

# Functional Cooperation between the Neural Adhesion Molecules L1 and N-CAM Is Carbohydrate Dependent

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**Abstract.** The neural cell adhesion molecules L1 and N-CAM have been suggested to interact functionally by formation of a complex between the two molecules (Kadmon, G., A. Kowitz, P. Altevogt, and M. Schachner. 1990. *J. Cell Biol.* 110:193–208). To determine the molecular mechanisms underlying this functional cooperation, we have studied the contribution of carbohydrates to the association of the two molecules at the cell surface.

Aggregation or adhesion between L1- and N-CAM-positive neuroblastoma N2A cells was reduced when the synthesis of complex and/or hybrid glycans was modified by castanospermine. Fab fragments of polyclonal antibodies to L1 inhibited aggregation and adhesion of castanospermine-treated cells almost completely, whereas untreated cells were inhibited by ~50%. Fab fragments of polyclonal antibodies to N-CAM did not interfere with the interaction between castanospermine-treated cells, whereas they inhibited aggregation or adhesion of untreated cells by ~50%.

These findings indicate that cell interactions depending both on L1 and N-CAM (“assisted homophilic” binding) can be reduced to an L1-dominated interaction (“homophilic binding”). Treatment of cells with the carbohydrate synthesis inhibitor swainsonine did not modify cell aggregation in the absence or presence of antibodies compared with untreated cells, indicating that castanospermine-sensitive, but swainsonine-insensitive glycans are involved. To investigate whether the appropriate carbohydrate composition is required for an association of L1 and N-CAM in the surface membrane (*cis*-interaction) or between L1 on one side and L1 and N-CAM on the other side of interacting partner cells (*trans*-interaction), an L1-positive lymphoid tumor cell line was coaggregated with and adhered to neuroblastoma cells in the various combinations of castanospermine-treated and untreated cells. The results show that it is the *cis*-interaction between L1 and N-CAM that depends on the appropriate carbohydrate structures.

**I**N the nervous system, the neural cell adhesion molecule L1 is expressed on a subset of postmitotic neurons, whose axons fasciculate with other L1-positive axons (Holley and Schachner as cited in Schachner et al., 1985; Stallcup et al., 1985; Martini and Schachner, 1986, 1988; Persohn and Schachner, 1987; Dodd et al., 1988; Bartsch et al., 1989). L1 is involved in neuron–neuron adhesion and neurite outgrowth on other neurites (Keilhauer et al., 1985; Chang et al., 1987). In the peripheral nervous system, L1 is also involved in neuron–glia interactions that underlie adhesion and neurite outgrowth (Martini and Schachner, 1986; Bixby et al., 1988; Seilheimer and Schachner, 1988). Ng-CAM, the chicken analogue of L1 (Grumet et al., 1984) has also been suggested to mediate neuron–glia adhesion in the central nervous system (Grumet and Edelman, 1988). The neural cell adhesion molecule N-CAM<sup>1</sup> has a much wider distribution (for review see Edelman et al., 1986) and within the nervous system, appears to be expressed by all L1-positive cells. First hints as to the functional cooperativity be-

tween the two molecules derived from observations on the colocalization of the two molecules at contact sites between cultured neurons (Pollerberg et al., 1987, manuscript submitted for publication), strong association within the surface membrane as revealed by copatching experiments (Thor et al., 1986), reduced lateral mobility within the surface membrane of both molecules upon neuronal differentiation (Pollerberg et al., 1986, manuscript submitted for publication) and synergistic effects of L1 and N-CAM antibodies in the inhibition of cell adhesion (Faissner et al., 1984; Rathjen and Rutishauser, 1984). Recent evidence from our laboratory indicates that L1 and N-CAM can indeed engage in a close functional association within the cell surface, which may result from formation of a putative molecular complex between the two molecules (Kadmon et al., 1990). This association appears to cause an increased interaction with L1 on the neighboring partner cell: a phenomenon that we have termed “assisted homophilic” binding (Kadmon et al., 1990). To characterize the molecular mechanisms underlying this association, we have investigated the role of carbohydrates. Here we report that the functional association between L1

1. *Abbreviations used in this paper:* N-CAM, neural cell adhesion molecule.

and N-CAM depends on particular carbohydrate structures and that the homophilic interaction of L1 is relatively unaffected by the specific composition of carbohydrates.

## Materials and Methods

### Antibodies

Production and specificity of mono- and polyclonal antibodies to L1 and N-CAM and preparation of IgG fractions and Fab fragments have been described (Rathjen and Schachner, 1984; Kadmon et al., 1990). Immunoaffinity-purified polyclonal N-CAM antibodies were isolated by means of an N-CAM antigen column according to the procedure of Tacke et al. (1987). The H28 N-CAM mAb reacts with a peptide sequence of a fusion protein derived from the cDNA clone pMI.3 containing a 580-bp stretch between the transmembrane domain and the amino terminus of the protein (Goridis et al., 1985). mAb D3 reacts with an intracellular domain specific for the largest form of N-CAM, N-CAM 180 (Schlosshauer, 1989). L1 mAbs were prepared against L1 from adult mouse brain in Lou x Sprague-Dawley F1 hybrid rats as described for the L1 mAb 324 (de St. Groth and Scheidegger, 1980; Rathjen and Schachner, 1984). The characterization of these antibodies will be described elsewhere (Kadmon et al., manuscript in preparation).

### Cells

Neuroblastoma N2A cells and cells of the ESb-MP lymphoid tumor line were maintained in culture as described (Fogel et al., 1983; Rathjen and Schachner, 1984; Lang et al., 1987). N2A cells express both L1 and N-CAM, whereas ESb-MP cells only express L1. For treatment of cells with the carbohydrate synthesis inhibitors castanospermine and swainsonine (Boehringer Mannheim, Mannheim, FRG), cells were supplied with 20  $\mu\text{g}/\text{ml}$  castanospermine every 12 h and 2  $\mu\text{g}/\text{ml}$  swainsonine every 48 h for 5 d. Untreated cells were maintained under the same conditions as castanospermine- or swainsonine-treated cells, except that inhibitors were omitted. No differences in morphology or proliferation rates were observed between cells under the different treatments (see also Ratner et al., 1986).

### Immunoprecipitation and Glycosidase Treatment

Cultures of ESb-MP and N2A cells maintained in vitro for five days were surface-labeled with  $\text{Na}^{125}\text{I}$  in the presence of lactoperoxidase. Detergent lysates were obtained and immunoprecipitation was performed using L1 and N-CAM mAbs as described. Immunoprecipitates were digested overnight at 37°C with 2 U/ml endoglycosidase H (Boehringer Mannheim) or for 1.5 h with 0.4 U/ml glycopeptidase F (Boehringer Mannheim) and separated by 7% SDS-PAGE.

