Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion

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Communicated by M. Schachner

Monoclonal and polyclonal L1 antibodies react by indirect immunofluorescence with the cell surface of cultured tetanus toxin-positive neurons from post-natal cerebella of mice, but not with glial fibrillary acidic protein-positive astrocytes, O4 antigen-positive oligodendrocytes or fibronectin-positive fibroblasts or fibroblast-like cells. During cerebellar development L1 antigen is detectable on tetanus toxin-positive cells as early as embryonic day 13 after 3 days in culture. In sections of the early post-natal cerebellum, L1 antigen is found on pre-migratory neurons in the internal, but not in the external part of the external granular layer. In the adult cerebellum, L1 antigen is predominantly localized in the molecular layer and around Purkinje cells. Fibers in white matter and the granular layer are also L1 antigen-positive. Granule cell bodies and synaptic glomeruli are weakly antigen-positive. Several cell lines derived from neuroblastoma C1300 also express L1 antigen. The antigen is not detectable by enzyme-linked immunosorbent assay in tissue homogenates of liver, kidney, lung, heart, sperm or thymus. With polyclonal L1 antibodies, cross-reactive determinants are found in brains of rat, guinea pig, hamster, chicken, rabbit and man, but not in frog, while monoclonal antibody reacts detectably only with mouse brain. The molecular species recognized by both monoclonal and polyclonal antibodies display two prominent bands by SDS-PAGE under reducing and non-reducing conditions with apparent mol. wts. of 140 and 200 kd. L1 antigen isolated from cultured cerebellar cells consists mainly of a band in the 200-kd range and a faint one at 140 kd. L1 antigen from neuroblastoma N2A shows two bands with slightly higher apparent mol. wts. All molecular forms of L1 antigen can be labeled by [³H]fucose and [³H]glucosamine. Ca²⁺-independent re-aggregation of cerebellar cells from early post-natal C57BL/6J mice and of the continuous cell line N2A derived from the murine neuroblastoma C1300 is inhibited by Fab fragments of the polyclonal, but not of monoclonal antibody, both of which are known to react with the surface membrane of these cells.

Key words: cell surface glycoprotein/cerebellum/cell adhesion/monoclonal antibody/neuronal marker

Introduction

To characterize the mechanisms underlying cell-cell interactions during development and regeneration of the mammalian nervous system, neurobiologists searched for cell surface components which are unique for a particular cell type and a particular developmental stage and which are involved in cell recognition. Immunological techniques have proved

useful in identifying specific cell surface components which may be involved in cell recognition (Brackenbury *et al.*, 1977; Hausman and Moscona, 1979; Jakoi and Marchase, 1979; Jorgensen *et al.*, 1980; Rutishauser and Edelman, 1980; Thomas and Steinberg, 1981).

As a model system to study cell-cell interactions we have chosen the cerebellar cortex of the mouse which has been well characterised in other respects (Eccles *et al.*, 1967; Llinas, 1969; Palay and Chan-Palay, 1974). The cerebellar cortex is composed of relatively few cell types arranged in repeating units of stereotypically organized cellular elements which are remarkably constant amongst vertebrate species. In mammals some major events in neuron formation and migration as well as overt synaptogenesis occur post-natally so they become easily amenable to analysis. This study was undertaken to characterize a new cell surface antigen recognized by monoclonal antibody L1 with regard to cell type specificity, molecular composition and involvement in cell adhesion.

Results

Production of monoclonal and polyclonal antibodies and determination of molecular nature of L1 antigen

Monoclonal antibody L1 was obtained from a fusion of Y3-Ag 1.2.3 rat myeloma with splenocytes from Sprague-Dawley rats immunized with a glycoprotein fraction from cerebella of 8- to 10-day-old C57BL/6J mice. The glycoprotein fraction was prepared from detergent-solubilized enriched plasma membranes and obtained by affinity chromatography on lens lentil lectin. This purification procedure was chosen to enrich for particular glycoproteins, such as, for instance, NS-4 antigen (Goridis *et al.*, 1978b; Rohrer and Schachner, 1980; Schachner *et al.*, 1975), in order to gear the immune response more rigorously towards the recognition of cell surface-related components.

