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The neural cell adhesion molecule L1 is distinct from the N-CAM related group of surface antigens BSP-2 and D2

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The neural cell adhesion molecule L1 and the group of N-CAM related molecules, BSP-2 and D2 antigen, are immunochemically distinct molecular species. The two groups of surface molecules are also functionally distinct entities, since inhibition of Ca^{2+}-independent adhesion among early post-natal mouse cerebellar cells by Fab fragments of both antibodies are at least additive, when compared with equal concentrations of the individual antibodies.
Key words: L1 antigen/N-CAM/BSP-2 and D2 antigens/cell adhesion/mouse cerebellum

Introduction

Specific nerve connections are generated by multiple steps in cell-cell interactions during development of the nervous system. These distinct, yet coordinated events include cell proliferation, migration, aggregation, cytodifferentiation, synapse formation, cell death and elimination of synapses (Cowan, 1982). It is likely that short-range cell interactions between surface components of adjacent cells play an important, although probably not exclusive role during proliferation, migration, aggregation and synapse formation. An understanding of the role of cell surfaces in cell recognition depends on the elucidation of the molecular mechanisms involved.

We have recently described a cell surface antigen designated L1 which is recognized by monoclonal and polyclonal antibodies (Rathjen and Schachner, 1984). L1 antigen has so far been found in the central nervous system only on neurons, and immunohistological evidence suggests that these are predominantly post-mitotic. The antigen is involved in a Ca^{2+}-independent adhesion mechanism (Rathjen and Schachner, 1984), and in migration of granule cells in the developing mouse cerebellar cortex (Lindner *et al.*, 1983). L1 antibodies do not influence the synaptic or electrical activities in cell cultures of central nervous system (Kettenmann *et al.*, 1984), or the formation of neuromuscular junction (Mehrke *et al.*, 1984) neither have they yet been found to interfere with fasciculation and extension of neurites (Orkand *et al.*, 1984; Mehrke *et al.*, 1984). The antigen can be resolved into two glycoprotein bands with apparent mol. wts. of 140 and 200 kd (Rathjen and Schachner, 1984) at both adult and early post-natal ages of normal and neurologically mutant mice (Schachner *et al.*, 1983).

L1 antigen shares with the neural cell surface molecules, N-CAM and BSP-2, the property of mediating Ca^{2+}-independent adhesion among neural cells (Edelman, 1983; Thiery *et al.*, 1977; Hoffman *et al.*, 1982; Chuong *et al.*, 1982; Sadoul *et al.*, 1983), but it differs from them in apparent molecular structure and several functional properties. A comparison of the molecular and functional properties of the two groups of molecules was therefore called for. Since the D2 protein is immunochemically related to both N-CAM (Jorgensen *et al.*, 1980) and BSP-2 (Hirn *et al.*, 1983) it was included in our study. Here we show that L1 antigen on the one hand and BSP-2 and D2 on the other are immunochemically different and distinct molecular entities. These findings imply that two different sets of cell surface constituents are involved in Ca^{2+}-independent adhesion among neural cells, in addition to a Ca^{2+}-dependent adhesion mechanism that has not yet been defined at the molecular level (Fischer and Schachner, 1982).

Results

Immunochemical studies were undertaken to compare L1 antigen with the N-CAM related group of antigens, BSP-2 and D2.

Immunospot-binding test

Monoclonal L1 and BSP-2 antibodies reacted with their respective immunopurified antigens immobilized on nitrocellulose filters, but did not cross-react over control values with the other antigen (see Figure 1 for BSP-2 antigen). Monoclonal M4 antibodies, which are also derived from rat, served as negative control (Figure 1c).

