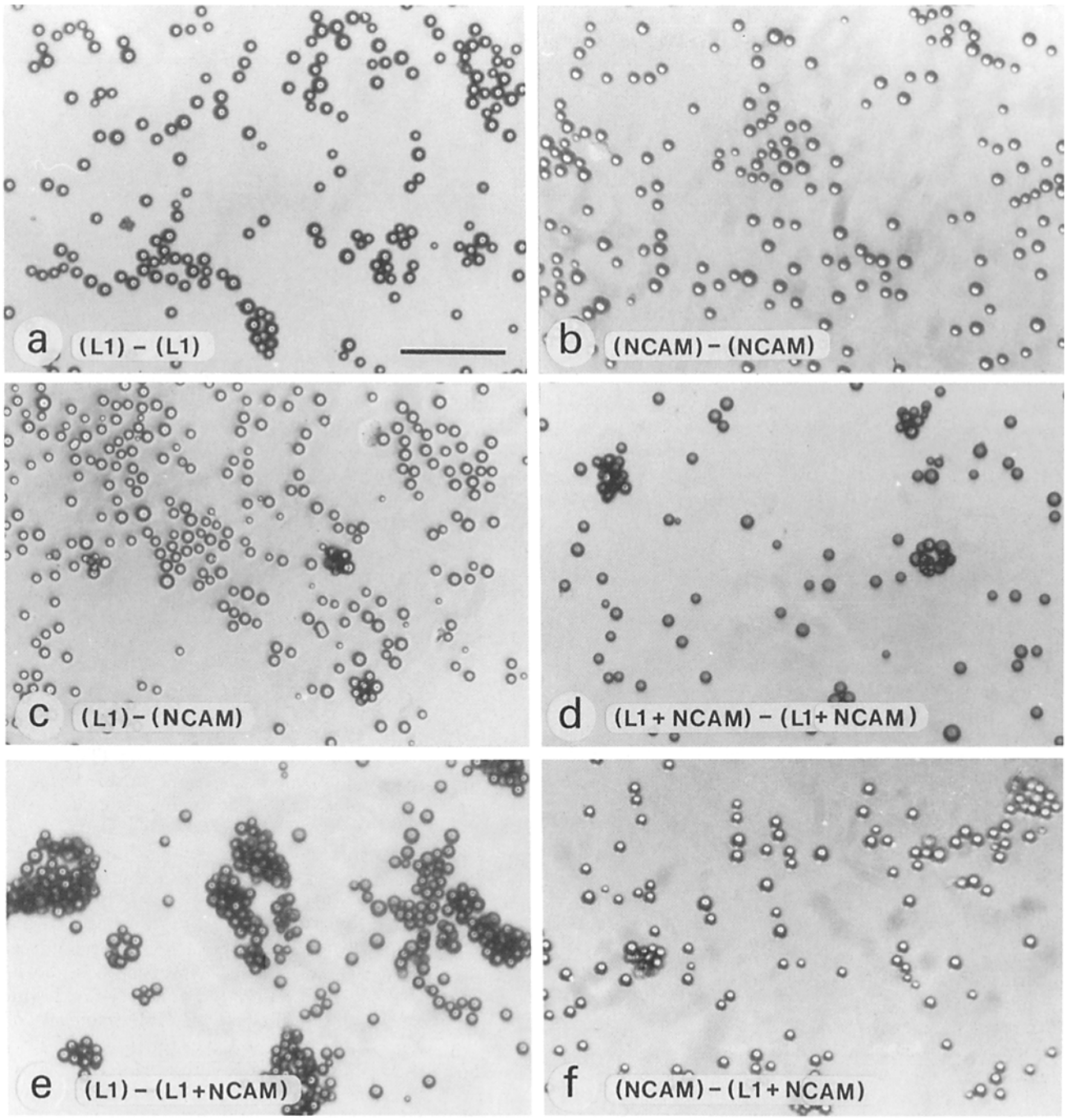


Figure 2. Light microscopic examination of the aggregation of L1- and N-CAM-coated Latex beads in the absence of Ca^{2+}. Latex beads were coated with L1 ([*L1*]; *a*, *c*, and *e*), N-CAM ([*NCAM*]; *b*, *c*, and *f*) or both L1 and N-CAM ([*L1+NCAM*]; *d*, *e*, and *f*) and were allowed to aggregate homotypically (*a*, *b*, and *d*) or heterotypically (*c*, *e*, and *f*) for 5 h at 20°C in the absence of Ca^{2+}. Bar (*a*) = 100 μm. *a–f* have the same magnification.

Binding of L1 and N-CAM to L1-coated Substrates

To characterize the concentration dependence of adhesion molecule interactions, a 1:1 mixture of L1 and N-CAM was preincubated for 4 h on ice and added to wells coated with serially diluted L1 (100–0.78 μg/ml), MAG or J1 (200 μg/ml). Binding of N-CAM to the coated wells was detected by an N-CAM mAb. Binding of the complex of N-CAM with L1 to immobilized L1 increased linearly on a semilogarithmic scale as a function of L1 concentrations (Fig. 5). A plateau was reached at 17 μg/ml, when 80% maximal binding was reached. This plateau may be due to saturation of the wells with coated L1. N-CAM alone did not bind to immobilized L1 in the range of concentrations tested. Moreover, binding of the N-CAM+L1 combination to J1 and MAG was considerably lower than to L1 (Fig. 5).