Following appropriate screening (see Materials and methods), an antibody was detected which reacted with most, but not all cultured cells as judged by indirect immunofluorescence methods. Monoclonal antibodies were obtained from supernatants of cloned cell cultures or from ascites fluid. Immunoaffinity purification of L1 antigen was achieved using monoclonal antibody which belongs to the IgG subclass (not shown) and was immobilized on Affi-Gel 10. Enriched plasma membranes were obtained from 8- to 10-day-old mouse brains solubilized by treatment with 0.5% Nonidet P-40 (NP-40) and passed over an L1 antibody-Affi-Gel 10 column. L1 antigen was eluted from the column by alkaline pH and shown by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis to consist of two prominent bands with apparent mol. wts. of 140 and 200 kd (Figure 1A). In some preparations the 200-kd band migrated as a diffuse zone or as two or three closely stacked bands. A weak band with the apparent mol. wt. of 70 kd was also frequently seen. Rat immunoglobulins were always present in the antigen preparation, possibly due to release from the solid-state carrier under alkaline elution conditions. The immunoglobu-

Fig. 1. SDS-PAGE and immunoblot showing L1 antigen. **(A)** L1 antigen was purified by immunoaffinity chromatography using monoclonal antibody from brains of 8- to 10-day-old C57BL/6J mice. The 8% SDS-polyacrylamide gel was stained by the reducing silver method (Oakley *et al.*, 1980). **Track 1:** L1 antigen. **Track 2:** detergent-solubilized crude membrane fraction which was loaded onto L1 antibody affinity column. The mol. wt. standards are indicated by arrows. H, indicates the heavy chain of monoclonal L1 antibody and L, the light chain. (Note: among several L1 antigen purifications, one preparation yielded a third band with an apparent mol. wt. of 240 kd.) **(B)** Immunoblot analysis of L1 antigen from cerebella of 10-day-old mice with polyclonal L1 antibody. Plasma membrane-enriched fractions were separated by SDS-PAGE (6–15% acrylamide gradient) and transferred electrophoretically onto nitrocellulose sheets (Towbin *et al.*, 1979). Binding of polyclonal L1 antibody was visualized by HRP-conjugated goat anti-rabbit antibodies and developed with chloronaphthol (Hawkes *et al.*, 1982).

lins could be seen as heavy and light chains by SDS-PAGE in the presence of reducing agents (Figure 1A).

The one-step purification procedure yielded a considerable enrichment over the solubilized total homogenate (compare tracks 1 and 2 in Figure 1A). The relative intensities of the two protein bands varied with different preparations (not shown). Supplementation of buffers with additional protease inhibitors such as 50 μ M leupeptin, 50 μ M pepstatin and 500 μ M phenylmethylsulfonyl fluoride (PMSF), did not influence the relative proportions of the two bands (not shown).

Polyclonal antibody, which was obtained from rabbits by immunization with immunoaffinity-purified L1 antigen, also reacted in immunoblotting tests (Towbin *et al.*, 1979) with the two protein bands with apparent mol. wts. of 140 and 200 kd (Figure 1B). Immunoblot analysis using monoclonal antibody could not be performed, since the antibody did not react with antigen denatured by SDS.

Immunoaffinity purification of L1 antigen from cultured cerebellar cells, which had been labeled biosynthetically with L-[3 H]fucose, resulted in a prominent band in the 200-kd range and a faint one at 140 kd (Figure 2). Biosynthetic labeling of cerebellar cells with [3 H]glucosamine or a radioactive amino acid mixture gave identical gel patterns (not shown). This pattern was independent of cell culture densities ($1.4\text{--}3.8 \times 10^7$ cells/Petri dish, 10 cm in diameter) and whether cells were labeled directly after seeding or after 3 days in culture.

Fig. 2. Autoradiogram of a SDS-PAGE of L-[3 H]fucose-labeled L1 antigen immunoaffinity purified using monoclonal L1 antibody. Monolayers of 6-day-old C57BL/6J cerebellar cells and logarithmically growing N2A cells were biosynthetically labeled for 48 h with [3 H]fucose. Cells were washed and then solubilized in the presence of 0.5% NP-40 and L1 antigen immunoaffinity purified. 15 000 c.p.m. were layered onto an 8% SDS polyacrylamide gel and visualized by fluorography. **Track 1:** L1 antigen from cerebellar cells. **Track 2:** L1 antigen from N2A cells. **Track 3:** mixture of L1 antigens from cerebellar and N2A cells.

