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Comparison of two cell surface molecules involved in neural cell adhesion

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Two cell surface molecules found in mouse brain, N-CAM and the L1 antigen, were compared in terms of their cell adhesion function, polypeptide structures, antigenic determinants and distribution in cerebellar tissue. Fab fragments of polyclonal antibodies to either N-CAM or L1 antigen only partially inhibited the rate of calcium-independent aggregation of neuroblastoma N2A cells, whereas complete and more efficient inhibition was obtained when they were used in combination. Despite the functional similarity, comparison of the electrophoretic behaviour of the purified molecules and of their proteolytic fragments shows that the L1 antigen polypeptide is distinct from that of N-CAM. In addition, no antigenic cross-reactivity was detected between the two molecules. In cryostat sections of cerebellum from young post-natal mice, N-CAM was found to be present in all cell and neurite layers, whereas L1 antigen was expressed only in regions containing post-mitotic cells. These results indicate that two chemically and histochemically distinct cell surface polypeptides can contribute to the calcium-independent adhesiveness of neural cells, and suggest that their differential expression might cause adhesive specificity among cells of developing neural tissues.

Key words: N-CAM/L1 antigen/calcium-independent adhesion/neuroblastoma cells/mouse cerebellum

Introduction

The developmental significance and molecular basis of cell-cell adhesion has been the subject of investigation since the 1940s. Over the past decade the application of immunological techniques to study cells from the chick nervous system has led to identification and characterization of a neural cell adhesion molecule (N-CAM) which is involved in calcium-independent adhesion among both neural and muscle cells (for review, see Edelman *et al.*, 1983; Rutishauser, 1983). The molecule has binding activity consistent with its proposed function as a ligand in the formation of cell-cell bonds (Rutishauser *et al.*, 1982), and monovalent rabbit antibodies to N-CAM specifically inhibit adhesion among neural cells and their processes (Rutishauser *et al.*, 1978a; Rutishauser *et al.*, 1978b), histotypic differentiation in cultured cell aggregates and retinal tissue (Rutishauser *et al.*, 1978a; Buskirk *et al.*, 1980), and interactions between spinal cord neurons and muscle cells *in vitro* (Rutishauser *et al.*, 1983).

More recently, the application of hybridoma technology to the study of mouse neural cell surfaces has led to the description of another membrane glycoprotein, called L1 antigen, which seems to be functionally related to N-CAM in that anti-L1 Fab fragments inhibit the calcium-independent aggregation of mouse cerebellar cells and neuroblastoma N2A cells (Rathjen and Schachner, 1983, 1984; Schachner *et al.*, 1983). These Fab fragments have also been shown to modify the migration of external granular cells from mouse cerebellum in an *in vitro* migration system (Lindner *et al.*, 1983).

Given the possibility that N-CAM and L1 antigen are similar in function, a study was undertaken to compare the two molecules both in terms of their chemical structure and biological properties. In addition, histological studies of young post-natal cerebellum have been carried out to evaluate the expression of L1 antigen and N-CAM in different layers of this tissue.

Results

Aggregation experiments

Fab fragments prepared from rabbit antisera to mouse N-CAM or to mouse L1 antigen inhibit the aggregation of mouse neural cells (Chuong *et al.*, 1982; Rathjen and Schachner, 1984). In the present study, the adhesion-blocking activities of these two Fab preparations were compared, both separately and in combination. Neuroblastoma N2A cells were used for these experiments because they are homogenous with respect to expression of the L1 antigen, whereas dissociated embryonic or post-natal mouse brain cells are not (Rathjen and Schachner, 1984). Fab fragments to N-CAM and L1 antigen each separately inhibited the calcium-independent aggregation of neuroblastoma N2A cells during a 40 min incubation (Figures 1 and 2). In both cases the degree of inhibition was similar and dependent on Fab concentration as well as the length of the incubation. For the individual antibodies, maximal inhibition (80%) was obtained with 1 mg/ml Fab and a 20 min incubation. In aggregation experiments where the two Fab preparations were combined in equal amounts, the degree and efficiency of inhibition increased dramatically, particularly at low Fab concentrations (Figure 1) and after longer time periods (Figure 2). In contrast to the individual Fabs, complete inhibition could be obtained with the combined antibodies and at 1 mg/ml total antibody this block persisted during long incubation periods.