When polyclonal rabbit antibodies to purified L1, BSP-2 and D2 were tested by the immunospot-binding technique with the purified BSP-2 and L1 antigens, the individual antibodies reacted with their respective antigens as expected

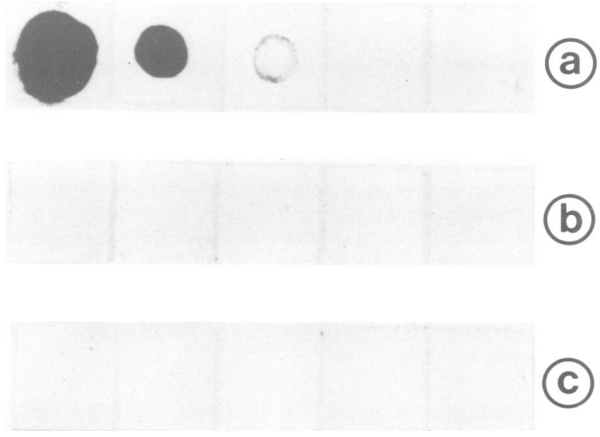

Fig. 1. Immunospot-binding test using monoclonal antibodies with BSP-2 antigen spotted in 1 μl volumes and steps of 1:5 dilution in horizontal lanes from left to right. The first spot on the left contains 0.2 μg protein. **(a)** Monoclonal BSP-2 antibody; **(b)** monoclonal L1 antibody; **(c)** monoclonal M4 antibody (negative control). All antibodies are derived from rat and used at dilutions of 1:25 **(a,b)** and 1:10 **(c)**.

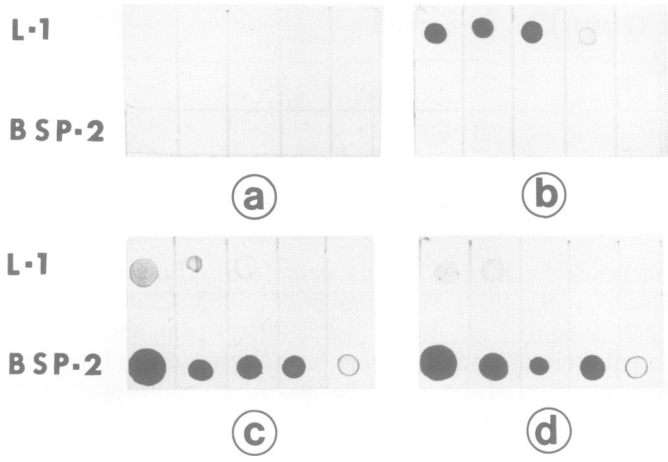

Fig. 2. Immunospot-binding test using polyclonal antibodies with L1 antigen and BSP-2 in the horizontal lanes as indicated. Antigens are spotted in 1 μl volumes and steps of 1:5 dilution in horizontal lanes from left to right. The first spot on the left contains 0.15 μg protein. **(a)** Polyclonal antiserum to human hemoglobin (negative control); **(b)** polyclonal L1 antiserum; **(c)** polyclonal BSP-2 antiserum; **(d)** polyclonal D2 antiserum. All antisera are from rabbits and are used at dilutions of 1:1000.

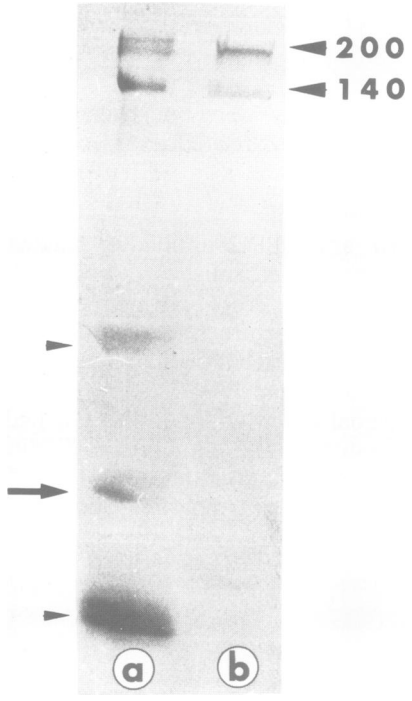

Fig. 3. Western blot analysis using polyclonal L1 antibodies with L1 antigen which was immunoaffinity-purified from adult mouse brain on a monoclonal L1 antibody column **(a)** and with crude membrane fraction of neonatal mouse brain **(b)**, SDS-PAGE was performed on a 11% gel. **(a)** Small arrows point to contaminations of heavy and light chains in the immunoaffinity purified antigen preparation which occasionally contains probable degradation products (large arrow). The 200 kd band is sometimes seen as a doublet.