L1 antigen isolated from C1300 mouse neuroblastoma clone N2A consisted of two bands in the 140- and 200-kd range but with slightly higher mol. wts. than for the antigen isolated from cerebellar cells (Figure 2). As with cerebellar cells, the 200-kd band was the predominant one.

Cell type-specificity of L1 antigen expression in the early post-natal cerebellum

Cell type-specificity of antigen expression was determined with mono- and polyclonal L1 antibodies by indirect immunofluorescence in viable monolayer cultures of cerebellar cells from early post-natal mice to assure cell surface reactivity only. Cell types were identified by double-immunolabeling procedures using established cell type-specific antigenic markers for the mouse cerebellum (Schnitzer and Schachner, 1981). In monolayer cultures of early post-natal mouse cerebellum, L1 antigen was not found on glial fibrillary acidic protein-positive astrocytes (not shown), on O4 antigen-positive oligodendrocytes (Figure 3) or on fibronectin-positive fibroblasts or fibroblast-like cells (not shown). However, all tetanus toxin-positive neurons were labeled by L1 antibodies (Figure 4). Even after prolonged culture periods (2–4 weeks) only neurons expressed detectable levels of L1 antigen. It is noteworthy that after culture periods of 1–2 days, cell bodies and processes were found to react well with L1 antibody as was observed for tetanus toxin. After longer culture periods, however, and in contrast to tetanus toxin, L1 antibody seemed to react more intensely with

Fig. 3. Double immunofluorescence labeling with L1 and O4 antibodies. Cerebellar cells from 4-day-old mice were grown on poly-L-lysine coated coverslips for 3 days *in vitro*. O4 antibody was directly conjugated with tetramethylrhodamine and polyclonal L1 antibody was visualized by indirect immunofluorescence. (a) phase contrast, (b) L1 antibody, (c) O4 antibody.

neurites than with cell bodies. Polyclonal and monoclonal antibodies reacted identically throughout these assays.

Tissue specificity of L1 antigen expression

Immunobinding tests (Hawkes *et al.*, 1982) with polyclonal and monoclonal L1 antibodies on homogenates of different mouse tissues revealed L1 antigen in enriched membrane

Fig. 4. Double immunofluorescence labeling with monoclonal L1 antibody and tetanus toxin. Cerebellar cells from 4-day-old mice were grown on poly-L-lysine coated coverslips for 3 days *in vitro*. Monoclonal L1 antibody and tetanus toxin were visualized by indirect immunofluorescence. (a) phase contrast, (b) monoclonal L1 antibody, (c) tetanus toxin.

preparations of adult and early post-natal cerebella and total brain, but not of liver, lung, testis, thymus, kidney and spleen of adult mice.

Expression of L1 antigen on tumors of neural origin

Monoclonal and polyclonal L1 antibodies reacted with C1300

neuroblastoma clones NB41A3 and N2A and the neuroblastoma-glioma hybrid 108-CC15 (not shown). Polyclonal L1 antibody, but not monoclonal L1 antibody, reacts with clones B103 and B104 which are of rat origin. (Monoclonal L1 antibody does not react with rat neural tissue.) The medulloepithelioma UAL-1 from the mouse did not express detectable levels of L1 antigen as tested by indirect immunofluorescence with monoclonal and polyclonal L1 antibodies.

Expression of L1 antigen during development

Monolayer cells from cerebella of 13-day-old mouse embryos were maintained for 3 days *in vitro* and examined by immuno-double-labeling methods for expression of tetanus toxin receptors and L1 antigen. Most, but not all tetanus toxin-positive cells also expressed L1 antigen as detected by polyclonal antibodies (not shown).