Biochemical comparison of N-CAM and L1 antigen

For these studies both N-CAM and L1 antigen were isolated by immunoaffinity chromatography from detergent-solubilized membranes of young post-natal mouse brains. In Figure 3a is shown the electrophoretic behavior of these molecules in a polyacrylamide gel (PAGE) containing SDS. N-CAM migrated as a broad, diffusely stained band (apparent mol. wt. 180–250 kd), which reflects its structural heterogeneity as previously described (Hoffman *et al.*, 1982; Chuong *et al.*, 1982). L1 antigen isolated from brains of the same age produced a very different pattern consisting of two bands of apparent mol. wt. 200 kd and 140 kd and a minor

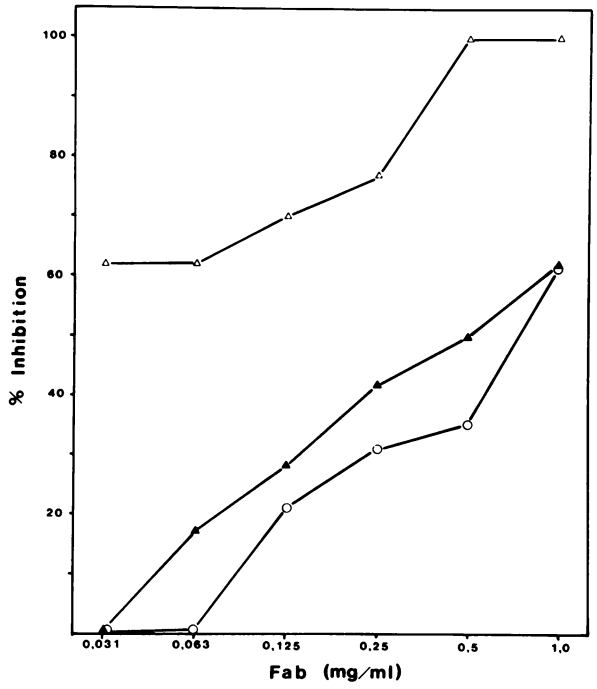

Fig. 1. Inhibition of aggregation of N2A cells after 40 min by Fab fragments of polyclonal antibodies to L1 antigen (▲), to N-CAM (○), and by an equal combination of both Fab preparations (△). Abcissa: concentration of total Fab, ordinate: percent inhibition of aggregation as determined by changes in the rate of decrease in particle number (see Materials and methods).

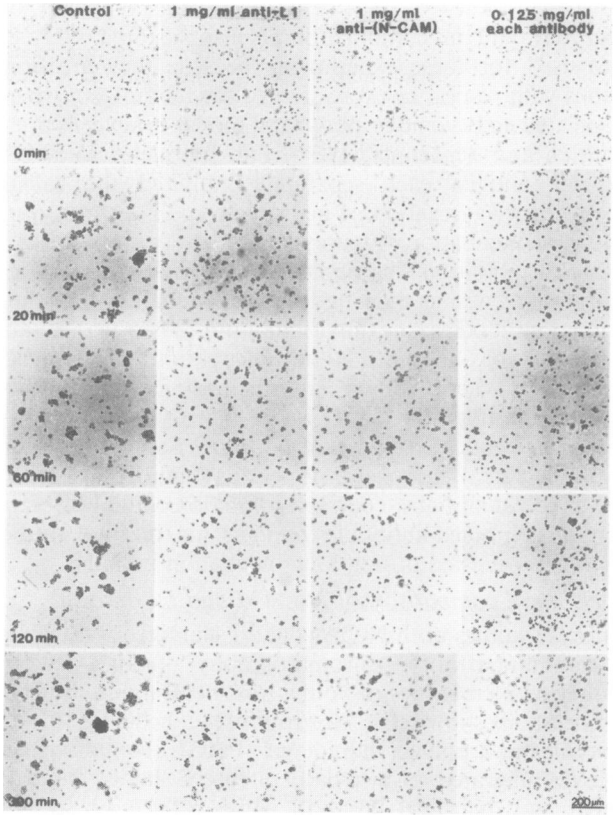

Fig. 2. Inhibition of aggregation of N2A cells with anti-L1 Fab and/or anti-(N-CAM) Fab after various time periods.