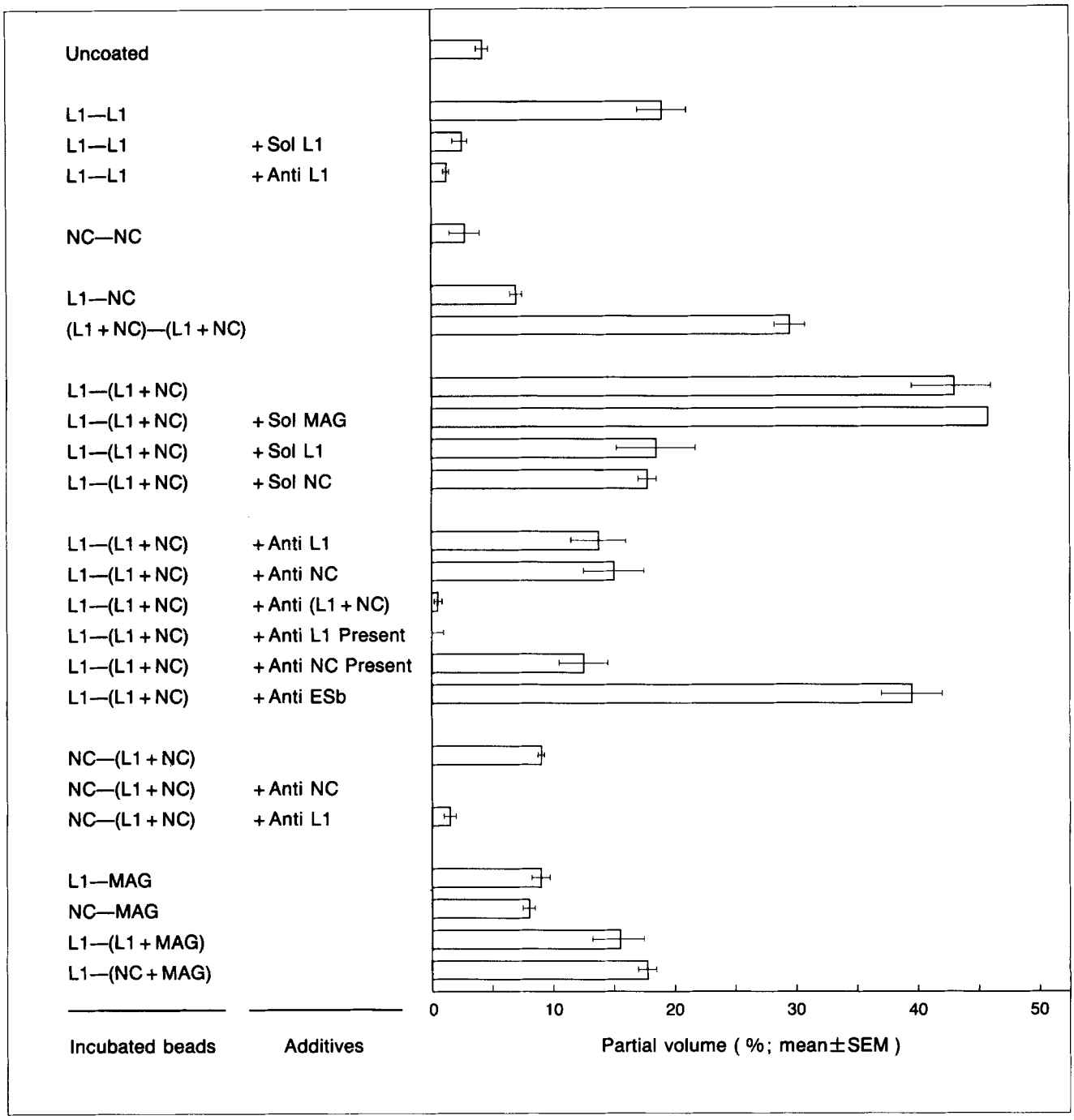


Figure 3. Coulter counter analysis of Ll-, N-CAM- and MAG-coated Latex beads in the absence of Ca^{2+}. Latex beads were coated with Ll (*Ll*), N-CAM (*NC*), Ll and N-CAM ([*Ll+NC*]), MAG (*MAG*), Ll and MAG ([*Ll+MAG*]), N-CAM and MAG ([*NC+MAG*]) or with BSA (*uncoated*) and were postcoated with BSA. The beads were incubated homotypically or heterotypically for 30 h at 4°C in the absence of Ca^{2+} as indicated. Where indicated (*additives*), coated beads had been preincubated with or incubated in presence (*present*) of Fab fragments (0.5 mg/ml) of polyclonal antibodies to Ll (*Anti Ll*), N-CAM (*Anti NC*), Ll and N-CAM, combined (*Anti* [*Ll+NC*]) or ESb cell membranes (*Anti ESb*), or in the presence of 50 μg/ml of the soluble forms of Ll (*Sol Ll*), N-CAM (*Sol NC*), or MAG (*Sol MAG*). Aggregation was measured by particle analysis. The bars represent the cumulative partial volume measured in channels corresponding to large aggregates. Values are means from at least three experiments performed in triplicates ± SEM.

Cell-Substrate Adhesion

The adhesion and neurite extension of cerebellar neurones to and on Ll and N-CAM was investigated to further characterize the cooperativity between the two molecules.

Small cerebellar neurones selectively bound to substrate-coated Ll (Fig. 6 *Ba*). They covered Ll spots uniformly (Figs. 6 *Ba* and 7 *Bc*) and sent out processes that often crossed over one another (Fig. 6 *Ba*). A similar pattern of adherent cell types and neurite outgrowth was observed on the mixture of Ll and N-CAM (Fig. 6, *Af* and *Ah*; Fig. 7 *Ba*). Adhesion and neurite outgrowth of cerebellar cells to

Table I. Inhibition of Heterotypic Aggregation of Cells and Beads that Carry L1 or L1+N-CAM

Additives	Antigen or antibody concentration	Inhibition of coaggregation
	μg/ml	*%, mean ± SEM*
A. Beads		
Soluble L1	50	58.5 ± 6.04
Soluble N-CAM	50	60.2 ± 1.74
Soluble MAG	50	−11.6 ± 0.14
L1 Fab (pre)	500	64.9 ± 5.38
L1 Fab	500	100.0 ± 2.13
N-CAM Fab (pre)	500	63.5 ± 4.41
N-CAM Fab	500	70.6 ± 4.74
L1 Fab + N-CAM Fab (pre)	500 + 500	99.1 ± 0.88
ESb Fab	1,000	6.1 ± 5.69
B. Cells		
L1 Fab	500	73.8 ± 6.59
N-CAM Fab	500	55.2 ± 3.20
Antiliver membranes Fab	1,000	−4.6 ± 3.61

(*A*) Latex beads were coated either with L1 alone or with both L1 and N-CAM and were mixed at a 1:1 bead ratio. The beads were allowed to coaggregate without Ca^{2+} in the absence of additives or in the presence of the soluble forms of L1, N-CAM, or MAG or Fab fragments of polyclonal antibodies to L1, N-CAM, or ESb cell membranes. Bead aggregation was also tested after preincubation with the antibodies and their subsequent removal (pre) and evaluated by Coulter counter particle analysis. Values are given as reduction in aggregate formation (percent inhibition) under the respective treatments as compared to the mean aggregate formation in the absence of soluble adhesion molecules and antibodies. Values are means from three independent experiments with each value carried out in triplicates ± SEM. (*B*) L1-positive ESb-MP cells and L1+N-CAM-positive N2A cells were mixed together in suspension in low Ca^{2+} in the absence or presence of Fab fragments of polyclonal antibodies to L1, N-CAM, or liver cell membranes. Inhibition of aggregation was evaluated by comparing the reduction in the partial volume measured in channel 7 when the two cell types were mixed before and after their aggregation as shown in Fig. 9. Values are means from three experiments with each value carried out in triplicates ± SEM.

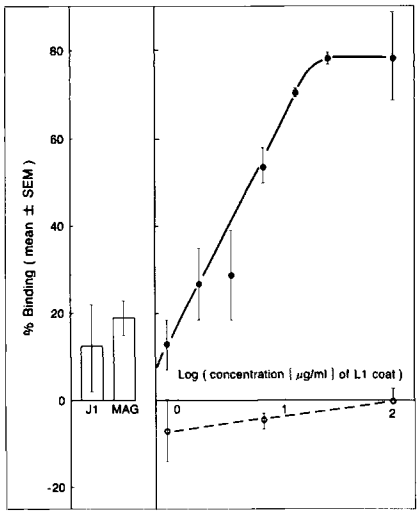


Figure 5. Binding of the L1 plus N-CAM complex to immobilized L1. Microtiter ELISA plates were coated in triplicates with 100 μl/well of serially diluted L1 (0.78–100 μg/ml; *curves*) or with 200 μg/ml J1 or MAG (*columns*). The coated wells were then incubated overnight at room temperature in the absence of Ca^{2+} with 10 μg/ml N-CAM (○) or 10 μg/ml of a preincubated 1:1 mixture of L1 and N-CAM (●; *columns*). Protein binding was determined by ELISA with monoclonal N-CAM antibody. Background and maximal binding levels were estimated in BSA-coated and uncoated wells, respectively. Binding values are given in a semilogarithmic plot as percentage of the maximal binding levels. Values are means ± SEM from two experiments performed in triplicates.

N-CAM was variable, but normally weak and often uneven within a spot (Fig. 6, *Ac*, *Ad*, and *Bb*; Fig. 7, *Be* and *Bf*).

We next evaluated adhesivity and neurite extension as a function of substrate protein concentration. For this purpose, the lowest concentrations of L1, N-CAM, and their combination that gave detectable cell adhesion were compared (Table II; Fig. 6). L1 was still active when coated at a concentration of 22 μg/ml (Table II), but no longer allowed attachment of cerebellar neurones at 7.5 μg/ml (Fig. 6 *Ab*). N-CAM did not bind cells at concentrations < 67 μg/ml. However, its adhesivity was already low and variable at 200 μg/ml (Fig. 6, *Ac*, *Ad* and *Bb*; Fig. 7, *Be* and *Bf*). The mixture of L1 and N-CAM at a 1:1 protein ratio retained adhesivity and neurite-promoting activity at a concentration as low as 1.2 μg/ml/antigen (Fig. 6, *Ae–Ah*).