In sagittal sections of neonatal mouse cerebella, the antigen was expressed in the internal, but not the external part of the external granular layer. Outlines of cells with larger cell bodies, possibly Purkinje and Golgi type II cells were also stained by L1 antibody. At post-natal day 8, the external granular layer showed an even more distinct gradient of immunofluorescence labeling, with the outer part of the layer devoid of antigen, while with increasing distance from the surface of the folium antigen expression became more prominent (Figure 5). The nascent molecular layer was also very strongly stained by antibody. The internal granular layer was weakly L1 antigen-positive. At adult ages the molecular layer

was found to be predominantly L1 antigen-positive (Figure 6). Immunostaining of the internal granular layer was less prominent. A diffuse labeling was seen in synaptic glomeruli. In white matter tracts antigen-positive fiber bundles could be discerned. These bundles were often observed to thin out into single fibers upon entry into the granular layer (not shown).

L1 antigen in different species

Total homogenates from several species were tested by immuno-binding for the presence of L1 antigen cross-reactive material. Antigenic site of the L1 molecules recognized by monoclonal antibody were less widely cross-reactive among species than those recognized by polyclonal antibody. While monoclonal antibody reacted only with neural tissue from mouse, polyclonal antibodies reacted also with rat, guinea pig, hamster and, more weakly, with chick, rabbit and human. Frog brain did not express cross-reactive sites.

Inhibition of cell aggregation by Fab fragments of polyclonal L1 antibody: cerebellar and neuroblastoma cells

To investigate the influence of L1 antigen on the short-term re-aggregation of single neural cells in suspension, ways had to be found to obtain cells which were capable of re-aggregation and also expressed sufficient levels of L1 antigen as detected both by poly- and monoclonal antibodies. When cerebella from 6-day-old mice, retinae from 4- to 13-day-old

Fig. 5. Indirect immunofluorescence labeling with polyclonal L1 antibody of a sagittal section from cerebellum of an 8-day-old mouse. EGL, external granular layer; ML, molecular layer; IGL, internal granular layer.

Fig. 6. Indirect immunofluorescence labeling with polyclonal L1 antibody of a sagittal section from cerebellum of a 25-day-old mouse. M, meninges; ML, molecular layer; PC, Purkinje cells; GL, granular layer.

mice or total brains from 15-day-old mouse embryos were dissociated by high and low trypsin concentrations in the presence or absence of Ca^{2+} or mechanically without enzyme treatment, single cell suspensions were obtained which aggregated, but did not express detectable levels of L1 antigen as tested by indirect immunofluorescence. It was therefore not surprising to observe that Fab fragments of polyclonal L1 antibody did not inhibit aggregate formation of freshly dissociated cells (not shown). When cerebellar cells from 7-day-old mice obtained by trypsinisation and maintained as monolayers for 18–24 h *in vitro* were detached from the culture plate and mildly dissociated by trypsin (20 $\mu\text{g}/\text{ml}$) in the presence of 1 mM EDTA, 40–50% of all cells were found to express immunologically detectable levels of L1 antigen. (After longer culture periods all tetanus toxin-positive neurons expressed L1 antigen. From these cultures it was, however, difficult to obtain viable single cell suspensions dispersed enough to be adequate for re-aggregation tests.) When this cell population was allowed to re-aggregate, Ca^{2+} -independent aggregate formation, as estimated by decrease in particle number, was found to range from 53 to 40% in eight experiments (Table I). Fab fragments of monoclonal antibodies (up to 500 $\mu\text{g}/\text{ml}$) did not inhibit aggregate formation (not shown). However, Fab fragments of polyclonal L1 antibodies (1 mg/ml) inhibited aggregate formation in the range of 22–30% in eight experiments. It should be pointed out that aggregation and inhibition values under strictly identical conditions showed some variation from experiment to experiment.

Since levels of inhibition by antibodies were not particularly large with cultivated cerebellar cells, possibly due to heterogeneity of L1 antigen expression, and since the dissociation of embryonic and early post-natal neural tissues into single cells yielded only cells without detectable levels of L1 antigen, we turned to mouse neuroblastoma C1300 clone N2A. Cells of this clone all express high levels of L1 antigen detectable with both monoclonal and polyclonal antibodies. L1 antigen from N2A cells consists of mol. wt. bands similar to cerebellar cells. Aggregation was higher with clone N2A than with neuroblastoma clone NB41A3 or the glioma-neuroblastoma hybrid 108-CC15, both of which were also found to express L1 antigen. Inhibition of Ca^{2+} -independent aggregate formation of clone N2A was seen with Fab fragments from polyclonal (Figure 7 and Table II), but not monoclonal L1 anti-

bodies (Table II). Inhibition of aggregate formation over a 20 min period ranged between 60% and 86% using a Fab concentration of 0.5 mg/ml (Table II) and was linearly proportional to the logarithm of the concentration of Fab fragments of polyclonal L1 antibody (Figure 8). The inhibition was higher after 20 than after 40 min of aggregation time (Figure 8). The Fab fragments of polyclonal L1 antibodies are neutralized by pre-incubation with purified L1 antigen (Figure 7d).