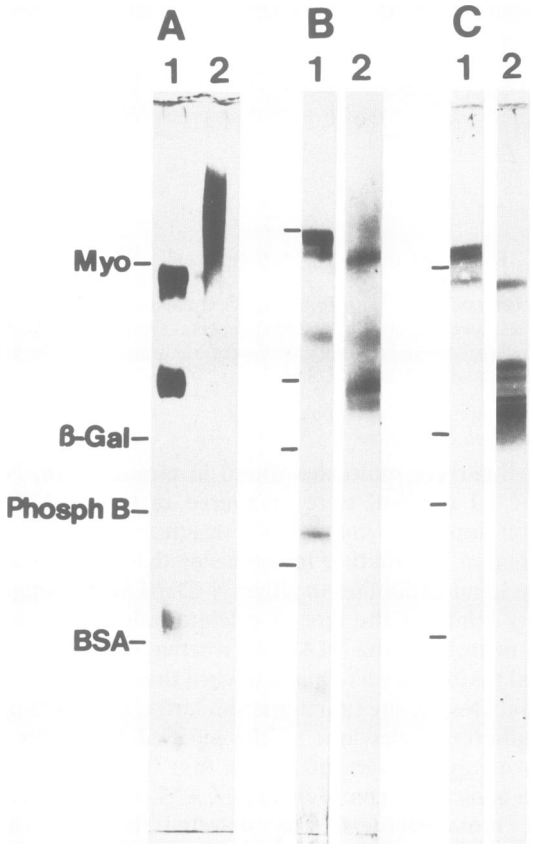

Fig. 3. Comparison of L1 antigen and N-CAM by SDS-PAGE (7% acrylamide); migration of the indicated protein standards is marked to the left of each group of gel lanes (see Materials and methods). **(A)** L1 antigen **(lane 1)** and N-CAM **(lane 2)** were purified by immunoaffinity chromatography from young post-natal mouse brains using monoclonal antibody to L1 antigen or monoclonal antibody to N-CAM. Proteins were visualized by the reducing silver method. Each lane contained 0.5 µg of protein. **(B)** Plasma membrane fractions from brains of adult mice which were separated by SDS-PAGE, transferred onto nitrocellulose filters, and the L1 antigen and N-CAM were detected by binding of rabbit anti-L1 **(lane 1)** or anti-(N-CAM) **(lane 2)** and staining by the horseradish peroxidase method. Each lane was loaded with 80 µg of membrane proteins. **(C)** Analysis of L1 antigen and N-CAM from plasma membrane fractions from neuroblastoma N2A cells, using the same procedure as in **B. Lane 1:** bands detected by polyclonal antibodies to L1 antigen, **Lane 2:** bands detected by polyclonal antibodies to N-CAM. In all cases samples were not boiled.

component of 70 kd. The 200-kd band sometimes migrated as a diffuse zone or split into two or three closely spaced bands (Rathjen and Schachner, 1984). Furthermore, L1 antigen from adult mouse brain produced the same pattern in SDS-PAGE (Figure 3b, lane 1), whereas the migration of N-CAM from adult mouse brain changed to three bands of apparent mol. wt. 180 kd, 140 kd and 120 kd (Figure 3b, lane 2). This change is known to reflect a decrease in the sialic acid content of N-CAM (Hoffman *et al.*, 1982; Rothbard *et al.*, 1982; Edelman and Chuong, 1982; Cunningham *et al.*, 1983).

Further structural comparison of L1 antigen and N-CAM was carried out by digestion of each protein with *Staphylococcus aureus* V8 protease and analysis of the cleavage products by SDS-PAGE (Figure 4). This peptide map clearly demonstrated that the N-CAM and L1 antigen polypeptide chains are distinct from each other.