As controls, other adhesion molecules were tested (Table II; Fig. 6 *B*). Only few cells adhered to the MAG (Fig. 6 *Bc*) and no cells attached to the enriched J1 glycoproteins (Fig. 6 *Bd*). Also, only few cerebellar cells adhered to mixtures of N-CAM and MAG (Fig. 6 *Bg*) or J1 (Fig. 6 *Bh*). In these cases, adherent cells were mostly nonneuronal as estimated by morphological appearance. Binding of neurones was more obvious to the mixtures of L1 and MAG (Fig. 6 *Be*) or J1 (Fig. 6 *Bf*). However, in these combinations adhesion was lower than on the L1 or the L1+N-CAM substrates (Table II) and neurite outgrowth patterns were different.

To evaluate whether L1 and N-CAM need to interact with each other to become a potent substrate, L1 and N-CAM (50 μg/ml) were mixed and incubated together for varying lengths of time prior to their immobilization on the substrate (Fig. 7). When L1 and N-CAM were substrate coated in <0.5 min after being mixed together, adhesion of cerebellar neurones was weak and frequently a division into cell-rich and cell-poor zones was seen (Fig. 7, *Aa* and *Ab*). If L1 and

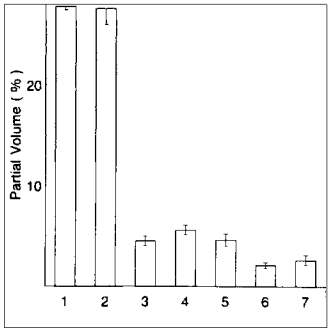
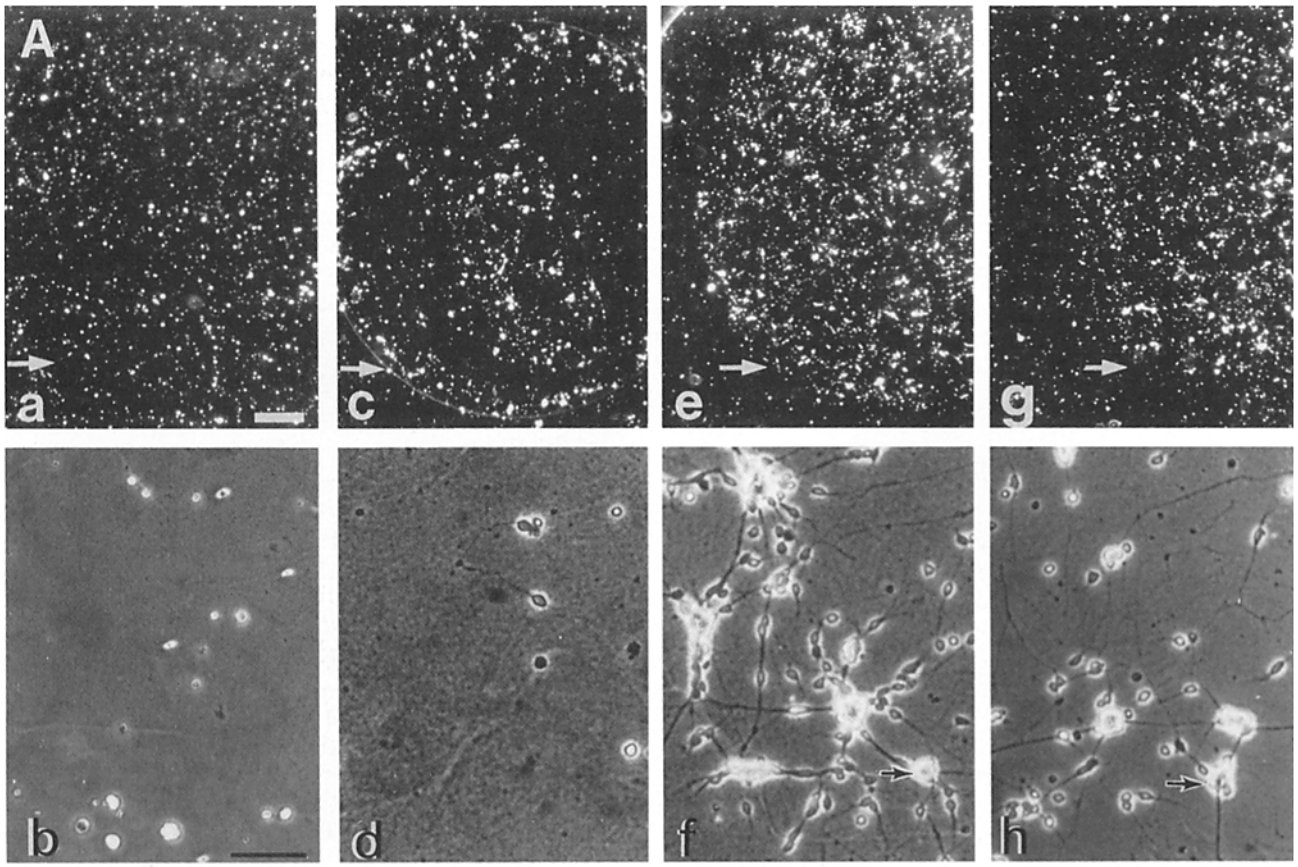


Figure 4. Coulter counter analysis of the aggregation of N-CAM-coated beads in the presence of heparin. Latex beads were coated with N-CAM (lanes *1–5*) or BSA (lanes *6* and *7*) and were post-coated with BSA. The beads were allowed to aggregate at room temperature for 8 h in the absence of Ca^{2+} and in the presence of 10^{-7} M heparin (lanes *1–3*, and *6*), 10^{-7} M chondroitin sulfate (lane *4*), and Fab fragments (0.5 mg/ml) of polyclonal antibodies to MAG (lane *2*) or N-CAM (lane *3*), or in the absence of additives (lanes *5* and *7*). Aggregation was measured by particle analysis. The bars represent the cumulative partial volume measured in channels corresponding to large aggregates. Values are means from two experiments, each comprising three vials measured in triplicates ± SEM.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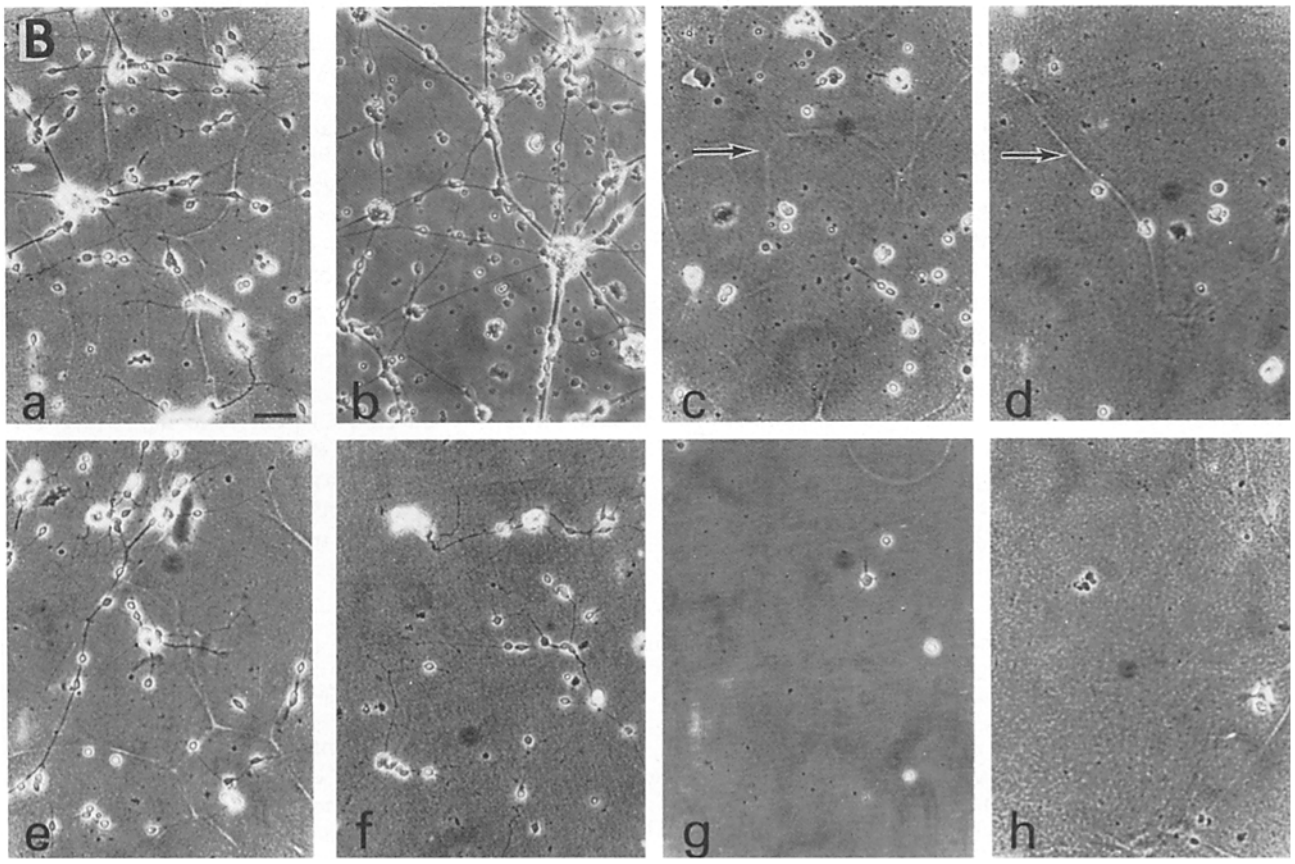
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N-CAM were preincubated together for 1 or 10 min, almost no adhesion was detected (for 10 min see Fig. 7, *Ac* and *Ad*). Nonetheless, the few adherent neurones exhibited the typical morphological features of neurones on an L1 substrate (Fig. 7 *Ad*). After 30 min of preincubation adhesion was detectable (Fig. 7, *Ae* and *Af*). Neurones adhered homogeneously to the whole surface of the spot and sent out long processes. Preincubations lasting 1 or 4 h resulted in very strong and uniform adhesion of neurones to the spots as well as in extensive neuronal outgrowth accompanied by some fasciculation and contact formation (for 1 h, see Fig. 7, *Ag* and *Ah*). Fab fragments of polyclonal antibodies to L1, but not to mouse liver membranes (both at 0.5 mg/ml) inhibited adhesion of cerebellar cells to L1 and L1+N-CAM (for L1+N-CAM, see Fig. 7, *Ba* and *Bb*).