### Indirect Immunofluorescence and Cytofluorography

Indirect immunofluorescence of N2A and ESb-MP cells was carried out on single cell suspensions using N-CAM and several L1 mAbs. Cytofluorography was performed on a FACSCAN (Becton-Dickinson & Co., Oxnard, CA) to determine relative fluorescence intensities. Forward angle light scatter was used as an estimate for relative changes in cell size, as for single cells it is closely related to cell volume (Shapiro, 1988).

### Patching and Copatching

Induction of patching was performed on N2A cells by incubation with monoclonal antibodies to N-CAM (undiluted hybridoma supernatant) and tetramethylrhodamine-labeled rabbit anti-rat IgG antibodies (diluted 1:50; Dianova, Hamburg). Cells were dissociated in PBS, pH 7.2 containing 1 mM EDTA and washed in PBS. Cells were then incubated for 40 min with the N-CAM mAb, washed, incubated for another 40 min with the secondary antibody, and washed again. All steps were carried out at 4°C. Cells were then incubated for 3 h at 37°C. Copatching of L1 was investigated after fixation of cells with 4% paraformaldehyde in PBS for 10 min at room temperature, extensive washing and blocking with BSA-containing buffers and addition of biotinylated L1 polyclonal antibodies (10  $\mu\text{g}/\text{ml}$ ) visualized by indirect immunofluorescence using fluorescein-labeled streptavidin (Sigma Chemical Co., Munich, FRG). Vice versa, patching was induced by L1 mAb 324, visualized by tetramethylrhodamine-labeled rabbit anti-rat IgG antibodies, and copatching was visualized by biotinylated polyclonal N-CAM antibodies using fluorescein-labeled streptavidin. Fluorescence was in-

spected with a Zeiss fluorescence microscope (Carl Zeiss, Inc., Oberkochen, FRG) equipped with the appropriate filters.

### Aggregation and Coaggregation

Aggregation of N2A cells was determined at a low  $\text{Ca}^{2+}$  concentration (0.1 mM) by allowing cells to aggregate for 35 min at 37°C and a rotation speed of 85 rpm. At these low  $\text{Ca}^{2+}$  concentrations,  $\text{Ca}^{2+}$ -dependent adhesion mechanisms have been found inoperative (Fischer and Schachner, 1988; Kadmon et al., 1990). Single-cell suspensions for aggregation were obtained by treatment of monolayer cultures with EDTA (1 mM in PBS for 2–5 min at room temperature). Aggregate formation was determined by Coulter counter analysis (Coulter Electronics, Hialeah, FL) of particle size distribution (Kadmon et al., 1990). The cumulative partial volume occupied by particles detected in channels no. 11–16 were used to evaluate aggregation. A smaller channel number was taken than previously used (channel no. 12; Kadmon et al., 1990), because aggregates of swainsonine- and castanospermine-treated cells were smaller than aggregates of untreated cells.

For determination of coaggregation, N2A and ESb-MP cells were harvested by EDTA treatment and mixed together before allowing them to aggregate for 60 min at 37°C and 85 rpm at a low  $\text{Ca}^{2+}$  concentration (0.1 mM). Coaggregation was determined in the Coulter counter by measuring the reduction of single ESb-MP cells (in channel 7). This reduction correlates with their appearance in coaggregates because ESb-MP cells maintained under rotation do not homotypically aggregate within 60 min, but require at least 2 h for aggregation (Kadmon et al., 1990).

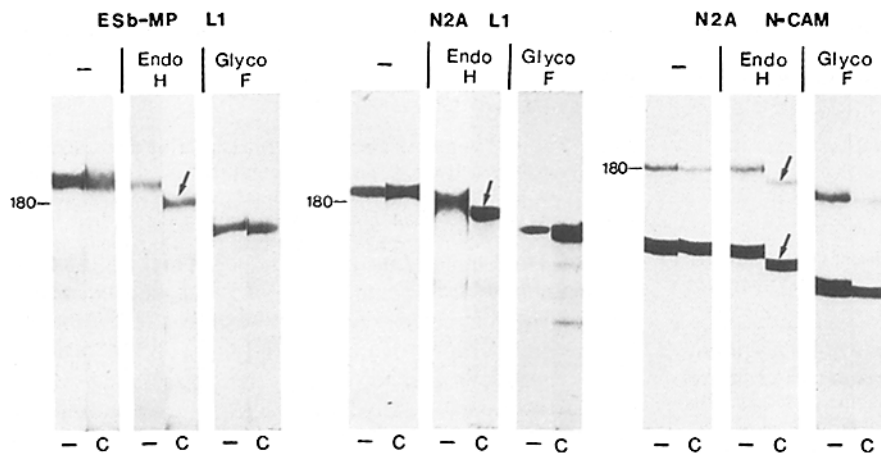
### Cell Adhesion

Adhesion of N2A and ESb-MP cells was determined using fluorescein-labeled probe cells and monolayer target cells as described (Kadmon et al., 1990). In brief, single cell suspensions of probe cells were added to confluent cultures of N2A or ESb-MP cells on glass coverslips. Cells were incubated for 60 min at 37°C and 45 rpm. Adhesion of fluorescein-labeled probe cells to target cell monolayer cultures was evaluated by microscopic examination of coverslips. Aggregates of adherent probe cells were scored as single particles and the few probe cells that adhered to cell-free areas were not scored. For each value, four coverslips were taken and three fields of 0.025 mm<sup>2</sup> were counted per coverslip.

## Results and Discussion

The influence of the carbohydrate composition on the adhesion properties of the neural cell adhesion molecules L1 and N-CAM was investigated by quantitative measurements of aggregation and adhesion between two cell types previously shown to interact by "assisted homophilic" interaction (Kadmon et al., 1990). Neuroblastoma N2A cells express both L1 and N-CAM, whereas the lymphoma-derived ESb-MP cells express only L1 (Kowitz et al., manuscript submitted for publication). At a low  $\text{Ca}^{2+}$  concentration (0.1 mM), ESb-MP cells exhibit adhesion that appears to be solely L1-dependent. This interaction can be mimicked by coating purified L1 glycoprotein onto Latex beads that then aggregate with slow kinetics. The homophilic interactions between L1-carrying Latex beads or L1-positive cells are drastically enhanced when one of the interacting partners carries also N-CAM in a close molecular association with L1.