Discussion

We have described a cell surface antigen detected by monoclonal antibody L1 which can be resolved as two bands with apparent mol. wts. of 140 and 200 kd. These two components have so far been detected in monolayer cultures of early post-natal mouse cerebellum, in brains of early post-natal mice and, with slightly higher apparent mol. wts., in clone N2A of murine neuroblastoma C1300. A similar composition and similar mol. wts. have been reported for cell surface antigens of neurons, such as NS-4 antigen (Goridis *et al.*, 1978b; Rohrer and Schachner, 1981), BSP-2 (Hirn *et al.*, 1981), a cell surface glycoprotein shared between muscle and brain (Lemmon *et al.*, 1982), D2 glycoprotein (Jorgensen and Bock, 1974; Jorgensen *et al.*, 1980) and N-CAM (Thiery *et al.*, 1977; Hoffman *et al.*, 1982; Chuong *et al.*, 1982; McClain and Edelman, 1982).

The antigenic determinants recognized by polyclonal and monoclonal L1 antibodies on the one hand, and by monoclonal and polyclonal BSP-2 antibodies and polyclonal D2 antibody on the other, are localized on different molecular entities which are immunochemically distinct from each other (Schachner *et al.*, 1983; Faissner *et al.*, in preparation). In preliminary experiments, a comparison between mouse N-CAM and L1 antigen also suggests that these molecules are immunochemically and biochemically distinct (Rathjen and Rutishauser, unpublished results). BSP-2, D2, and N-CAM were found to be similar if not identical to each other (Sadoul *et al.*, 1983; Hirn *et al.*, 1983; Jorgensen *et al.*, 1980).

In the early post-natal mouse cerebellum, L1 cell surface antigen has so far been recognized on all tetanus toxin-positive neurons, but not on glia and fibroblasts or fibroblast-like cells. A neuronal location in the central nervous system has been found also for NS-4 antigen, and suggested for D2

Table I. Inhibition of aggregation of single cerebellar cells after maintenance *in vitro* by Fab fragments of polyclonal L1 antibody

Experiment	Percent aggregation ^a in the presence of Fab fragments from non-immunized rabbits (1 mg/ml)	Percent aggregation ^a in the presence of Fab fragments from polyclonal L1 antibody (1 mg/ml)	Percent inhibition of aggregation ^b by Fab fragments of polyclonal L1 antibody (1 mg/ml)
1	51 ± 2.2	40 ± 1.4	22
2	49 ± 1.6	38 ± 1.4	22
3	50 ± 1.6	38 ± 2.5	24
4	45 ± 1	33 ± 1.6	27
5	40 ± 2.1	31 ± 1.7	22
6	46 ± 1.4	33 ± 2.8	28
7	53 ± 1.6	40 ± 1	25
8	43 ± 4.2	30 ± 3.5	30

Monolayers of cerebellar cells from 6-day-old C57BL/6J mice were maintained for 18–24 h *in vitro*. Cells were then detached by trypsinization (20 $\mu\text{g}/\text{ml}$) in the presence of 1 mM EDTA. After washing in CMF-HBSS, cells were incubated with Fab fragments on ice and subsequently rotated in a shaking water bath for 30 min. Particle numbers were counted in a Coulter Counter at 0 and 30 min.

^aAggregation is expressed as percentage decrease in particle number after 30 min of rotation at 37°C. The standard deviation in triplicate assays is shown.

^bInhibition of aggregation is expressed as % inhibition = $\frac{\text{aggregation (control)} - \text{aggregation (plus Fab fragments of polyclonal L1 antibody)}}{\text{aggregation (control)}} \times 100$.