L1- and N-CAM-dependent Homo- and Heterotypic Cell Aggregation

To verify the results obtained with purified adhesion molecules, L1- and N-CAM-dependent interactions between live cells were measured under low Ca^{2+}. First, homotypic aggregation of N2A cells, which express both L1 and N-CAM, was compared with that of ESb-MP cells, which express L1, but not N-CAM. Eb 288 cells that express neither adhesion molecule served as controls.

Small aggregates of N2A cells could be detected already after 5–10 min of incubation under rotation (not shown). After 30 min of incubation, almost all cells were observed in aggregates (Fig. 8 *Aa*) with ~20% of the cells present in large aggregates, as evaluated by Coulter counter particle analysis (Fig. 8 *B*). In the presence of 0.5 mg/ml Fab fragments of polyclonal antibodies to L1 or N-CAM, aggregation was reduced by 35 ± 2.9% (mean ± SEM) or 46 ± 2.8%, respectively. These values are similar to those reported previously (Rathjen and Rutishauser, 1984; see also Faissner et al., 1984). When Fab fragments of L1 and N-CAM polyclonal antibodies were added at a 1:1 protein ratio and final concentration of 0.5 mg/ml antibody, only about 3.5% of the cells formed large aggregates, resulting in a reduction of aggregation by 83 ± 2.5% with respect to the control.

Contrary to N2A cells, ESb-MP cells that express only L1 did not form aggregates within 1 h of incubation (Figs. 8 *B* and 9 *A*). Small aggregates were first seen after 3 h of incubation (Fig. 8, *Ab* and *B*) and by the end of the fifth hour, ~25% of the cells were measured as large aggregates (Fig. 8 *B*). To probe whether cells were still competent for aggregation after 5 h, Ca^{2+} concentrations were increased to the normal level of 1.25 mM and 50 nM TPA were added to activate their Ca^{2+}-dependent aggregation (see also Patarroyo et al., 1985; Kowitz et al., manuscript submitted for publication). This resulted in a further increase of aggregation to 46 ± 3.4% (Fig. 8 *C*) within 30 min, indicating that the cells were still viable and capable of aggregation.

Eb 288 cells express neither L1 nor N-CAM, but are closely related to ESb-MP cells. They did not aggregate significantly within 5 h of incubation under low Ca^{2+} (Fig. 8, *Ad* and *B*). The aggregation value of Eb 288 cells was not significantly different from that obtained for ESb-MP cells in the presence of 0.5 mg/ml Fab fragments of polyclonal antibodies to L1 (Figs. 8, *Ac* and *B*). Fab fragments of polyclonal antibodies to ESb cell membranes, which also react with ESb-MP cells, did not perturb aggregation of ESb-MP cells (Fig. 8 *B*).

Heterotypic incubation of ESb-MP with N2A cells resulted in the formation of pronounced coaggregates already within 35 min (Fig. 9 *B*; Table III *A*). The addition of 0.5 mg/ml Fab fragments of polyclonal antibodies to L1 resulted in a partial (35%) reduction in the aggregation of N2A cells (Fig. 8 *B*), but in a large reduction (65–80%) in the coaggregation between the two cell types (Tables I *B* and III *A*). ESb-MP cells now appeared as single cells that no longer coaggregated with N2A cells, indicating that their adhesion to N2A cells would be mediated by L1. A similar result was obtained when Fab fragments of polyclonal antibodies to N-CAM were tested (Tables I *B* and III *A*). Furthermore, after treatment of ESb-MP cells with the soluble form of N-CAM, their homotypic aggregation was significantly increased after one hour of incubation, whereas soluble MAG did not have this effect (Table IV). Aggregation of ESb-MP cells treated with soluble N-CAM was inhibited by Fab fragments (0.5 mg/ml) of polyclonal N-CAM and L1 antibodies but not of MAG antibodies (Table IV).

L1- and N-CAM-dependent Homo- and Heterotypic Cell Adhesion

To verify the results obtained in the aggregation experiments, the adhesion of suspended to immobilized cells was also measured (Table III *B*). ESb-MP cells did not adhere homotypically, but when confronted with N2A cells significant adhesion was seen. This heterotypic adhesion was almost completely inhibitable by antibodies to L1 (~90%) and strongly by antibodies to N-CAM (~80%). Homotypic adhesion of N2A cells was partially inhibited by L1 (52%) and N-CAM (61%) antibodies.

Discussion

In this study, evidence has been gathered to suggest that the cell adhesion molecule L1 engages in a so-called homophilic binding mechanism and that N-CAM enhances the L1-mediated interaction. In this interaction, the most adhesive combination appears to be L1 on one of the interacting part-

Figure 6. Light microscopic examination of adhesion and neurite extension of cerebellar neurones on L1 and N-CAM substrates. Dispase-dissociated cerebellar cells were prepared from 8-d-old mice and plated onto nitrocellulose-treated Petri dishes prespotted with cell adhesion molecules. Cell adheison and neurite extension were examined 20 h after plating. (*A*) *a* and *b*: L1, diluted 1:27 to 7.5 μg/ml; *c* and *d*: 200 μg/ml N-CAM; *e* and *f*: L1+N-CAM diluted 1:9 to 11 μg/ml for each antigen; *g* and *h*: L1+N-CAM diluted 1:81 to 1.2 μg/ml for each antigen. Bar, 0.5 mm (in *a*), where *a*, *c*, *e*, and *g* are equally magnified, and 50 μm (in *b*), where *b*, *d*, *f*, and *h* are equally magnified. Spot margins (*white arrows*) and macrophages that adhere to neurones (*black arrows*) are marked. *B*: *a–d*: 200 μg/ml L1 (*a*), N-CAM (*b*), MAG (*c*), or J1 (*d*). Notice the variable binding to N-CAM, as depicted by *Ad*, *Bb*, and 7*Bf*. *e–f*: 100 μg/ml L1 mixed with 100 μg/ml MAG (*e*) or J1 (*f*); *g* and *h*: 100 μg/ml N-CAM mixed with 100 μg/ml MAG (*g*) or J1 (*h*). Bar (in *a*), 50 μm; *a–h* have the same magnification. (*Arrows*) Lines produced by the texture of nitrocellulose.