To investigate the carbohydrate dependence of this close molecular association, N2A and ESb-MP cells were cultured in the presence of castanospermine, an alkaloid known to interfere with the processing of the high-mannose type glycans to hybrid or complex types (for review, see Elbein, 1987). To assure the complete inhibition of the conversion of high mannose to complex types, cells were maintained for 5 d in the presence of castanospermine or swainsonine, which were added freshly to the cultures every 12 or 48 h, respectively. During this time, castanospermine and swain-

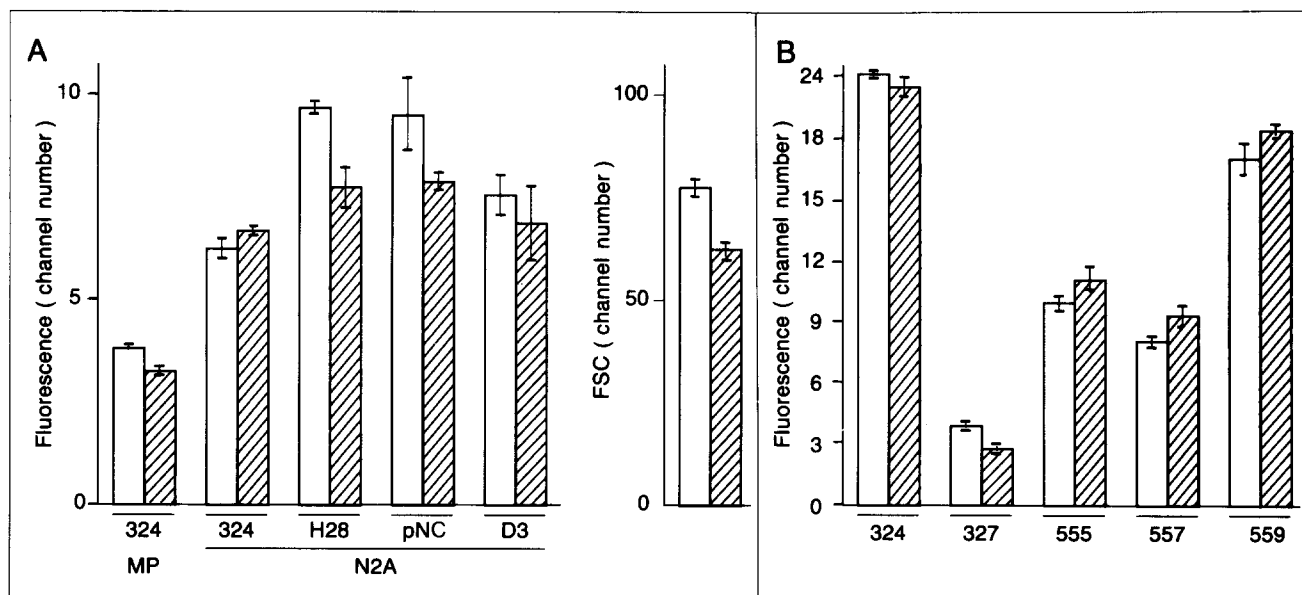


**Figure 1.** Live cultures of ESb-MP and N2A cells were surface-labeled with  $^{125}\text{I}$  and detergent extracts immunoprecipitated with L1 and N-CAM mAbs as indicated. Cells had been maintained in the absence (-, *bottom*) or presence (c, *bottom*) of castanospermine. Immunoprecipitates were separated by SDS-PAGE without (-, *top*) or after digestion with endoglycosidase H (*Endo H*) or glycopeptidase F (*Glyco F*). Radioactivities in each lane were not matched to represent the identical numbers of cells.

sonine did not affect proliferation of N2A or ESb-MP cells nor did they change the cell shape or viability, although the adhesivity of both cell types to tissue culture plastic was slightly reduced.

To show that castanospermine and swainsonine treatments resulted in a change in the carbohydrate composition of L1 and N-CAM, the carbohydrate structure of the two glycoproteins was investigated using glycosidases. For this purpose, N2A and ESb-MP cells maintained in the presence of castanospermine or swainsonine, were surface-labeled with  $^{125}\text{I}$  and the detergent extract of these cells was immunoprecipitated using L1 or N-CAM mAbs. Immunoprecipitates were digested with endoglycosidase H or glycopeptidase F before their separation by SDS-PAGE under reducing conditions

(Fig. 1 for castanospermine-treated cells). The molecular weights of L1 and N-CAM were similar in castanospermine- or swainsonine-treated and untreated cells. In castanospermine-treated and untreated N2A and ESb-MP cells, treatment with glycopeptidase F reduced the apparent molecular mass of L1 from 200 to 150 kD. N-CAM was reduced in castanospermine-treated and untreated N2A cells from 180 and 140 to 160 and 115 kD, respectively. In contrast, endoglycosidase H cleaved L1 and N-CAM differently in castanospermine-treated versus untreated cells. In both cell types, endoglycosidase H reduced the apparent molecular weight of L1 in castanospermine-treated cells (Fig. 1), whereas in untreated cells the enzyme produced only a small reduction in molecular weight. Endoglycosidase H also reduced the molecular



**Figure 2.** Relative expression of L1 and N-CAM on ESb-MP (MP) and N2A cells maintained in the absence and presence of castanospermine. Live N2A and ESb-MP cells were labeled by indirect immunofluorescence with L1 and N-CAM mAbs. Cells had been cultured in the absence (*white columns*) or presence (*shaded columns*) of castanospermine. Immunofluorescence intensities per cell were evaluated by cytofluorography. (A) Cells were labeled using the L1 mAb (324) and immunoaffinity-purified polyclonal N-CAM antibodies (pNC), an N-CAM mAb (H28), and an mAb specific for N-CAM 180 (D3). The forward angle light scatter (FSC) of cells is related to their size by an approximate linear relationship (Shapiro, 1988). (B) N2A cells were labeled with different monoclonal L1 antibodies. Values are triplicate means  $\pm$  SEM from samples of 10,000 cells.

weight of N-CAM in castanospermine-treated, but not in untreated cells. Similar results were obtained when L1 and N-CAM were immunoprecipitated from swainsonine-treated cells (not shown), indicating that the two glycoproteins were also modified by swainsonine. These results show that in both cell types, castanospermine and swainsonine interfere with the processing of the nascent high mannose glycans of L1 and N-CAM.