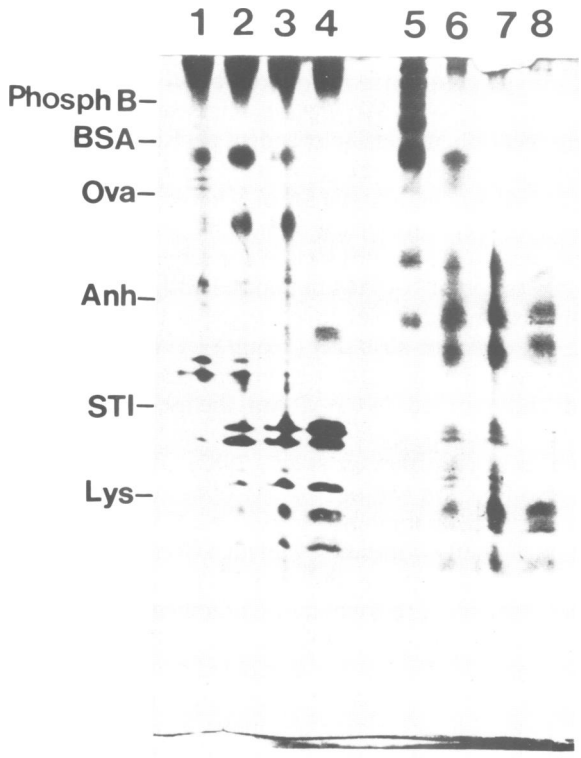

Fig. 4. Comparison by SDS-PAGE of peptides generated by treatment of purified N-CAM **(lanes 1–4)** or L1 antigen **(lanes 5–8)** with different amounts of *S. aureus* V8 protease. Each lane contained 3 μg of protein. **Lanes 1** and **5**: 1 ng protease, **lanes 2** and **6**: 10 ng protease, **lanes 3** and **7**: 25 ng protease, **lanes 4** and **8**: 100 ng protease. The gel was 15% acrylamide and peptides were stained by the silver method; the migration of the indicated protein standards is marked to the left of gel lanes (see Materials and methods).

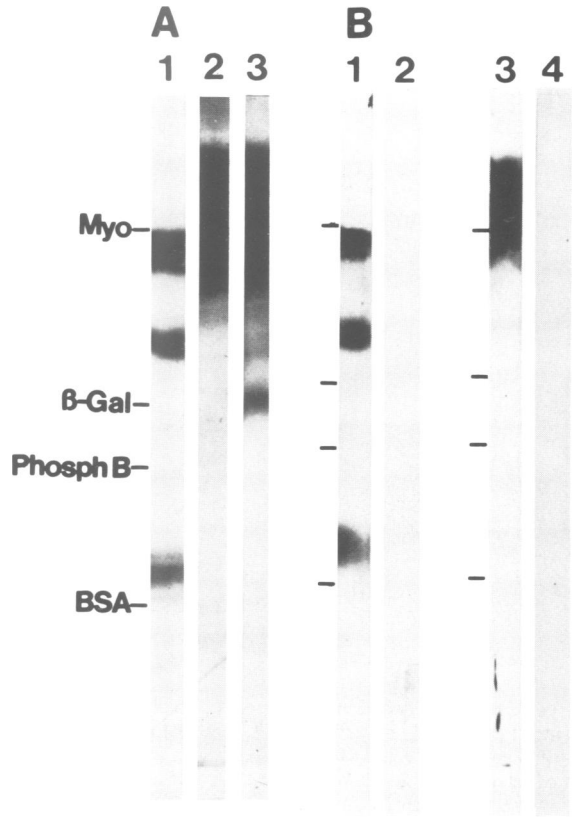

Fig. 5. Analysis of antigenic determinants on L1 antigen and N-CAM. **(A)** Binding of polyclonal antibodies against L1 antigen **(lane 1)**, of monoclonal antibody to N-CAM **(lane 2)**, and of polyclonal antibodies against N-CAM **(lane 3)** with membrane proteins from brains of young post-natal mice after separation by SDS-PAGE and transfer to nitrocellulose. Each lane was loaded with 80 μg of membrane proteins. **(B)** Binding of polyclonal anti-L1 to purified L1 antigen **(lane 1)** and to purified N-CAM **(lane 4)**, and of polyclonal anti-(N-CAM) to purified L1 antigen **(lane 2)** and to purified N-CAM **(lane 3)** after SDS-PAGE and transfer to nitrocellulose. Each lane was loaded with 0.5 μg of protein. The migration of the indicated protein standards is marked to the left of each group of gel lanes (see Materials and methods). Samples were not boiled. Binding of antibodies was visualized by the horseradish peroxidase method.