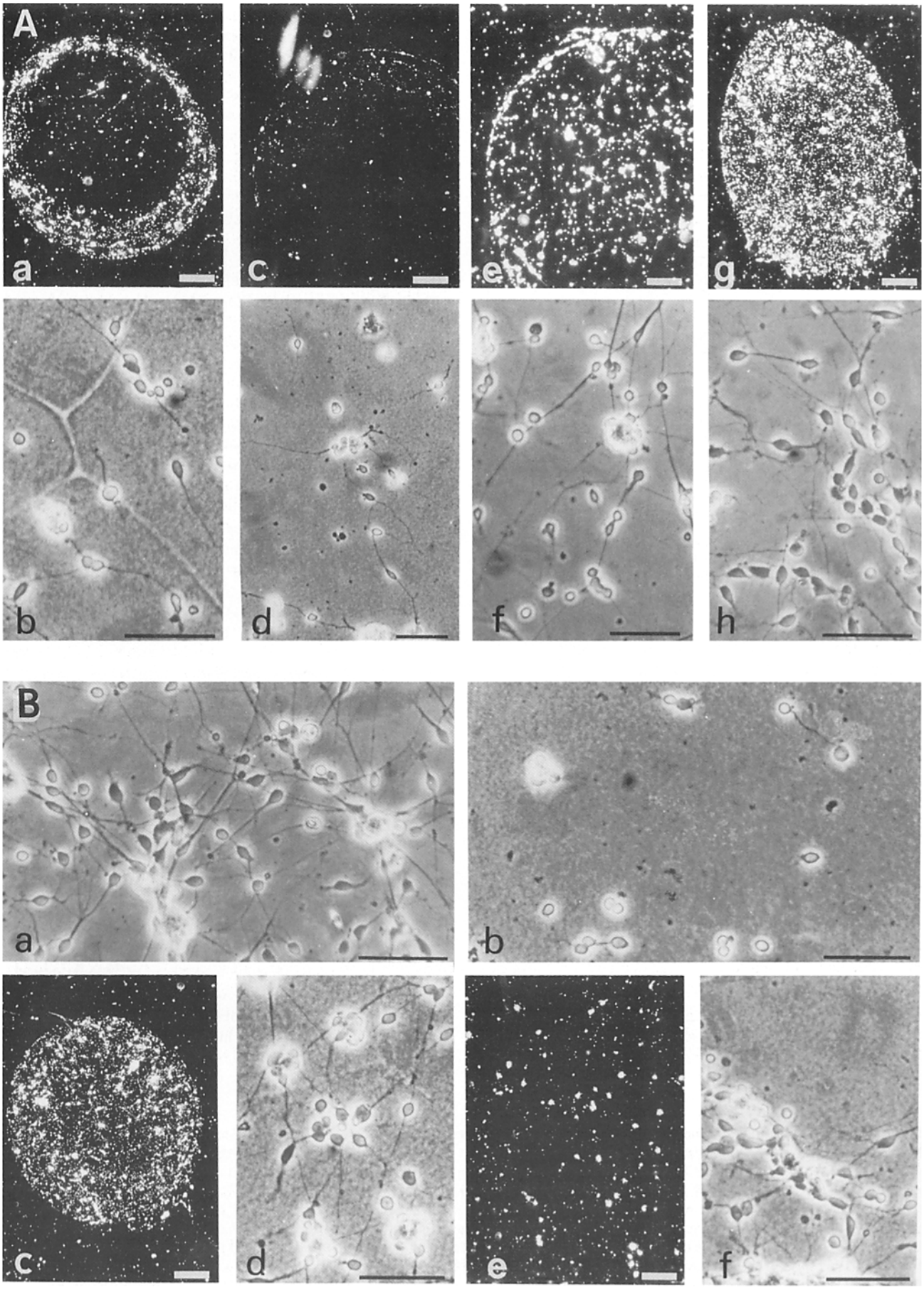
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Table II. Adhesion of Cerebellar Neurones to Immobilized Adhesion Molecules

Adhesion molecule	Protein concentration					
			μg/ml			
	200	67	22	7.4	2.5	0.4
L1	+++	+++	+	−	−	−
N-CAM	++/−	+/−	−	−	−	−
MAG	±	−	−	ND	ND	ND
J1	−	−	−	ND	ND	ND
Adhesion molecule combination	**Protein concentration per adhesion molecule**					
			μg/ml			
	100	33	11	3.7	1.2	0.2
L1+N-CAM	+++	+++	+++	++	++	−
L1+MAG	+	+	−	ND	ND	ND
L1+J1	++	+	−	ND	ND	ND
N-CAM+MAG	−	−	−	ND	ND	ND
N-CAM+J1	−	−	−	ND	ND	ND

Dispase-dissociated cerebellar cells were prepared from 8-d-old mice and plated onto nitrocellulose-treated Petri dishes prespotted with serially diluted adhesion molecules (L1, N-CAM, MAG, J1) or their combinations. Combined proteins were mixed 1:1 and preincubated for 4 h on ice. Adhesion of neurones to the spots was examined microscopically 20 h after plating. Adhesion was considered homogeneous and dense, whereby all neurites reached other neurones (+++); homogeneous but not dense (few cell aggregates and many neurites not reaching other neurones) (++); homogeneous but sparse (neurones equally distributed on all replicate spots, but <10% of the neurites reaching other neurones) (+); heterogeneous and mostly weak (++/− or +/−); weak, and only some of the replicate spots positive (±) or negative (−).

ners and the L1+N-CAM complex on the other. Furthermore, the L1+N-CAM complex appears to require preformation to be active.

Homophilic Binding of L1

The study was instigated by the availability of a tumor cell variant of a lymphoid tumor cell line that expressed L1, but not N-CAM (Kowitz et al., manuscript submitted for publication). This L1-positive variant showed slow Ca^{2+}-independent aggregation that could be strongly inhibited by L1 antibodies. The observation that the L1-negative parent cell line did not aggregate under low Ca^{2+} with itself or with the L1-positive variant (Table III *A*), suggested a homophilic binding mechanism for L1. To test this hypothesis, immunoaffinity-purified L1 glycoprotein was adsorbed onto beads and their aggregation was characterized. Aggregation of the L1-coated beads did indeed occur and could be inhibited by the soluble form of L1 and by L1 antibodies. This result is compatible with previous observations using purified L1 and Ng-CAM (Grumet and Edelman, 1988; Sadoul et al., 1988) and supports the possibility that the L1-dependent aggregation of L1-positive lymphoid cells is mediated by homophilic binding of L1. Furthermore, these results strengthen the view that L1 can be isolated in a functional state.

N-CAM Binding Depends on Heparin

In contrast to previous observations (Cunningham et al., 1983; Hoffman and Edelman, 1983; Moran and Bock, 1988), but in agreement with others (Sadoul et al., 1988; Becker et al., 1989), we could not demonstrate homophilic binding of N-CAM beads with each other under the conditions of this study. We therefore investigated the possibility that N-CAM was altered by our isolation procedure. N-CAM could indeed be observed to mimic self-binding when N-CAM beads were allowed to aggregate in the presence of heparin. It is, therefore, conceivable that heparin, a carbohydrate polymer chain, cross-links single N-CAM molecules via their heparin binding sites (Cole et al., 1986; Cole and Akeson, 1989). Alternatively, heparin may induce conformational changes in N-CAM that enable N-CAM to engage in true homophilic binding. Our observations thus render a gross impairment of N-CAM under our isolation procedures unlikely and raise the possibility that previous observations on N-CAM self-binding may have been due to the presence of heparin, heparan sulfate or heparan sulfate proteoglycans as it has recently been reported (Cole and Burg, 1989). Furthermore, it is possible that N-CAM self-binding may have been caused by the presence of contaminating levels of L1 or to nonspecific interactions between hydrophobic domains (Grumet et al., 1984*b*; Hall and Rutishauser, 1987; Becker et al., 1989). However, it should be pointed out that it cannot be excluded that the N-CAM forms in our preparation do not contain the molecular species involved in homophilic binding in sufficient quantity and adequate configuration.