As a basis for the comparison of castanospermine-treated and untreated cells in aggregation and adhesion, the relative levels of L1 and N-CAM cell surface expression were measured by indirect immunofluorescence and cytofluorography (Fig. 2). N2A cells were labeled with immunoaffinity-purified polyclonal and monoclonal N-CAM antibodies and both N2A and ESb-MP cells were labeled with L1 mAbs. The intensities of L1 immunofluorescence were the same in castanospermine-treated or untreated cells (Fig. 2). Binding sites for N-CAM antibodies recognizing the protein backbone of the different forms of N-CAM (unpublished results) were reduced by 18–20% in N2A cells treated with castanospermine versus untreated cells (Fig. 2 A). Forward-angle light-scatter measurements showed that castanospermine-treated cells were slightly smaller in diameter than untreated cells (Fig. 2 A), indicating that the reduction in intensity of N-CAM immunofluorescence per cell may be due to a decreased cell size. Binding of antibodies specific for the largest form of N-CAM, N-CAM 180, was not significantly reduced in castanospermine-treated versus untreated cells (Fig. 2 A). To probe whether the epitope configuration of L1 was altered by castanospermine treatment, several L1 mAbs (324, 327, 555, 557, and 559) were used for cytofluorometric analysis (Fig. 2 B). These antibodies recognize at least three independent conformation-dependent epitopes (unpublished observations). No significant differences were seen in the immunofluorescence intensity profile between castanospermine-treated and untreated N2A cells. The combined observations indicate that castanospermine treatment alters the carbohydrate composition of L1 and N-CAM without drastically affecting protein structure and surface expression (see also Ratner et al., 1986).

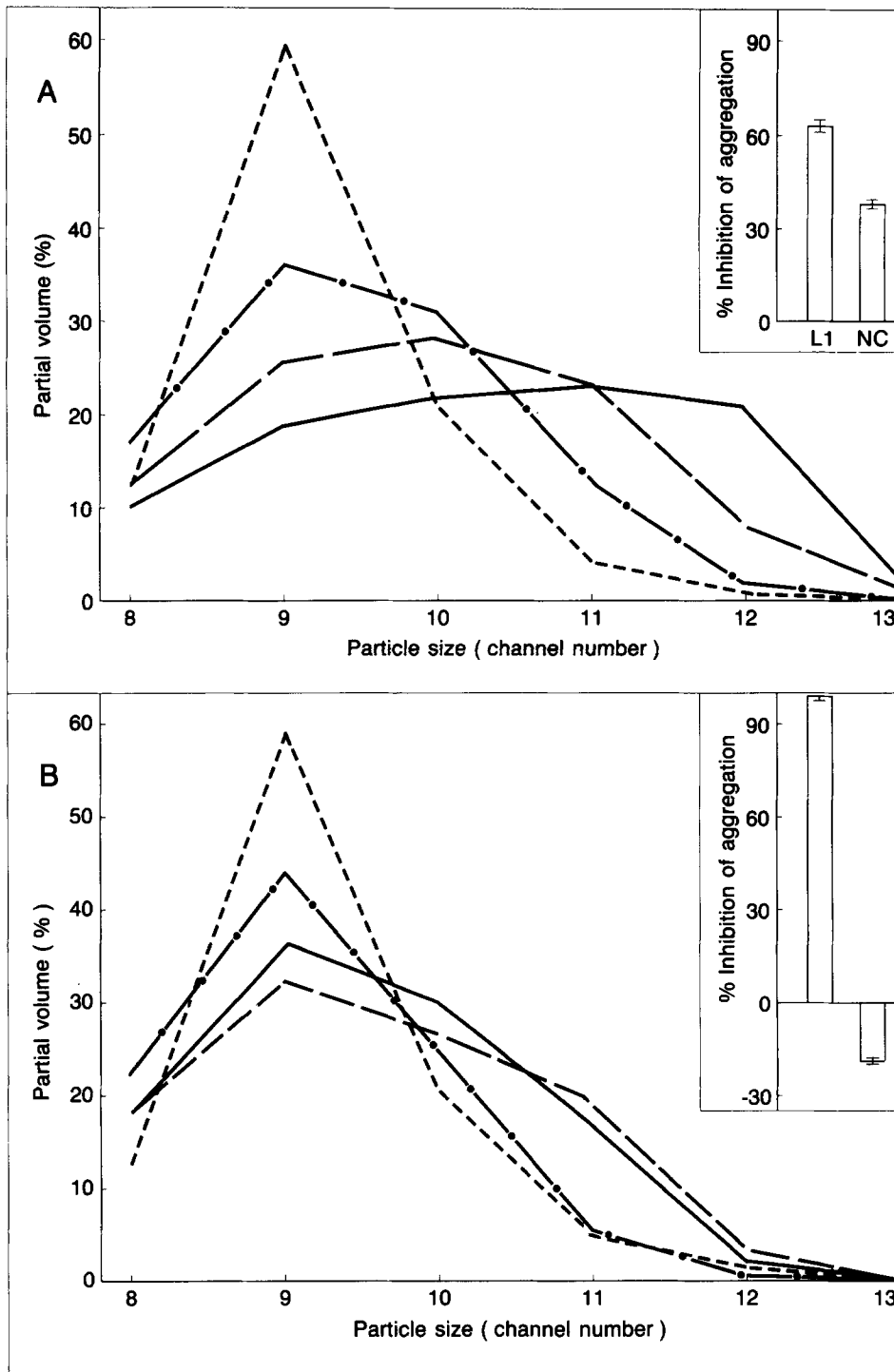
Ca<sup>2+</sup>-independent aggregation of castanospermine-treated and untreated N2A cells was then measured (Fig. 3). Aggregation of castanospermine-treated cells was reduced by ~60% in castanospermine-treated versus untreated N2A cells (compare B with A in Fig. 3; Fig. 4 A). Inhibition of aggregation of untreated cells in the presence of Fab fragments of polyclonal L1 or N-CAM antibodies was ~60 and 40%, respectively, in this experiment (Fig. 3 A). In contrast, following treatment with castanospermine, the aggregation of N2A cells was no longer inhibitable by Fab fragments of polyclonal N-CAM antibodies, whereas it was completely inhibitable by L1 antibodies (Fig. 3 B). Thus, with the predominance of high-mannose type glycans on L1 and N-CAM, N2A cells become less adhesive and their adhesivity depends predominantly on L1 and no longer on N-CAM. This strong reduction in N-CAM-dependent aggregation is most likely not due to a decrease in cell surface density of N-CAM in castanospermine-treated versus untreated cells nor to a significant reduction in N-CAM 180 expression (Fig. 2 A). Our observations thus suggest that an altered carbohydrate composition of N-CAM prevents it from participating in aggregation. Furthermore, our experiments suggest that, if

N-CAM would be involved in homophilic binding, carbohydrate structures other than the unusual polysialic acid of the so-called embryonic form play an important role in this interaction.