Fig. 7. Inhibition of aggregation of N2A cells by Fab fragments of polyclonal L1 antibody as observed by light microscopy. **(a)** Cell suspension at 0 min. **(b)** Cell suspension after 20 or 40 min of aggregation in the presence of Fab fragments from non-immunized rabbits (1 mg/ml). **(c)** Cell suspension after 20 or 40 min of aggregation in the presence of Fab fragments from polyclonal L1 antibody (1 mg/ml). **(d)** Cell suspension after 20 or 40 min of aggregation in the presence of Fab fragments from polyclonal L1 antibody (1 mg/ml) neutralized with 25 µg L1 antigen. The left column shows the cell suspension after 20 min, the right column after 40 min.

Table II. Inhibition of aggregation of neuroblastoma clone N2A cells by Fab fragments of polyclonal antibody L1

Experiment	Percent aggregation ^a in the presence of Fab fragments from non-immunized rabbits (0.5 mg/ml)	Percent inhibition of aggregation ^a by Fab fragments of polyclonal L1 antibody (0.5 mg/ml)	Inhibition of aggregation ^a by Fab fragments of monoclonal L1 antibody (50 µg/ml)
1	37 ± 2.8	84	n.t.
2	55 ± 4.2	62	n.t.
3	39 ± 2.8	60	n.t.
4	38 ± 2.8	81	n.t.
5	51 ± 4.2	86	n.d.
6	56 ± 4.2	n.t.	n.d.
7	34 ± 2.8	n.t.	n.d.

Confluent monolayer cells of clone N2A were dissociated by trypsin (20 µg/ml) in CMF-HBSS containing 1 mM EDTA. After washing in CMF-HBSS, cells were incubated with Fab fragments on ice and subsequently rotated at 37°C for 20 min on a shaking water bath. Particle numbers were counted at 0 and 20 min.

^aSee Table I. The standard deviation in duplicate assays is shown.

n.t. — not tested.

n.d. — not detectable.

Fig. 8. Inhibition of aggregation of N2A cells as a function of concentration of Fab fragments of polyclonal L1 antibody. ▲ Aggregation time, 20 min, △ aggregation time, 40 min. Concentration of Fab fragments is plotted on a logarithmic scale. Percent inhibition is calculated as indicated in Table I. Percentages are mean values from two determinations in one experiment.

glycoprotein and N-CAM. BSP-2 has been found on both glia and neurons (Langley *et al.*, 1982). Interestingly, not all tetanus toxin-positive neurons are L1 antigen-positive in cultures of cerebella from 13-day-old mouse embryos after 3 days *in vitro*. This raises the question of heterogeneity among neuronal populations and specificity of markers for particular cell types.

In the early post-natal mouse cerebellum, L1 antigen expression follows a spatio-temporal gradient. In histological sections, L1 antigen was not detectable in regions of high proliferative activity of neurons, i.e., the external part of the external granular layer, but was found in the inner part of this layer, where neurons are mostly post-mitotic. We therefore suggest that the expression of L1 antigen is related to the developmental stage of neurons. A similar spatio-temporal distribution has been found for NS-4 antigen, tetanus toxin

receptors and G_{M1} ganglioside (Schnitzer and Schachner, 1981; Willinger and Schachner, 1980). However, in contrast to these cell surface components, L1 antigen is less prominent in the internal granular layer, both in the developing and adult cerebellum. It is possible that the topographically distinct regulation of L1 antigen expression at the surface membrane of neurons depends on their state of differentiation. Our observations *in situ* and *in vitro* support the notion that neuronal precursors, which have just reached their post-mitotic stage, express L1 antigen on cell bodies and processes, whereas in a more differentiated stage L1 antigen is more prominent in cellular processes than on cell bodies. However, further investigation is needed to correlate the topography and cell type-specificity of L1 antigen expression *in situ* and *in vitro* with respect to cell cycle and stage of differentiation. In addition, it will be pertinent to investigate whether other regions in the central nervous system with a laminated cyto-architecture also show a spatio-temporal gradient of L1 antigen expression.