N-CAM Enhances L1-dependent Adhesion

When coated on beads, L1 and N-CAM cooperated maximally when closely associated with each other on one side of the interacting partners, i.e., in *cis* position. Aggregation of beads was best when beads coated with the L1+N-CAM complex were incubated together with L1-coated beads. That this combination of reactive partner molecules is also operative on cells is indicated by the observation that preincubation of L1-positive tumor cells with soluble N-CAM increases their aggregation with L1-positive cells. When beads coated with both L1 and N-CAM were allowed to aggregate homotypically, aggregation was slow and, even after 30 h, weaker than when L1 beads coaggregated with L1+N-CAM beads. This observation suggests that the *trans*-interaction

Figure 7. Light microscopic examination of the effect of preincubation durations of L1 and N-CAM on their combined substrate properties. Dispase-dissociated cerebellar cells were prepared from 8-d-old mice and plated onto nitrocellulose-treated Petri dishes prespotted with L1, N-CAM, or L1 plus N-CAM. Cell adhesion and neurite extension were examined 20 h after plating. (*A*) L1 and N-CAM were diluted and mixed together in CMF-HBSS (each at 25 μg/ml) and immediately spotted on nitrocellulose (*a* and *b*) or preincubated for 10 (*c* and *d*), 30 (*e* and *f*) or 60 (*g* and *h*) min before spotting. Bar, 0.5 mm in *a*, *c*, *e*, and *g* and 50 μm in *b*, *d*, *f*, and *h*. (*B*) For controls, L1 and N-CAM were preincubated together for 4 h before spotting. Cells were plated in the presence of Fab fragments (0.5 mg/ml) of polyclonal antibodies to liver membranes (*a*) or L1 (*b*). Additionally, unmixed L1 (*c* and *d*) and N-CAM (*e* and *f*) were also spotted. Notice that the field shown in *f* lies within an N-CAM spot demonstrating the restricted and uneven adhesion and neurite extension characteristic for N-CAM substrates. Cell-dense areas cover only small parts of the spots. Bar, 50 μm in *a*, *b*, *d*, and *f* and 0.5 mm in *c* and *e*.

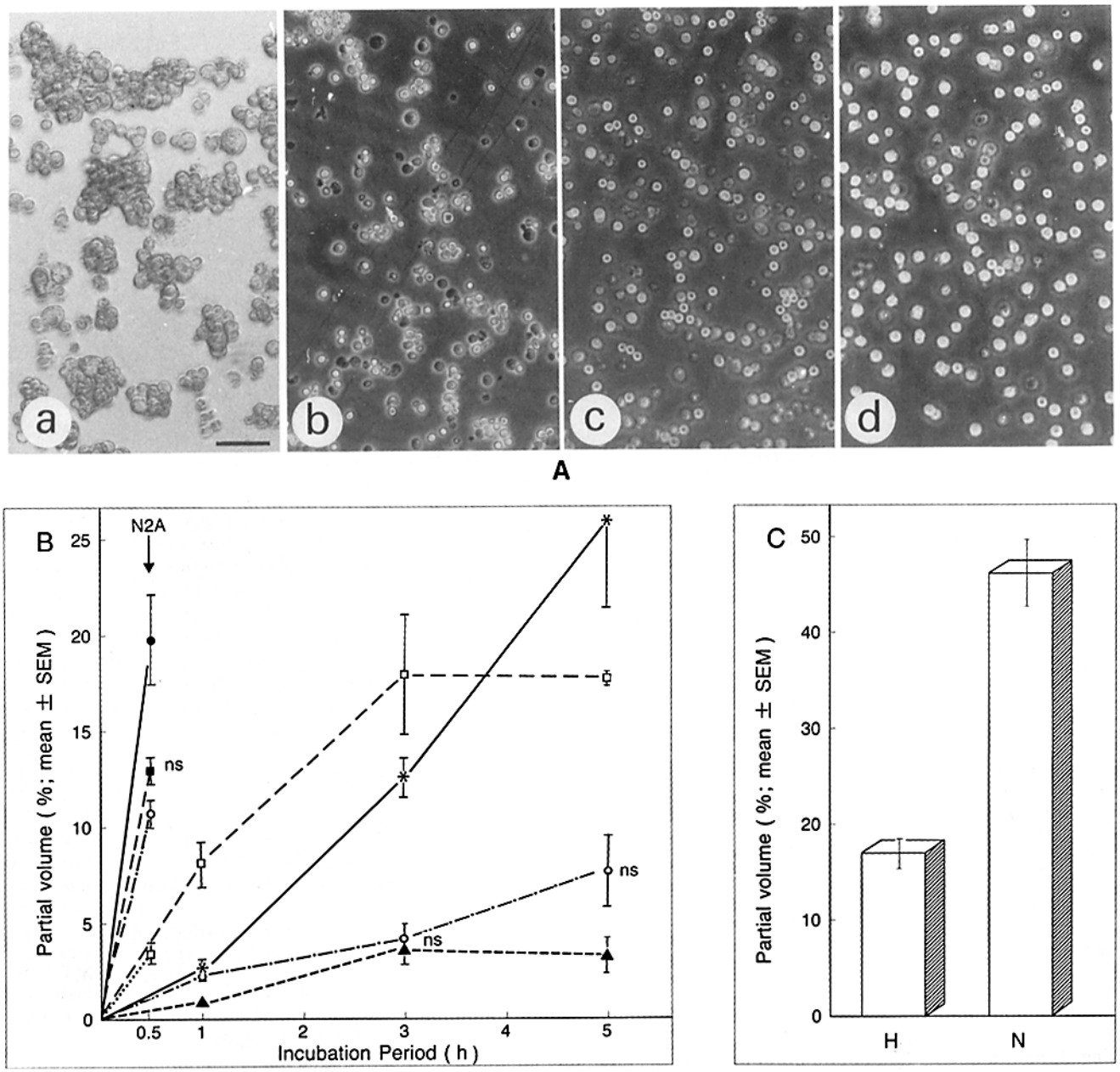

Figure 8. Homotypic aggregation of N2A, ESb-MP and Eb 288 cells in low Ca^{2+}. (*A*) Light microscopic examination of N2A cells allowed to aggregate for 20 min (*a*), and of ESb-MP (*b*) and Eb 288 (*d*) allowed to aggregate for 3 h. ESb-MP cells were also incubated for five hours in the presence of Fab fragments (0.5 mg/ml) of polyclonal antibodies to L1 (*c*). Bar (*a*) = 50 μm; *a–d* have the same magnification. (*B*) N2A cells were allowed to aggregate for 30 min in the absence of antibodies (●) or with Fab fragments of polyclonal antibodies to L1 (–·–○–·–), N-CAM (■), or to L1 and N-CAM (···□···). ESb-MP cells were allowed to aggregate for 1, 3, or 5 h in the absence of antibodies (*) or with Fab fragments of polyclonal antibodies to L1 (–·–○–·–) or ESb cell membranes (– – –□– – –). Eb 288 cells (▲) were allowed to aggregate for 1, 3, or 5 h in the absence of antibodies. Fab fragments were at a concentration of 0.5 mg/ml. Mean values measured in a representative experiment in quadruplicates ± SEM are given. For each aggregation period, the experimental values were compared by the SNK method. Values for aggregation of Eb 288 cells and of ESb-MP cells in the presence of Fab fragments of polyclonal L1 antibodies are not significantly different from each other. (*C*) ESb-MP cells were allowed to aggregate for 5 h in low Ca^{2+} and aggregation continued for another 30 min after addition of the phorbol ester TPA (50 nM) in 0.1 mM (*H*) or 1.25 mM (*N*) Ca^{2+}. Mean values ± SEM measured in triplicates are given from one representative experiment.

between L1+N-CAM complexes is weak, possibly even resulting from the presence of uncomplexed L1 in the L1+N-CAM mixture.