To evaluate whether the alteration in the aggregation behavior of N2A cells was due to a general, rather than a more specific modification of the adhesion molecules, we studied the aggregation of N2A cells treated with swainsonine, an inhibitor of  $\alpha$ -mannosidase (Elbein, 1987) (Fig. 4). Also the aggregation of swainsonine-treated cells was reduced in comparison with untreated cells, but it was less affected than by the castanospermine treatment (Fig. 4 A). When swainsonine-treated cells were allowed to aggregate in the presence of Fab fragments of polyclonal L1 or N-CAM antibodies, both antibodies inhibited aggregation to approximately the same extent as with untreated cells (Fig. 4 B). Thus, the loss of N-CAM-dependent aggregation seen with castanospermine-treated cells suggests that the participation of N-CAM in mediating aggregation of N2A cells depends on complex or hybrid type glycans.

In a previous paper (Kadmon et al., 1990) we postulated that N-CAM participates in aggregation by forming a complex with L1 in the surface membrane, which more effectively interacts with L1 on the partner cell surface than does L1 alone. In this work, we therefore examined whether alterations in the carbohydrate compositions of the glycoproteins would perturb the formation of this putative functional complex. N2A and ESb-MP cells were allowed to coaggregate with each other in the various combinations of castanospermine-treated and untreated cells (Fig. 5). When coaggregation of untreated N2A and ESb-MP cells was determined, ~65% of ESb-MP cells were measured as coaggregated with N2A cells (Fig. 5 A). When untreated N2A cells were allowed to coaggregate with castanospermine-treated ESb-MP cells, a reduction of coaggregation to 50% was seen (Fig. 5 A), indicating a reduction in coaggregation efficiency of ~20% (Fig. 5 B). When castanospermine-treated N2A cells were allowed to coaggregate with untreated ESb-MP cells, coaggregation was reduced to ~30% (Fig. 5 A), amounting to a reduction of ~60% in comparison with untreated cells (Fig. 5 B). Similar values were seen when castanospermine-treated N2A cells were allowed to coaggregate with castanospermine-treated ESb-MP cells (Figs. 5, A and B). These observations indicate that carbohydrates may be more involved on the side of the N2A cells that express both L1 and N-CAM.

The dependence of N-CAM function on the correct glycosylation pattern became more apparent in antibody inhibition experiments of coaggregation using the various combinations of castanospermine-treated and untreated N2A and ESb-MP cells (Fig. 6). Fab fragments of polyclonal L1 antibodies always interfered with coaggregation of untreated or castanospermine-treated N2A and ESb-MP cells. Coaggregation of untreated cells was inhibited in this experiment by 70% (Fig. 6, *white bar 1*). Inhibition was complete, although not significantly higher, when either cell line had been treated with castanospermine (Fig. 6, *white bars 2 and 3*). The strongest inhibition by L1 antibodies was seen when both N2A and ESb-MP cells had been exposed to castanospermine (Fig. 6, *white bar 4*). In this case, inhibition was up to 120%, suggesting that the few aggregated cells present in the initial single cell suspensions were dispersed under the influence of L1 antibodies. Very different results were ob-

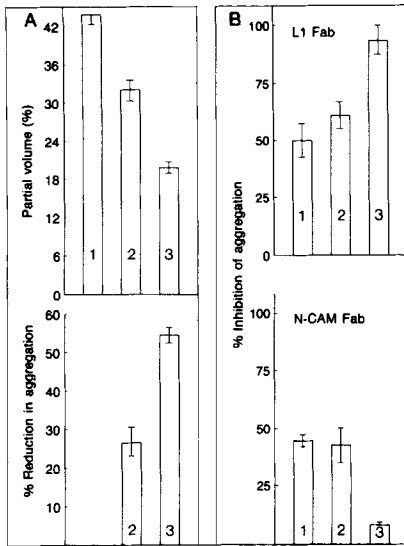


**Figure 3.** Influence of L1 and N-CAM antibodies on  $\text{Ca}^{2+}$ -independent aggregation of N2A cells maintained in the absence or presence of castanospermine. N2A cells cultured in the absence (A) and presence (B) of castanospermine were allowed to aggregate at low  $\text{Ca}^{2+}$  concentrations (0.1 mM) in the absence (—) or presence of Fab fragments (0.5 mg/ml) of polyclonal antibodies to N-CAM (---) or L1 (----). Distribution of particle sizes before aggregation (.....) shows single cells in channels 8 and 9. Channels 10, 11, 12, and 13 represent cell aggregates. Aggregation was evaluated by Coulter counter analysis. Inset in upper right hand corner indicates the inhibition of aggregate formation (in percent) as measured cumulatively in channels  $\geq 11$  in the presence of Fab fragments of polyclonal L1 or N-CAM (NC) antibodies (inset columns in B correspond to A). Values are means from four experiments performed in triplicates  $\pm$  SEM.

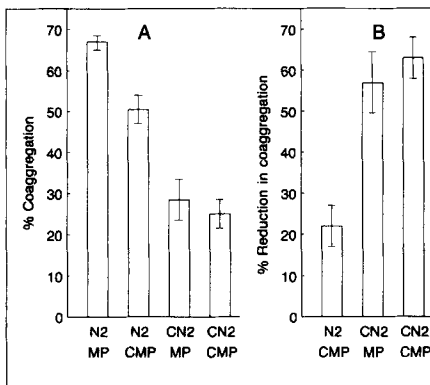
tained with N-CAM antibodies. They interfered with coaggregation of untreated N2A cells with untreated or castanospermine-treated ESb-MP cells (Fig. 6, *striped bars 1 and 2*), but had no effect on coaggregation when N2A cells were castanospermine-treated (Fig. 6, *striped bars 3 and 4*). In the presence of both antibodies, coaggregation was almost completely inhibited in all combinations of untreated and castanospermine-treated cells (Fig. 6, *checkered bars 1, 2, 3, and 4*). The slightly smaller inhibition of coaggregation seen between castanospermine-treated N2A and ESb-MP cells

(Fig. 6, *checkered bar 4*) remains at present unexplained. These combined observations suggest that an altered carbohydrate composition interferes with the functional association of N-CAM with L1, suggesting that the function of N-CAM depends on particular glycans.