(Figure 2). Since cross-reactivity between BSP-2, D2 and N-CAM had been established previously (Hirn *et al.*, 1983; Sadoul *et al.*, 1983; E. Bock and C. Goridis, unpublished results) the latter two antigens were omitted from these tests. A strong reaction of D2 antibodies with purified BSP-2 (Figure 2) supported these observations (and see below). On the other hand, a very weak reaction was observed when

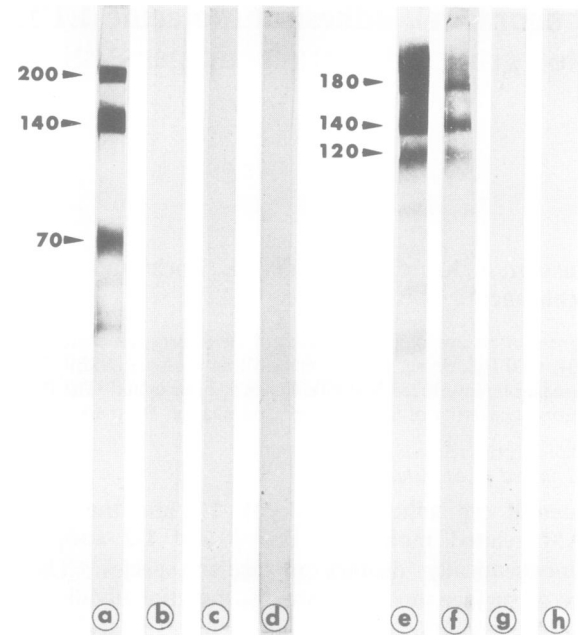

Fig. 4. Western blot analysis of immunoaffinity purified L1 antiserum **(a–d)** and BSP-2 **(e–h)** after SDS-PAGE (linear 7–15% gel) using polyclonal antibodies to L1 **(a,g)**, to BSP-2 **(b,e)**, and to D2 **(c,f)**; **(d,h)** without first antibodies. All antibodies are used at dilutions of 1:1000.

polyclonal BSP-2 or D2 antibodies were incubated with L1 antigen (Figure 2c, d), or when polyclonal L1 antibodies were reacted with BSP-2 (Figure 2b). Rabbit antibodies to human hemoglobin served as controls and did not show any detectable binding (Figure 2a).

The sensitivity of the immunospot-binding test with polyclonal antibodies was higher than that with monoclonal ones. Impurities in the antigen preparation of ~1% could be detected. It is, therefore, possible that small contaminations of one antigen preparation with another antigen are detected with this method. Alternatively, but less probably, cross-reactivities between the antigens exist which were not consistently detected by any of the other methods employed in this study (see below).

Western blot analysis

The reactivity of polyclonal antibodies with purified L1 and BSP-2 antigens and crude membrane fractions was assayed by immunoblot transfers after separation by SDS-PAGE. Polyclonal L1 antibody reacted with the two glycoprotein bands of 140 and 200 kd in both the purified L1 antigen preparation (Figure 3a) and crude membrane fractions (Figure 3b). Sometimes the bands in the 200 kd range split into two adjacent bands (Figure 3b) both at adult and early post-natal ages. A strong reaction of polyclonal D2 and BSP-2 antibodies was seen with purified BSP-2 (Figure 4e, f). Polyclonal L1 antibody gave no detectable reaction with purified BSP-2 (Figure 4g). Similarly, polyclonal BSP-2 and D2 antibodies did not react with purified L1 antigen (Figure 4b, c). Pre-immune rabbit sera, and antisera to human hemoglobin and omission of first antibodies never showed a reaction in these tests.

Monoclonal antibody L1 could not be used in Western blotting techniques, since antigen reactivity was lost after exposure to SDS. Monoclonal BSP-2 antibody reacted with BSP-2, but never with purified L1 antigen.