L1 antigen is involved in adhesion among neural cell bodies since Fab fragments of polyclonal L1 antibodies inhibit a short-term Ca²⁺-independent re-aggregation mechanism of early post-natal cerebellar and C1300 neuroblastoma cells. Specificity of antibody-mediated interference with adhesion is indicated by the observation that polyclonal, but not monoclonal antibodies are effective. Both antibodies react with L1 antigen, but presumably cover distinct sites or a site on the molecule, which in the case of the monoclonal antibody we used is not involved in adhesion.

Our results indicate that the Ca²⁺-independent adhesion mediated by L1 antigen is common to both normal cerebellar cells and a clonal cell line derived from mouse neuroblastoma C1300, a tumor of the peripheral nervous system (Augusti-Tocco and Sato, 1969; Nelson *et al.*, 1969; Schubert *et al.*, 1969). Only a partial inhibition of aggregation (22–30%) among cultivated early post-natal cerebellar cells is obtained. This incomplete inhibition could be a result of cellular heterogeneity in L1 antigen expression. (Only 40–50% of the obtained cerebellar cells do express L1 antigen.) A more substantial inhibition of aggregate formation (between 60 and 86%) is seen using neuroblastoma N2A; a cell population homogenous in L1 antigen expression.

The incomplete inhibition of aggregation of cerebellar cells might also be due to the existence of other adhesion mechanisms among L1 antigen-positive cerebellar cells

prepared at low trypsin concentrations in the presence of EDTA. Differences in adhesive properties exist among cerebellar cells obtained by different procedures (Fischer and Schachner, 1982). Even among early post-natal cerebellar cells prepared under the conditions of this study, a considerable degree of heterogeneity is evident. These cells consist of several populations of neuronal and glial cell types at different developmental stages. *In vitro*, L1 antigen is detectable in the cerebellum on neurons only. After dissociation of the tissue only L1 antigen-negative cells are obtained. However, after 18 h *in vitro* 40–50% of these cells express L1 antigen which *in situ* is not detectable on the precursors of neurons in the proliferative zone of the external granular layer. It is not known which features in the molecular and cellular heterogeneity of the developing mouse cerebellum lead to the apparent heterogeneity in re-aggregation experiments.

Two types of adhesion systems have been postulated for fibroblasts and neural cells, which differ in their sensitivity to trypsin and in their requirement for Ca^{2+} . These have been designated Ca^{2+} -dependent and -independent mechanisms and seem to be mediated by different molecules (Takeichi *et al.*, 1979; Magnani *et al.*, 1981; Brackenbury *et al.*, 1981; Fischer and Schachner, 1982). N-CAM is involved in a Ca^{2+} -independent adhesion mechanism (Thiery *et al.*, 1977; Hoffman *et al.*, 1982; Chuong *et al.*, 1982; McClain and Edelman, 1982). D2 glycoprotein is involved in the fasciculation of neurites (Jorgensen *et al.*, 1980) as is N-CAM (Rutishauser *et al.*, 1978). An influence of Fab fragments of polyclonal L1 antibody on fasciculation has not yet been demonstrable (unpublished observations), but an interference in a more complex cell interaction system, the translocation of granule cells in the cerebellar cortex has been described (Lindner *et al.*, 1983).

If L1 antigen functions as a ligand in cell-cell adhesion and if the corresponding receptor (or receptors) is also localized on neurons, then L1 antigen could be responsible for adhesion between neuronal cells only, and not between neurons and glia. It is conceivable that L1 antigen mediates adhesion among neurons in the form of a 'self-aggregation', as postulated for N-CAM (Rutishauser *et al.*, 1982), and hence contributes to the sorting out of post-mitotic pre-migratory neurons from the proliferative zone in the external granular layer of the cerebellum. Alternatively, L1 antigen might mediate adhesion of neurons to other cell types carrying different receptors. This mode of interaction may lead to an association between post-mitotic pre-migratory neurons with glial processes which have been postulated to guide migrating granule cell neurons (Rakic and Sidman, 1973). Interactions of L1 antigen with components of the extracellular matrix are also conceivable and might involve distinct functional sites on the L1 molecule, as is the case with fibronectin (Colonna *et al.*, Fukuda and Hakomori, 1979; Wagner and Hynes, 1979; Ruoslahti *et al.*, 1979; Balian *et al.*, 1980; Hahn and Yamada, 1979; Sekiguchi and Hakomori, 1980). We now need a detailed chemical characterization of the two molecular species of L1 antigen and their cellular receptors.