Specificity of L1 Binding to the L1+N-CAM Complex

To ascertain that L1 interacts specifically with the L1+N-CAM complex, several control experiments were carried out.

First, inhibition experiments were performed with antibodies and soluble forms of adhesion molecules. Reduction of bead aggregation was seen when L1 and L1+N-CAM beads were preincubated with antibodies to L1 or N-CAM. Complete inhibition of aggregation was achieved by incubat-

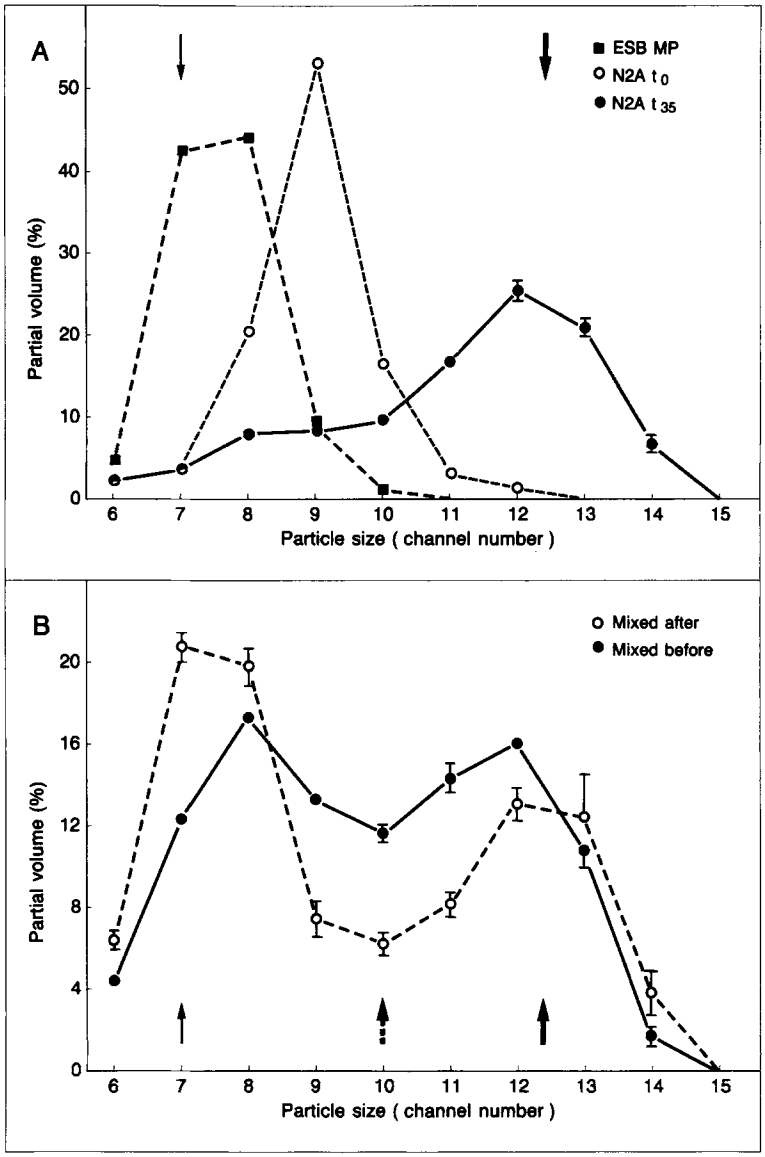

Figure 9. Coaggregation of ESb-MP and N2A cells as evaluated by Coulter counter analysis. ESb-MP and N2A cells were allowed to aggregate for 35 min in low Ca^{2+} concentrations either separately or mixed together. Aggregation profiles were evaluated by Coulter counter particle analysis. (*A*) Distribution of particle sizes of ESb-MP or N2A cells after aggregation (t_{35}) and of N2A cells before aggregation (t_0). Whereas N2A cells form large aggregates (*thick arrow*), ESb-MP cells do not aggregate (t_0 is not shown for ESb-MP cells). Note that single ESb-MP cells (channel 7, *thin arrow*) are smaller than N2A cells, which are not detected below channel 8. (*B*) ESb-MP and N2A cells were mixed together either before (*mixed before*) or after (*mixed after*) aggregation. Coaggregation of ESb-MP with N2A cells is indicated by a reduction in the partial volume occupied by single ESb-MP cells in channel 7 (*thin arrow*) and an increase in the volume occupied by aggregates (*broken* and *thick arrows*). Values are means from three experiments performed in triplicates ± SEM.

ing these beads with L1 antibodies alone or preincubating them with the mixture of L1 and N-CAM antibodies. The capacity of both antibodies to perturb aggregation indicates that both L1 and N-CAM are involved. That L1 antibodies fully inhibit bead aggregation would suggest that the glycoprotein is necessary on both sides of the interacting partners. Aggregation is not only inhibited by L1 antibodies, but also by the soluble form of L1, which thus acts as a competitive inhibitor. The inability of N-CAM antibodies to completely block aggregation results probably in part from the presence of beads coated with L1, but not with N-CAM, which remain free to aggregate homotypically. Soluble N-CAM also interrupts the adhesion of L1 to the L1+N-CAM complex, possibly by associating with uncomplexed L1, thus saturating the L1 beads in the L1+N-CAM complex, which is less adhesive to itself. That N-CAM did not bind detectably to L1 in the ELISA, but appears to interact with L1 in the bead and cell aggregation assays, may result from removing unbound N-CAM in ELISA, thus dissociating weak associations, whereas it remained associated in the aggregation assays. Alternatively, N-CAM may be able to interact with L1 in solution or on cells, but not when L1 is bound to artificial surfaces.

Second, other glycoproteins were tested for their ability to modify the interaction of L1 with L1 or N-CAM. Of the control glycoproteins used, the MAG appeared to be closest to L1 and N-CAM since, like L1 and N-CAM, it belongs to the immunoglobulin superfamily. The aggregation of L1 beads with beads coated with the L1+N-CAM complex was not reduced in the presence of the soluble form of MAG. Furthermore, only weak aggregation was observed when L1 beads were incubated together with beads coated with N-CAM or MAG. The stronger interaction of L1 with the complexes between L1 and MAG or N-CAM and MAG may be due to the possibility that L1 binds homophilically to L1 in the L1+MAG complex and that members of the immunoglobulin superfamily may form cooperative molecular associations with each other. Nevertheless, neither N-CAM and MAG individually nor the combinations of N-CAM and MAG or L1 and MAG have an affinity for L1 that is comparable to that seen with the L1+N-CAM complex and strong enough to produce similarly strong aggregation of beads.