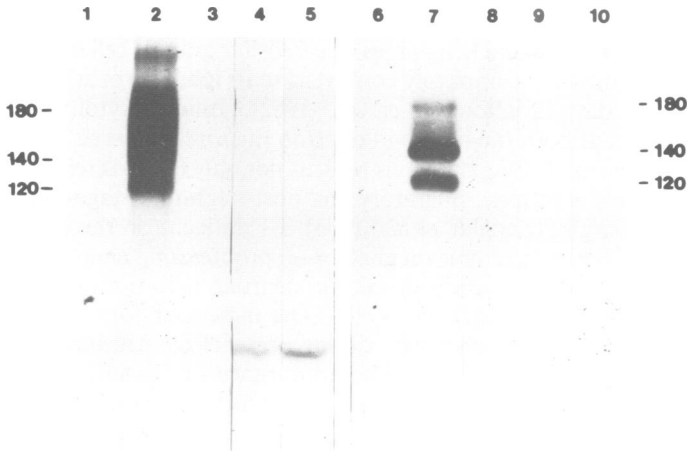

Fig. 5. Immunoprecipitation of purified BSP-2 by L1 mono- and polyclonal antibodies. BSP-2, immunoaffinity purified from embryonic (**lanes 1–5**) and adult (**lanes 6–10**) mouse brain, was iodinated with ^{125}I and immunoprecipitations were performed with BSP-2 monoclonal antibody (**lanes 2** and **7**), L1 monoclonal antibody (**lanes 3** and **8**), polyclonal L1 antiserum (**lanes 4** and **9**), control monoclonal antibody (**lanes 1** and **6**) and normal rabbit serum (**lanes 5** and **10**). The positions of the 180, 140 and 120 kd bands of unlabelled, purified adult BSP-2 are indicated. An autoradiograph of a 6.7% gel is shown.

Immunoprecipitation studies on purified BSP-2

The relationship between L1 and BSP-2 antigens was further explored by immunoprecipitation of purified BSP-2 with monoclonal and polyclonal L1 antibodies. Since BSP-2 exists in two distinct molecular forms, one characteristic of embryonic and the other one of adult mouse brain (Rougon *et al.*, 1982), both forms of the antigen were used for these experiments. As expected, the monoclonal BSP-2 antibodies recognized the embryonic and the adult molecules equally well (Figure 5, lanes 2 and 7). By contrast, immunoprecipitates prepared with poly- and monoclonal L1 antibodies did not contain any labeled band in this region of the gels, irrespective of whether embryonic or adult BSP-2 was used as antigen (Figure 5, lanes 3+4 and 8+9). The labeled band in the 40-kd mol. wt. region seen in the precipitates of embryonic BSP-2 with polyclonal rabbit anti-L1 serum was also present in the control precipitate. There is thus a complete lack of recognition of affinity-purified BSP-2 by both mono- and polyclonal L1 antibodies.

Sequential affinity chromatography of solubilized brain membrane fraction on BSP-2 and L1 monoclonal antibody columns

To establish that L1 and BSP-2 antibodies do indeed recognize distinct molecular species Nonidet P-40 solubilized membrane fractions from adult mouse brain were passed sequentially first over a monoclonal L1 antibody column and the run-through of this column was then passed over a monoclonal BSP-2 antibody column. The eluates of each column were analyzed by SDS-PAGE (Figure 6). The profiles of each eluate showed that they are different from each other, representing the two distinct bands of 140 and 200 kd for the L1 eluate (Figure 6a) and a more diffuse appearance of BSP-2 which is sometimes observed in preparations from adult brain (Figure 6b).