On the neuroblastoma N2A cells used in our aggregation experiments, N-CAM existed primarily in a desialated, adult-like form with a band at 180 kd and several bands in the 140-kd range (Figure 3c, lane 2). L1 antigen from these cells produced bands of 210 kd and 180 kd (Figure 3c, lane 1), which is different from L1 antigen isolated from mouse brain. The reason for this difference in mol. wt. of L1 antigen from brain tissue and N2A cells has so far not been determined.

Immunological comparison

These experiments were carried out to determine if N-CAM and L1 antigen might share antigenic determinants and to exclude the possibility that the adhesion-blocking Fab fragments might cross-react with both antigens. The antibodies were tested for binding to membrane proteins that had been separated by SDS-PAGE and transferred to nitrocellulose, and to purified N-CAM and L1 antigen from young post-natal mice.

With the separated membrane proteins from young post-natal mice (Figure 5a), polyclonal antibodies to L1 antigen bound to bands characteristic of L1 antigen, and both serum and monoclonal anti-(N-CAM) preparations recognized only N-CAM (compare with Figure 3a). The monoclonal anti-(N-CAM) did not bind to a 120-kd band recognized by the anti-(N-CAM) serum antibody; this difference probably reflects the narrow specificity of the monoclonal for an antigenic determinant found on sialic acid-rich N-CAM, and the absence of this determinant in the smaller fragment.

Binding of the two serum antibodies to purified L1 antigen and N-CAM is illustrated in Figure 5b and 5c. Anti-L1 bound to the L1 antigen but not to N-CAM, and anti-(N-CAM) bound to N-CAM but not to L1 antigen. In both cases all bands present in the antigen preparations (compare with Figure 3a) were recognized by the appropriate antibody.

Histological distribution of L1 antigen and N-CAM in mouse cerebellum

To evaluate the expression of these two functionally-related molecules in nerve tissue, we examined their distribution by immunohistochemical methods in cryostat sections of young post-natal mouse cerebellum. The staining patterns obtained with anti-L1 and anti-(N-CAM) were found to be clearly distinct (Figure 6). Staining of L1 antigen was observed primarily in the post-mitotic inner granular layer, the developing molecular layer and in the inner part, but not in the outer part, of the external granular layer. In contrast, N-CAM seemed to be almost uniformly distributed in all regions of the cerebellum.

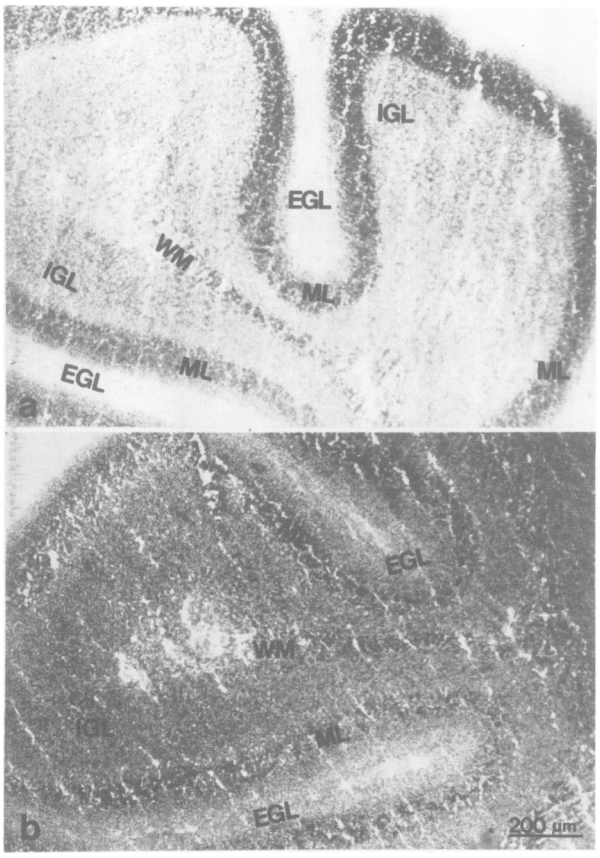

Fig. 6. Distribution of L1 antigen **(a)** and N-CAM **(b)** in sagittal cryostat sections from cerebellum of a 9-day-old mouse as visualized by binding of polyclonal anti-L1 or anti-(N-CAM) followed by staining with horseradish peroxidase-conjugated goat anti-rabbit $F(ab')_2$ and 5-chloronaphthol. EGL: external granular layer, IGL: internal granular layer, ML: molecular layer, WM: white matter. Note that L1 antigen is concentrated in the IGL, the neighboring molecular layer and in the inner part but not in the outer part of the EGL, whereas N-CAM is distributed more uniformly throughout the tissue.