Table III. Microscopic Evaluation of N2A–ESb-MP Cell Coaggregation and Adhesion

A. Coaggregation		Inhibition	
	Colocalization	L1 Fab	N-CAM Fab
	%		
N2A + ESb-MP	31.7 ± 9.36*	76.6 ± 5.93	80.1 ± 5.80
Eb 288 + ESb-MP	6.3 ± 7.41		

B. Adhesion			Inhibition	
Probe cells	Target cells	Bound cells	L1 Fab	N-CAM Fab
N2A	N2A	385 ± 8.9*	52.1 ± 1.98	60.9 ± 1.17
N2A	ESb-MP	165 ± 4.9*	90.9 ± 1.50	81.5 ± 1.11
ESb-MP	N2A	195 ± 3.6*	87.5 ± 0.75	77.0 ± 0.77
ESb-MP	ESb-MP	9 ± 1.7	ND	ND

Inhibition of interaction between L1-positive ESb-MP cells and L1 and N-CAM-positive N2A cells by Fab fragments (0.5 mg/ml) of polyclonal antibodies to L1 (L1 Fab) and N-CAM (N-CAM Fab) was examined microscopically in two assay systems. (*A*) Rhodamine-labeled N2A or EB 288 and bisbenzimide-labeled ESb-MP cells were mixed in suspension and allowed to aggregate together in low Ca^{2+} for 35 min in the presence or absence of antibodies. Individual ESb-MP cells were scored as colocalized, i.e., in direct contact with a N2A or Eb 288 cell, or not colocalized with N2A or Eb 288 cells and the percentage of all ESb-MP cells colocalized with N2A or Eb 288 cells (percent colocalization) determined. Inhibition of colocalization by antibodies is given in percent. Values are means from four experiments performed in triplicates ± SEM. Control (without antibody) is significantly different from each of the values scored in the presence of Fab fragments with $p \leq 0.001$ (*) (SNK method). (*B*) Fluorescein-labeled N2A or ESb-MP cells (probe cells) were added in suspension to confluent ESb-MP or N2A cells (target cells). Cells were incubated in low Ca^{2+} concentrations for 1 h at 37°C and 45 rpm in the presence or absence of antibodies. Bound probe cells were counted. Inhibition of adhesion is given in percent. Values are means from four coverslips ± SEM. Three fields of 0.025 mm^2 per coverslip were measured. *As in *A*.

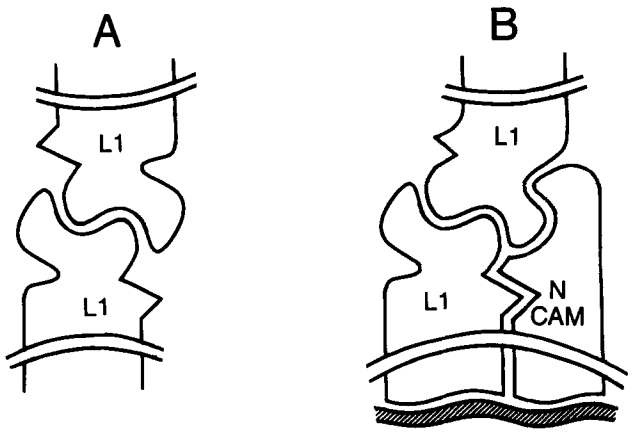

Figure 10. "Homophilic" and "assisted homophilic" adhesion: schematic interpretation. (*A*) "Homophilic" adhesion. L1 in the surface membrane of one cell mediates adhesion by interaction with L1 in another cell. This adhesion is slow, possibly reflecting a low affinity between L1 molecules, or necessity for conformational modifications before binding can be established. (*B*) "Assisted homophilic" adhesion. L1 associates with N-CAM on the surface of one cell to form a highly reactive binding site for L1 from another cell. This adhesion occurs rapidly and forms very stable contacts. It is conceivable that L1 and N-CAM may interact with the cytoskeleton (*striped band*) (see also Pollerberg et al., 1987, and Thor et al., 1986). This model suggests that the kinetics and affinity of L1-mediated homophilic adhesion can be modulated by association of L1 with N-CAM, which acts as a modulatory molecule. Interestingly, it suffices when N-CAM is present with L1 on the cell surface of only one cell.

Third, we probed whether the L1+N-CAM complex is a better substrate also for live cells, as compared with L1 or N-CAM alone or in combination with other adhesion molecules. Both L1 and the L1+N-CAM complex were good substrates for adhesion and neurite outgrowth, whereas N-CAM was not consistently active. Neither L1 nor N-CAM could form more adhesive substrates in combination with the other cell adhesion molecules J1 or MAG. Furthermore, the L1+N-CAM complex was better than either molecule alone as a substrate for neuronal adhesion. The complex could be reduced > 10-fold in concentration over L1 alone while retaining its capacity for cerebellar cell adhesion and neurite outgrowth. At this low concentration of the L1+N-CAM complex, the concentrations of L1 and N-CAM were 20- and 55-fold lower, respectively, than their lowest individual adhesive concentrations. It is interesting that mostly cells with a neuronal morphology adhered to L1 or the L1+N-CAM complex, indicating that L1 and even the L1+N-CAM complex are less attractive for glia under the conditions of this study.

Table IV. Short-Term Ca^{2+}-independent Aggregation of ESb-MP Cells in the Presence of Soluble N-CAM

Soluble adhesion molecule added	Concentration	Aggregation
	μg/ml	*%, mean ± SD*
None		10.9 ± 8.79
MAG	20	31.4 ± 6.16
N-CAM	5	136.3 ± 6.14
N-CAM	20	190.2 ± 14.31
N-CAM in the presence of N-CAM Fab	20	27.3 ± 12.61
N-CAM in the presence of L1 Fab	20	62.0 ± 17.53
N-CAM in the presence of MAG Fab	20	183.4 ± 9.11

ESb-MP cells were preincubated on ice in the presence or absence of the soluble form of N-CAM or MAG. They were then allowed to aggregate in low Ca^{2+} concentrations at 37°C and 85 rpm. After 20 min cells were added which had not been exposed to N-CAM or MAG and aggregation was allowed to continue for another 40 min in the absence or presence of Fab fragments (0.5 mg/ml) of polyclonal N-CAM, L1, or MAG antibodies. In each well, 4 × 10^5 preincubated cells and 8 × 10^5 nonpreincubated cells were incubated in a final volume of 800 μl. Aggregation was measured in octaplicates.

In the final set of control experiments, purified cell adhesion molecules have been tested in a binding assay. The L1+N-CAM complex specifically binds to L1 in a concentration-dependent manner, but does not bind to J1 or MAG. In contrast, N-CAM alone did not bind to immobilized L1, reaffirming the view that it is indeed the complex produced by L1 and N-CAM in the cell membrane or in solution which binds to L1.

Activity of the L1+N-CAM Complex Requires Preformation

An interesting feature of the association of L1 with N-CAM is the requirement for preincubation of the two molecules with each other before offering them as a complex to cells. This observation could be explained by the possibility that

the two molecules help each other to assume a molecular conformation that is supportive of interaction with L1 (Fig. 10). It is possible that weak molecular forces may have to come into play for the complex to order itself and perhaps undergo a change in configuration.

Conclusion

Our combined findings show that L1–L1 interactions are markedly and specifically increased by N-CAM, indicating that L1 and N-CAM cooperate with each other in adhesion. This is a novel mode of adhesion-mediating mechanism that we would like to call "assisted homophilic" interaction. This type of interaction could result either from formation of a new, compound binding site for L1 as depicted in Fig. 10, or from activation of one adhesion molecule by the other. In the immune system, several cell surface glycoproteins, CD3, CD8, or CD4 functionally cooperate with the T cell receptor to mediate T cell adhesion, possibly by molecular association (reviewed by Springer et al., 1987; Emmrich, 1988; Fleischer and Schrezenmeier, 1988). It is noteworthy that all four T cell molecules are, like L1 and N-CAM, members of the immunoglobulin superfamily (reviewed by Williams and Barclay, 1988). It will now be important to investigate the molecular determinants of the association between L1 and N-CAM, the auxiliary effect N-CAM might exert on other adhesion molecules and whether N-CAM may play both roles, that of a cell adhesion molecule and an adhesion-assisting molecule. It will have to be studied whether all or only some L1 and N-CAM molecules are associated with each other on the cell surface and whether L1 interacts primarily with one or more components of N-CAM. However, a preference for an L1–N-CAM 180 interaction has previously been suggested by copatching experiments and by their coaccumulation at sites of cell contact (Thor et al., 1986; Pollerberg et al., manuscript submitted for publication).

Versatility of adhesion mechanisms would be of obvious benefit for a complex tissue, such as the nervous system. Our study has shown that adhesion molecules can function either individually or in conjunction, with a concomitant change in their adhesive affinities, allowing for an economic use of few adhesion molecules in their various combinations.

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