Immunospot-binding tests with monoclonal antibodies showed that no L1 antigen was detectable in the eluate of the BSP-2 antibody column. Likewise, BSP-2 antigen was not detectable in the eluate or from an L1 antibody column. The

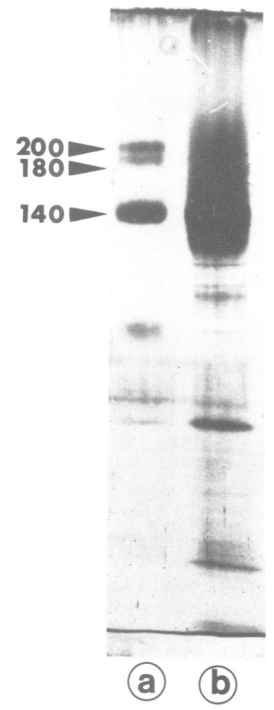

Fig. 6. SDS-PAGE analysis of eluates from monoclonal antibody columns L1 (**a**) and BSP-2 (**b**) obtained from Nonidet P-40 solubilized crude membrane fraction of adult mouse brain. The columns were run in tandem, with the L1 column first and followed by the BSP-2 column. Eluates were run on a linear 7–15% gel. The gel was developed by reduction of silver ions according to Oakley *et al.* (1980). Positions of the mol. wt. markers are indicated.

results were the same when antibody columns were run in reverse order, i.e., the BSP-2 preceding the L1 antibody column (not shown).

Demonstration of two functionally distinct adhesion mechanisms among early post-natal cerebellar cells

To investigate whether the two biochemically distinct groups of cell adhesion molecules are also functionally distinct from each other, adhesion among neural cells was measured in the presence of Fab fragments from polyclonal L1 and BSP-2 antibodies, either individually or in combination. Early postnatal cerebellar rather than C1300 neuroblastoma cells were used, since they are derived from normal brain tissue at a developmental stage when migration occurs. Furthermore, C1300 neuroblastoma cells express a biochemically different form of L1 antigen (Rathjen and Schachner, 1984).

Single cell suspensions were obtained from 8-day-old mouse cerebellum by dissociation with dispase. More than 95% of these cells were BSP-2 antigen-positive and ~70% were L1 antigen-positive by indirect immunofluorescence. Inhibition of aggregation depended on the concentrations of Fab fragments from polyclonal L1 and BSP-2 antibodies (Figure 7 and Table I). When the two antibodies were applied simultaneously in equal amounts, the inhibitory effect on aggregation was more than additive at antibody concentrations up to 0.4 mg/ml, when compared with equal concentrations of each antibody alone. Saturation levels were reached at concentrations of ~0.75 mg/ml) for each antibody alone (not shown) and approached at ~0.5 mg/ml for the combined antibodies at 20 min of aggregation time. At 40 min, saturation was no longer detectable and inhibitory efficiency less pronounced. Fab fragments of monoclonal L1 antibody

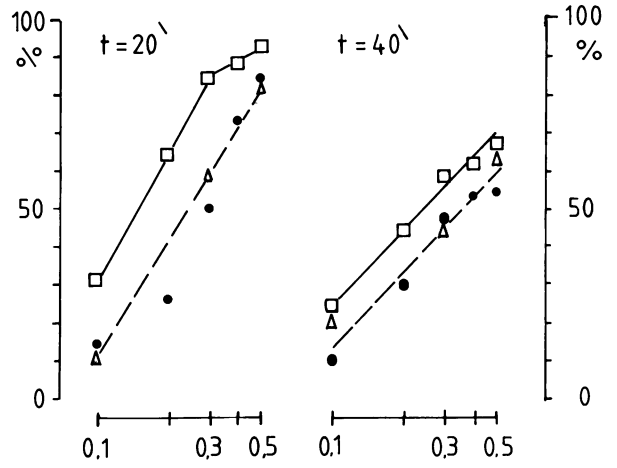

Fig. 7. Inhibition of aggregation of 8-day-old cerebellar cells as a function of concentration of Fab fragments from L1 and BSP-2 antibodies alone and in combination at 20 and 40 min (t' = 20; t' = 40), ●, L1; △, BSP-2; □, L1 and BSP-2. Concentrations of Fab fragments are plotted on a logarithmic scale and represent final concentrations. When used in combination, L1 and BSP-2 antibodies are present in equal amounts. Inhibition of aggregation is expressed as % inhibition = aggregation (control) − aggregation (in presence of Fab fragments)/aggregation (control). Aggregation is expressed as percentage decrease in particle number and varied between 55 and 70% after 20 and 40 min. Percentages are mean values from duplicates in three independent experiments. For standard deviations see Table I.