Discussion

The inhibition of calcium-independent aggregation of neuroblastoma N2A cells by Fab fragments of polyclonal antibodies to either L1 antigen or N-CAM suggests that these two cell surface molecules are associated with a similar function. The observations that a combination of the two Fab preparations was more effective than either antibody alone, and that their combined effect was synergistic rather than additive, suggests that the antibodies are reacting with distinct components of the adhesion process. Accordingly, the biochemical and immunological comparison of L1 antigen and N-CAM indicate that their polypeptide chains have different amino acid sequences and that the two molecules do not have common antigenic determinants.

The results of these studies raise several questions concerning the molecular mechanism(s) of calcium-independent cell-cell adhesion. N-CAM is believed to be a ligand in the formation of membrane-membrane adhesions, and the available evidence is consistent with a bond containing one N-CAM from each cell and possibly a direct affinity between those two molecules (Rutishauser *et al.*, 1982). Far less is known about the chemistry of the L1 antigen, and its primary link to function is the inhibition of adhesion by anti-L1 Fab (Rathjen and Schachner, 1984). Such inhibition of cell-cell binding by Fab has proved so far to be a reliable criterion for cell adhesion molecules (Brackenbury *et al.*, 1977; Gallin *et al.*, 1983) and the synergistic effect produced by a combination of anti-L1 and anti-(N-CAM) suggests that each is an important component of calcium-independent neural cell adhesion. The question is therefore raised whether the L1 antigen is part of the N-CAM adhesion system or whether it contributes to a completely separate mechanism. The differences in L1 antigen and N-CAM expression in the cerebellum, in particular the expression of N-CAM but not L1 antigen in the outer part of the external granular layer, suggests that the function of N-CAM does not absolutely require L1 antigen. This is consistent with the fact that membrane vesicles containing only lipid and purified N-CAM can themselves aggregate and display a binding specificity that is similar to that of intact neural cells (Rutishauser *et al.*, 1982). Whether L1 antigen can also function without N-CAM has not been established. In any case their combined roles in N2A cell aggregation suggests that the two molecules might affect each other's function, either directly or indirectly. This possibility, coupled with the apparent restriction of L1 antigen expression to post-mitotic cells (Rathjen and Schachner, 1984), leads us to propose that variations in the combined activities of these two molecules contribute to the specification of adhesive properties involved in generating histotypic structures.

Clearly this hypothesis will require further documentation, particularly with respect to the role of L1 antigen in adhesion and the possibility of a direct interaction between N-CAM and L1 antigen. In any case the potent inhibition of adhesion obtained by combining the effects of anti-(N-CAM) and anti-L1 Fab will be of considerable practical value in studies of cell adhesion during development *in vivo*.

Materials and methods

Isolation of L1 antigen and N-CAM

L1 antigen or N-CAM from young post-natal mouse brains were isolated by immunoaffinity chromatography with monoclonal antibodies against L1 antigen (Rathjen and Schachner, 1984) or the carbohydrate moiety of chick N-CAM (Thanos *et al.*, 1984) coupled to Affi-Gel 10 (Bio-Rad, USA) by methods described previously (Hoffman *et al.*, 1982; Chuong *et al.*, 1982; Rathjen and Schachner, 1984). Briefly, membranes were prepared according to Brunette and Till (1971) and were solubilized in 1% Nonidet P-40 (NP-40, Sigma, USA), 50 mM Tris, 150 mM NaCl, 1 mM EDTA and 40 U aprotinin/ml (Sigma, USA), pH 7.2. Non-solubilized material was removed by centrifugation at 100 000 *g* and 4°C for 1 h. The supernatants were added to affinity columns containing monoclonal antibody to L1 antigen or to N-CAM. After washing of the columns with solubilization buffer, the columns were eluted with a pH 11.5 buffer containing 0.1 M diethylamine (Sigma, USA), 0.5% NP-40, 1 mM EDTA.