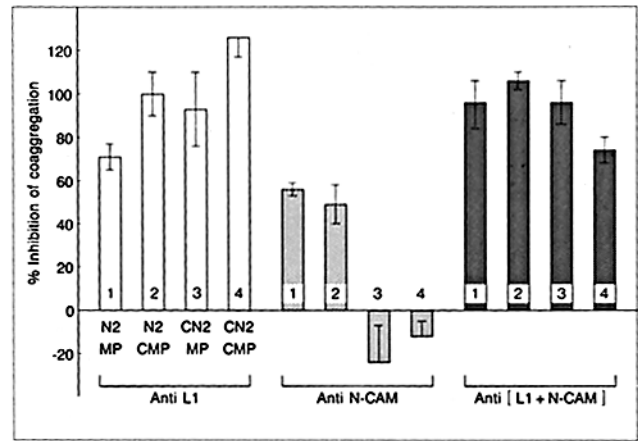
To verify the results obtained in coaggregation experiments, we also performed adhesion tests in the various combinations of castanospermine-treated and untreated N2A and ESb-MP cells. For this purpose, single cell suspensions of either N2A or ESb-MP cells, as probe cells, were confronted



**Figure 4.** Effects of castanospermine and swainsonine treatment of N2A cells on L1- and N-CAM-dependent aggregation. N2A cells maintained in the absence (1) and presence of swainsonine (2) or castanospermine (3) were allowed to aggregate under low  $Ca^{2+}$  concentrations (0.1 mM). Aggregation was evaluated by Coulter counter analysis using the cumulative partial volume in channels  $\geq 11$  as indicator of aggregation (see Fig. 3). (A) Aggregation (top) and reduction in aggregation as compared with cells maintained in the absence of carbohydrate synthesis inhibitors (bottom). (B) Aggregation in the presence of Fab fragments (0.5 mg/ml) of polyclonal antibodies to L1 (top) or N-CAM (bottom). Values are means from three experiments performed in triplicates  $\pm$  SEM. Inhibition of aggregation was evaluated from the cumulative partial volume measured in channels  $\geq 11$ .



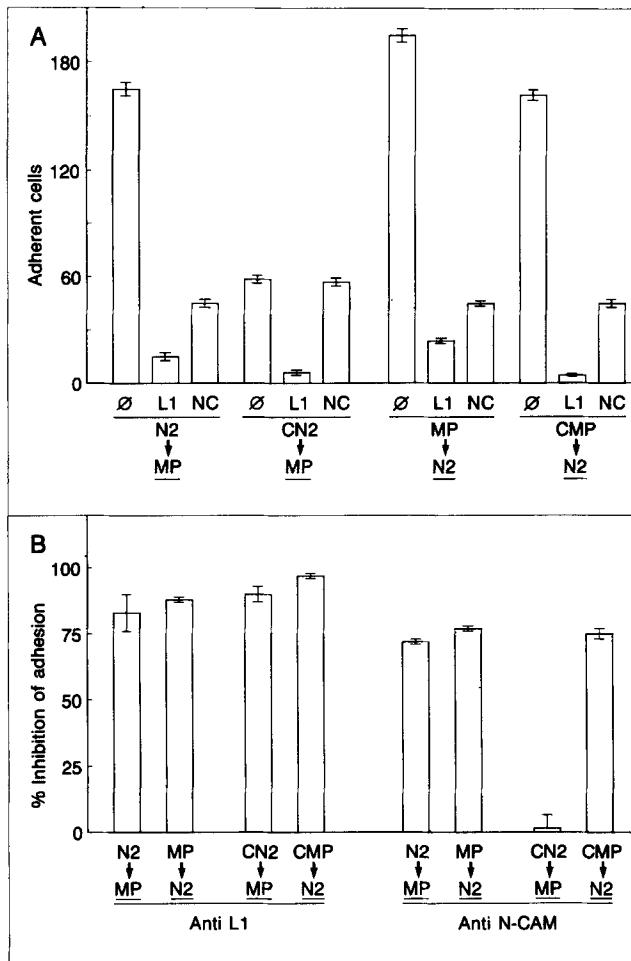
**Figure 5.** Coaggregation of untreated and castanospermine-treated N2A with ESb-MP cells. L1- and N-CAM-positive N2A cells were mixed with L1-positive ESb-MP cells before coaggregation or after separate aggregation. Coaggregation was determined by a reduction of single ESb-MP cells (particles in channel 7) after mixed aggregation as compared with this value after separate aggregation. This is a specific indicator for coaggregation of N2A and ESb-MP cells, since aggregation of ESb-MP cells alone does not occur within the duration of this assay (60 min) (Kadmon et al., 1990). (A) Coaggregation of different combinations of cells. Untreated N2A cells (N2); untreated ESb-MP cells (MP); castanospermine-treated N2A cells (CN2); castanospermine-treated ESb-MP cells (CMP). (B) Reduction in coaggregation compared with untreated N2A and ESb-MP cells in percent. Values are means from four experiments performed in triplicates  $\pm$  SEM.



**Figure 6.** Effect of L1 and N-CAM antibodies on coaggregation of untreated and castanospermine-treated N2A and ESb-MP cells. Untreated and castanospermine-treated N2A and ESb-MP cells were allowed to coaggregate in different combinations (see Fig. 5 A). (1) N2 and MP cells; (2) N2 and CMP cells; (3) CN2 and MP cells; (4) CN2 and CMP cells. Co-aggregation was determined as described in Fig. 5 in the presence and absence of Fab fragments (0.5 mg/ml) of polyclonal antibodies to L1 (white bars), N-CAM (hatched bars) and both antibodies at 0.25 mg/ml (checked bars). Inhibition of coaggregation (in percent) was calculated by comparison of reduction of particle numbers in channel 7 between cells in the presence of antibodies or their absence (0% inhibition of coaggregation). Values are means from four experiments in triplicates  $\pm$  SEM. The negative inhibition by N-CAM antibodies is not significantly different from zero.

with monolayers of N2A or ESb-MP target cells (Fig. 7). Castanospermine-treated ESb-MP cells were only marginally reduced (17%) in their adhesion to untreated N2A cells, when compared with adhesion of untreated ESb-MP cells to untreated N2A monolayer target cells (Fig. 7 A). However, when N2A cells were treated with castanospermine, a significant reduction (65%) was seen in comparison to untreated N2A cells (Fig. 7 A), again pointing at the importance of appropriate glycosylation on the N-CAM carrying N2A cells. L1 antibodies were always effective in inhibiting the heterotypic adhesion between untreated and castanospermine-treated N2A and ESb-MP cells, irrespective of which cell type was castanospermine treated (Fig. 7 B). Thus, L1-mediated adhesion, which has been suggested to operate by a homophilic mechanism on these cells (Kadmon et al., 1990), does not appear to depend on appropriate glycosylation. On the other hand, N-CAM antibodies only blocked adhesion when N2A cells had not been treated with castanospermine (Fig. 7 B), suggesting that the heterotypic adhesion is no longer dependent on N-CAM when N2A cells are castanospermine-treated. These results confirm those obtained by the coaggregation experiments and indicate a crucial role for carbohydrates in validating N-CAM function, possibly by allowing the formation of a complex between L1 and N-CAM.