Table I. Inhibition of aggregation (in percent) of 8-day-old cerebellar cells in the presence of Fab fragments of L1 and BSP-2 antibodies alone and in combination after 20 min aggregation

Concentration of Fab fragments (mg/ml)	L1	BSP-2	L1 and BSP-2
0.1	15.0 ± 11.5	10.5 ± 10.1	31.5 ± 12.1
0.2	26.4 ± 11.0		64.0 ± 11.2
0.3	50.9 ± 17.8	58.6 ± 14.8	84.6 ± 9.3
0.4	73.7 ± 7.9		88.1 ± 6.5
0.5	84.7 ± 11.6	83.1 ± 12.5	92.5 ± 6.8

Experimental procedures and calculations are given in the legend to Figure 7. The values are mean values of duplicates from three independent experiments and standard deviations.

which were shown to bind to cerebellar cells (Rathjen and Schachner, 1984) did not inhibit aggregation.

Discussion

The L1 antigen on one hand and the N-CAM-related molecules BSP-2 and D2 on the other are immunochemically different from each other and are distinct molecular entitites. Similar differences have been observed for L1 antigen and N-CAM from adult mouse brain (Rathjen and Rutishauser, personal communication). These observations show that neural cells and, in particular, early post-natal cerebellar cells at crucial stages of cell interaction, express two types of adhesion molecules.

The two groups of antigens are also functionally distinct molecular species, since the action of their respective antibodies on adhesion are more than additive. This type of synergistic effect is difficult to interpret in a complex cell interaction system, but clearly suggests that the two groups of adhesion molecules are functionally distinct entities.

It is noteworthy that BSP-2 is present in its embryonic form before and during times of cerebellar granule cell migration, until it is completely converted to its adult form at post-natal day 12 (Rougon *et al.*, 1982). Immunocytological studies at both the light and electron microscopic levels have shown that BSP-2 antigen is present not only on all cerebellar neurons at mitotic, migratory and post-migratory stages, but also on glia (Langley *et al.*, 1983). L1 antigen, on the other hand, is not detectable on glia nor on proliferating neurons; it is first expressed on post-mitotic neurons before migration (Rathjen and Schachner, 1984). The molecular form of L1 antigen is not changed during cerebellar development (Schachner *et al.*, 1983). The appearance of L1 antigen on pre-migratory BSP-2 positive granule cell neurons and the final conversion of the embryonic to the adult form of BSP-2 after cessation of migration are likely to have particular functional implications. N-CAM has been shown to mediate cell adhesion by a 'self aggregating' mechanism (Hoffman and Edelman, 1983). The receptor for L1 antigen and its cellular localization on cerebellar cell types is presently unknown.

Although L1 antibodies have been shown to affect the migration of granule cell neurons from the external to the internal granular layer (Lindner *et al.*, 1983), it has so far not been possible to disturb other types of cell interaction such as neurite extension and fasciculation (Orkand *et al.*, 1984; Mehrke *et al.*, 1984), synapse formation between nerve and muscle and cerebellar cells (Orkand *et al.*, 1984; Mehrke *et al.*, 1984), and synaptic activity (Kettenmann *et al.*, 1984; Mehrke *et al.*, 1984). On the other hand, N-CAM has been implicated in the fasciculation and extension of neurites (Rutishauser and Edelman, 1980; Rutishauser *et al.*, 1978), formation of neuromuscular junction (Grumet *et al.*, 1982; Rutishauser *et al.*, 1983) and ordered segregation of cell bodies in the retina (Buskirk *et al.*, 1980). From these observations it is tempting to speculate that the two groups of molecules act in a complementary fashion in different sets of cell interaction phenomena. On the other hand, the present experiments show that the two Ca^{2+}-independent adhesion systems can act in conjunction. Furthermore, in addition to the two Ca^{2+}-independent adhesion systems, another molecularly yet undefined Ca^{2+}-dependent adhesion mechanism has been found on early post-natal cerebellar cells (Fischer and Schachner, 1982; and unpublished results). The functional significance of the combined adhesion systems for cell interactions during early post-natal development of the cerebellar cortex will have to be elucidated.