Rabbit antibodies to L1 antigen and N-CAM

Polyclonal rabbit antibodies to L1 antigen and N-CAM were raised by injection of 30 µg purified L1 antigen or N-CAM at fortnightly intervals. The first injection was prepared with complete Freund's adjuvant and all consecutive immunizations with incomplete Freund's adjuvant. The IgG fractions from rabbit anti-L1 and anti-(N-CAM) were isolated by ion-exchange chromatography on DEAE-cellulose (DE52, Whatman) according to Fahey and Terry (1979). Fab fragments of IgG fractions were prepared with merucipapain (Sigma) as described (Porter, 1959).

Analytical procedures

SDS-PAGE was performed with 7% or 15% acrylamide concentration in slab gels. Staining of gels by reduction of silver ions was carried out according to Merril *et al.* (1982). The following proteins served as mol. wt. markers (in daltons): lysozyme (14 400; Lys), soybean trypsin inhibitor (21 500; STI), carbonic anhydrase (31 000; Anh), ovalbumin (45 000; Ova), bovine serum albumin (66 200; BSA), phosphorylase B (92 500; Phosph B), β-galactosidase (116 250; β-Gal), and myosin (200 000; Myo) (Bio-Rad, USA). After fractionation by SDS-PAGE, solubilized proteins of enriched mouse membranes, L1 antigen or N-CAM were transferred onto nitrocellulose filters (0.45 µm pore

size, type HA, Millipore, Neu-Isenburg, FRG) at 9 V/cm for 5 h according to Towbin *et al.* (1979). After saturation of protein binding sites with 3% BSA (Sigma, USA) and 0.05% Tween 20 (Sigman, USA), the filters were incubated with the IgG fraction (25 – 50 μg/ml) of rabbit anti-L1 or rabbit anti-(N-CAM), and the bound antibody visualized using horseradish peroxidase-conjugated goat anti-rabbit IgG (1:500 to 1:1000, Miles, UK) and 5-chloronaphthol (Hawkes *et al.*, 1982). Monoclonal antibody to N-CAM was visualized by horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:1000, Miles, UK). Protein determinations were carried out according to Lowry *et al.* (1951) or by the micromethod of Neuhoff *et al.* (1979), using BSA as a standard. Peptide mapping in slab gels was carried out according to Cleveland *et al.* (1977) using *S. aureus* V8 protease (Miles, UK).

Aggregation of neuroblastoma N2A cells

Neuroblastoma N2A cells were obtained from Flow Laboratories (FRG) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum. The cells were detached from culture flasks using low trypsin concentrations (Rathjen and Schachner, 1984). Calcium-independent reaggregation of N2A cells was performed in scintillation vials (Beckman, USA) as described (Brackenbury *et al.*, 1977; Rathjen and Schachner, 1984) using a shaking water bath (New Brunswick Co., USA) at 37°C and 85 r.p.m. 8 x 10^5 cells/ml calcium- and magnesium-free Eagle's Medium were pre-incubatd for 20 min on ice with various concentrations of Fab fragments from rabbit anti-L1 or anti-(N-CAM). In controls, Fab fragments (1 mg/ml) from unimmunized rabbits were used.

Aggregation was expressed as percent decrease in particle number, and percent inhibition of aggregation was calculated as: aggregation (control) – aggregation (Fab of anti-L1, anti-N-CAM or both)/aggregation (control) x 100 (Brackenbury *et al.*, 1977). Particle number was determined in a Coulter Counter (Model ZF with 200 μm capillary, Coulter Electronics GmbH, FRG) at 0, 20 and 40 min.

Histological procedures

Cryostat sections (10 μm thick) from fresh frozen mouse brains were performed according to Goridis *et al.* (1978). The dried sections were incubated with the IgG fraction (50 μg/ml) of rabbit anti-L1 or rabbit anti-(N-CAM) for 30 min at room temperature, washed twice and fixed with 4% formaldehyde for 5 min on ice. Anti-(N-CAM) or anti-L1 antibodies were visualized using $F(ab')_2$ fragments of horseradish peroxidase-conjugated goat anti-rabbit $F(ab')_2$ (Medac, FRG; diluted 1:20) and 5-chloronaphthol (Hawkes *et al.*, 1982).

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