To visualize the association of L1 and N-CAM within the surface membrane by another method, patching and co-patching experiments were carried out on live cultured N2A cells using L1 and N-CAM antibodies (Fig. 8). When patching was induced by mAb to N-CAM together with antibodies



**Figure 7.** Adhesion of untreated and castanospermine-treated N2A and ESb-MP cells. (A) Adhesion of untreated and castanospermine-treated N2A (N2 and CN2, respectively) or ESb-MP (MP and CMP, respectively) probe cells to monolayer cultures of untreated ESb-MP (MP) or N2A (N2) target cells. Adhesion was measured in the absence or presence of Fab fragments (0.5 mg/ml) of polyclonal antibodies to L1 (L1) or N-CAM (NC). Adhesion was evaluated by counting fluorescein-labeled probe cells that had adhered to target cells. (B) Inhibition of probe to target cell adhesion by Fab fragments of polyclonal antibodies (0.5 mg/ml) to L1 (Anti L1) and N-CAM (Anti N-CAM). Values are means from four coverslips  $\pm$  SEM. Three fields of 0.025 mm<sup>2</sup> per coverslip were measured.

to rat IgG conjugated with tetramethylrhodamine during a 3-h incubation of N2A cells at 37°C, L1 coreistributed together with N-CAM as seen by labeling of L1 with biotinylated polyclonal L1 antibodies and fluorescein-labeled streptavidin. As reported previously (Thor et al., 1986), copatching occurred also when patching was induced with an L1 mAb and copatching was examined with polyclonal N-CAM antibodies. However, the codistribution effect was weaker than when patching was induced with N-CAM antibodies, possibly because L1 may coreistribute predominantly with the cytoskeleton-bound N-CAM 180 and not with N-CAM 140 (Pollerberg et al., 1986, 1987; Thor et al., 1986). In castanospermine-treated cells, no coredistribution of L1 and N-CAM was observed. When patching of N-CAM was induced, L1 tended to remain at cell-cell contact sites and did

not copatch (Fig. 8, c and d), suggesting that L1 remained at contact sites because of its ability to interact homophilically between apposing cell surfaces without the assistance of N-CAM. Conversely, castanospermine-treated cells without cell contacts showed uniform L1 expression on the entire cell surface even after N-CAM patching (not shown). When L1 was induced to patch in castanospermine-treated cells, N-CAM did not coreistribute. These experiments further demonstrate that the molecular association between L1 and N-CAM within the surface membrane of N2A cells is disturbed by altering their carbohydrate composition.

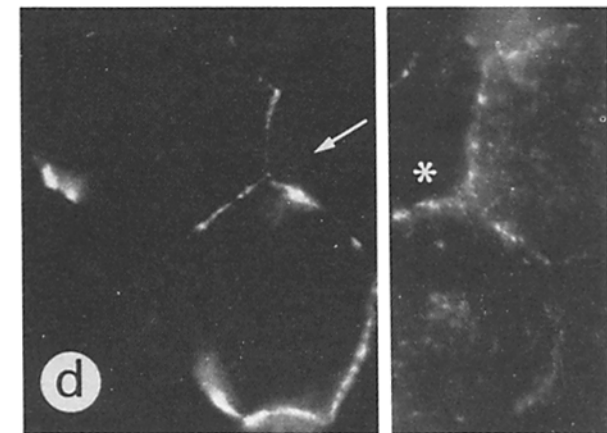
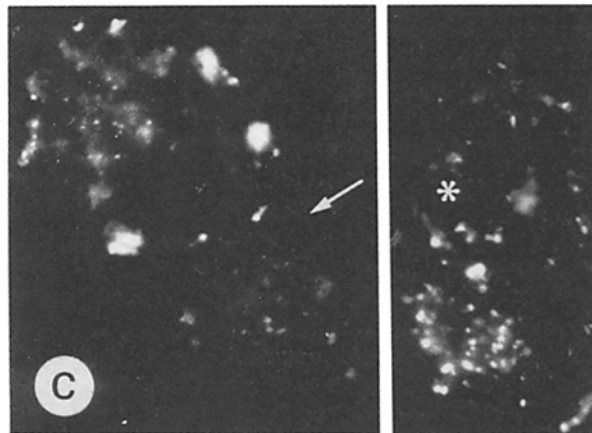
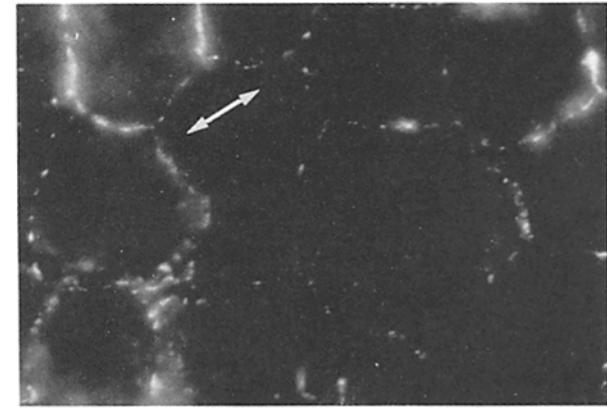
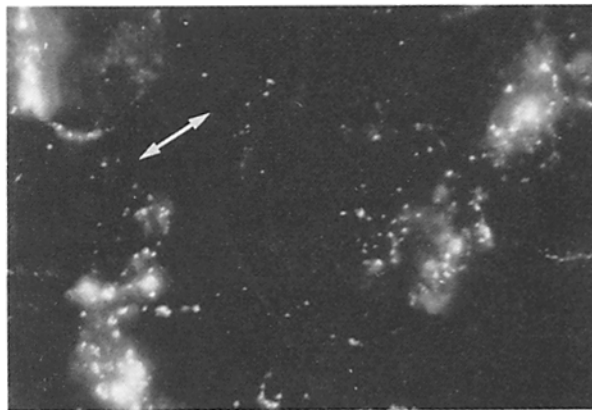
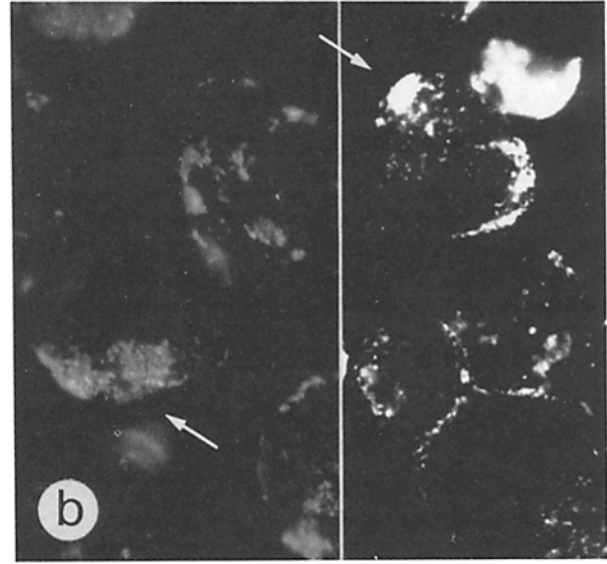
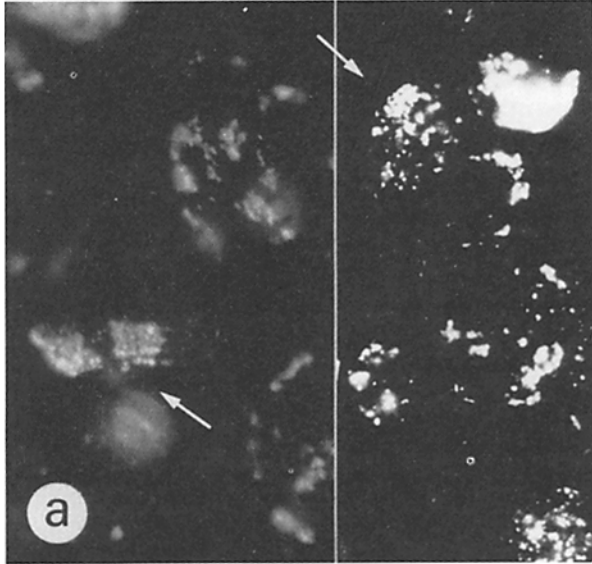
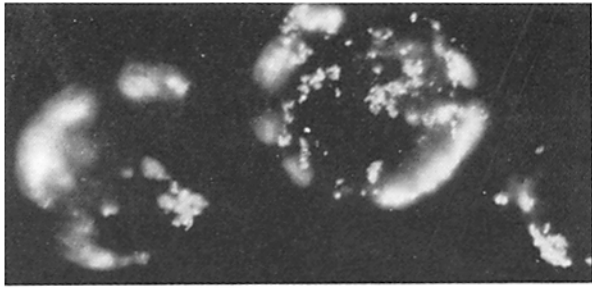
### Conclusions