Materials and methods

Antibodies

Production and preparation of monoclonal L1 antibody from rat has been described in detail previously (Rathjen and Schachner, 1984). Polyclonal antibodies to L1 antigen which was immunoaffinity purified from mouse brain with a monoclonal antibody column were prepared in rabbits and analyzed as described (Rathjen and Schachner, 1984). Antibodies were purified by $(NH_4)_2SO_4$ precipitation and DEAE column chromatography (Rathjen and Schachner, 1984). Polyclonal antibodies to D2 purified from rat brain (Rasmussen *et al.*, 1982) were prepared in rats and BALB/c mice by three injections of 2−5 μg of D2 per injection and animal. Injections were given at intervals of 7 days. Animals were bled 7 days after the last injection. Monoclonal rat and polyclonal rabbit BSP-2 antibodies were obtained as described previously (Hirn *et al.*, 1981). Goat anti-rabbit Ig and rabbit anti-rat Ig, both coupled with peroxidase were purchased from Miles. Fab fragments were prepared as described previously (Rathjen and Schachner, 1984).

Antigens

BSP-2 and L1 antigens were purified from adult mouse brain by immunoaffinity chromatograpy on monoclonal antibody columns as described

previously (Rougon *et al.*, 1982; Rathjen and Schachner, 1984). Crude membrane fractions from neonatal and adult C57BL/6J or NMRI mice were prepared as described (Rathjen and Schachner, 1984).

Immunospot-binding techniques

This technique was carried out as described in detail previously (Hawkes *et al.*, 1982). In brief, immunopurified antigens were spotted onto nitrocellulose filters. After reaction with first antibodies, peroxidase-conjugated second antibodies were applied and visualized by reaction with chloronaphthol. Staining intensities were scored by eye.

Western blot procedures

These techniques were carried out essentially as described by Towbin *et al.* (1979). Immunopurified BSP-2 and L1 antigens were subjected to SDS-PAGE on the same gel in 2 cm wide pockets, each antigen (20 μg) individually. Blotting was carried out for 2 h at 0.4 A to nitrocellulose filters. These were washed once in Tris-buffered saline (TBS, pH 7.4) and subsequently bathed in blocking buffer for ~1 h. Stripes were cut from the filter sheet corresponding to each gel lane and incubated individually with antibodies for ~1 h at room temperature. Application of second antibody and immunoperoxidase procedures using chloronaphtol as substrate were carried out as described in detail previously (Rathjen and Schachner, 1984).

Immunoprecipitation

BSP-2 purified from embryonic and adult mouse brain was iodinated with ^{125}I (Jensenius and Williams, 1974) and aliquots (5 x 10^5 c.p.m.) immunoprecipitated as described (Hirn *et al.*, 1983) with various antibodies. Monoclonal L1 antibody (1 μl) was used in the form of 10 times concentrated ascites (5.3 mg/ml), monoclonal BSP-2 (40 μl) in the form of hybridoma supernatant and rabbit L1 antibodies as serum (6 μl). 10 μl of ascites containing an irrelevant monoclonal rat antibody and 10 μl of normal rabbit serum were used as controls. Immune complexes were either formed with rabbit anti-rat Ig serum or with formalin-fixed *Staphylococcus aureus* Cowan I bacteria. The precipitates were analysed on 6.7% polyacrylamide gels.

Aggregation of cerebellar cells

Adhesion among single cell suspensions of cerebellar cells from 8-day-old C57BL/6J or NMRI mice under reaggregation conditions was measured essentially as described previously (Fischer and Schachner, 1982). Ca^{2+}-independent aggregation was carried out at 85 r.p.m. at 37°C in CMF-HBSS containing 10 mM Hepes and 0.1 mM Ca^{2+} at concentrations of 2 x 10^6 cells/ml. Single cell suspensions were obtained by a combination of enzymatic and mechanical dissociation (Schnitzer and Schachner, 1981). However, instead of trypsin, dispase II (Boehringer, Mannheim, FRG) was used at 0.4 mg/ml for 30 min at room temperature.

Acknowledgements

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