Our study provides evidence that aggregation and adhesion of L1- and N-CAM-positive neuroblastoma cells with each other or with L1-positive, but N-CAM-negative lymphoma-derived cells depend on the presence of properly biosynthesized complex or hybrid glycans. Alterations in carbohydrate composition of these glycoproteins appear to affect predominantly the functionality of N-CAM. Without the proper carbohydrate composition, it no longer appears to contribute to the adhesive mechanisms between these cells. Thus, if N-CAM indeed interacts with itself via a homophilic binding mechanism, specific alterations in its carbohydrate composition would modify this homophilic binding behavior in neuroblastoma cells. However, although this possibility may partially explain our observations, we favor another interpretation, namely that a glycan-dependent association between L1 and N-CAM within the surface membrane may form a functional complex. Previous evidence has suggested that such a complex may potentiate the homophilic binding of L1 in what we have called "assisted homophilic" interaction (Kadmon et al., 1990). This notion is supported by the observation that alteration of the carbohydrate composition of only L1 does not significantly modify the L1-dependent adhesive behavior. The association between L1 and N-CAM appears to be based on the particular glycan structures that are modified by castanospermine, but not by swainsonine. When L1 and N-CAM no longer associate, the adhesive interactions between cells are reduced to an L1-dependent mechanism. It is unlikely that our data can be explained in terms of a small reduction in N-CAM expression in castanospermine-treated cells, unless the interaction of N-CAM with L1 is very sensitive to N-CAM concentration or a particular form of N-CAM is specifically depleted in castanospermine-treated cells.

The molecular mechanisms whereby glycans may direct the association between L1 and N-CAM remain at present elusive and will have to be studied by probing the isolated glycoproteins in functional assay systems similar to those described (Kadmon et al., 1990). However, modification of L1 and N-CAM by biosynthetic or biochemical alteration of glycosylation in large enough quantities for such experiments is presently beyond financial and experimental possibilities. Nevertheless, it is tempting to speculate that functionally important carbohydrate structures that are shared by various adhesion molecules, such as the L2/HNK-1 epitope (Künemund et al., 1988) or the L3 and L4 oligosaccharide domains (Fahrig et al., 1989; Kücherer et al., 1987) may mediate such interactions.

The functional association between two cell surface glyco-







proteins in the nervous system is reminiscent of the situation of the CD3 and T<sub>i</sub> components of the T cell receptor complex in the immune system (for review, see Clevers et al., 1988; Morley et al., 1988). Here, the CD3 component does not directly participate in the *trans*-interaction with MHC molecules, but requires association with the T<sub>i</sub> heterodimer for its expression and function. Even more suggestive are the similarities between our observations and those on the functional association between CD4 and the T cell receptor. CD4 can interact independently of the T cell receptor with MHC class II molecules (Doyle and Strominger, 1987), but may recognize these molecules also in conjunction with the T cell receptor (for review, see Emmrich, 1988). Interestingly, all interacting partners in the nervous and immune systems belong to the immunoglobulin superfamily (Williams and Barclay, 1988). It is, therefore, tempting to speculate that interactions between individual members of the immunoglobulin superfamily may be mediated by glycans at structurally important sites, such as, for instance, the hinge region of the heavy chains of immunoglobulins. Such oligosaccharides may be responsible for the correct alignment of two molecules by interdigitation of glycans into complementary pouches on the partner protein (for review, see Rademacher et al., 1988). Such interactions need to be directly shown.

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**Figure 8.** Determination of copatching of L1 with N-CAM on untreated (*a* and *b*) and castanospermine-treated (*c* and *d*) N2A cells. N2A cells were patched by monoclonal N-CAM antibodies and visualized by indirect immunofluorescence using rhodamine-labeled second antibodies (*a* and *c*). Copatching of L1 was determined after patching with N-CAM antibodies by incubation of the cells with biotinylated polyclonal L1 antibodies visualized by fluorescein-labeled streptavidin (*b* and *d*). In each panel, results from two independent experiments are shown. Arrows and asterisks mark identical positions in *a* and *b* or *c* and *d*.

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