Materials and methods

Preparation of monoclonal L1 antibody

Female Sprague-Dawley rats (4–6 weeks old) were immunized with 80 μg of a glycoprotein fraction from a solubilized enriched membrane fraction from early post-natal mouse cerebellum isolated by affinity chromatography with lens lentil lectin (see below) in complete Freund's adjuvant by s.c. and i.p. in-

jection. All consecutive immunizations were carried out in incomplete Freund's adjuvant. Splenocytes of animals with high titers were chosen for fusion with the rat myeloma clone Y3-Ag 1.2.3 (Galfre *et al.*, 1979) according to De St. Groth and Scheidegger (1980). Fusions were first screened by the modified enzyme-linked immunosorbent assay (ELISA) immunobinding test (Hawkes *et al.*, 1982). Positive hybridoma supernatants were then screened immediately for cell surface reactivity on viable monolayer cultures of early post-natal mouse cerebellar cells cultured on multitest slides (Flow Lab., Bonn, FRG) by indirect immunofluorescence (Schnitzer and Schachner, 1981) using biotin-conjugated rabbit anti-rat antibodies (Vector Laboratories, Burlingame, USA) which were visualized by fluorescein-conjugated avidin (Sigma, St. Louis, USA). Cloning of hybridoma cells was performed according to Lagenaur *et al.* (1980).

Preparation of polyclonal L1 antibodies

Polyclonal antibodies to immunoaffinity-purified L1 antigen (see below) were prepared in New Zealand white rabbits by i.p. and s.c. injections. For the first injection, 20 μg of L1 antigen was emulsified in complete Freund's adjuvant. All consecutive immunizations were carried out in incomplete Freund's adjuvant at fortnightly intervals.

Preparation of Fab fragments of monoclonal and polyclonal antibodies

IgG fraction from rabbit anti-L1 serum and serum from naive rabbits was obtained according to Fahey and Terry (1979). Fab fragments of polyclonal and monoclonal IgG fractions were prepared with mercuripapain (Sigma) according to Porter (1959).

Preparation of an enriched cerebellar membrane fraction and isolation of glycoproteins

An enriched membrane fraction from 8- to 10-day-old C57BL/6J mice was prepared by a polyethylene glycol-dextran system according to a modification of the procedures of Brunette and Till (1971) and Stuhlfauth and Seeds (1976) as described in detail by Rathjen (1982). Lentil lectin was purified from lens culinaris seeds according to Kinzel *et al.* (1976). Affinity chromatography using lens lentil lectin coupled to Affi-Gel 10 (Bio-Rad, Munich, FRG) was carried out according to Lotan *et al.* (1977) and Hayman and Crumpton (1972).

Isolation of L1 antigen by immunoaffinity chromatography

L1 antibody purified from ascites was coupled to Affi-Gel 10 (Bio-Rad, Munich, FRG) at a concentration of 3.3 mg protein per ml of gel matrix. Similarly, serum proteins from non-immune rats obtained by precipitation with 50% ammonium sulphate were coupled to Affi-Gel 10. For isolation of L1 antigen, crude membranes were prepared from brains of 8- to 10-day-old C57BL/6J mice and solubilized in a buffer containing 0.5% NP-40, 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA and 40 U aprotinin/ml, pH 7.2. Protein concentrations were adjusted to 1 mg/ml. Non-solubilized material was removed by centrifugation at 100 000 g and 4°C for 1 h. The supernatant was added first to the column containing immobilized rat serum proteins. The flow-through of this column was connected directly to the L1 antibody-containing column (8 ml of gel matrix volume). The column was then washed with 30 column volumes of solubilization buffer, subsequently treated with 10 volumes of solubilization buffer with elevated NaCl concentration (0.3 M), and finally washed with solubilization buffer containing NaCl at a concentration of 0.15 M. Bound material was eluted with an alkaline buffer containing 0.1 M diethylamine, 0.5% NP-40, 1 mM EDTA, pH 11.5. Two column volumes were collected and quickly neutralized by addition of 0.5 M Tris buffer, pH 6.8.

Analytical procedures

SDS-PAGE analysis was performed with linear (6–15%) or without (8%) acrylamide concentration gradients in slab gels according to Laemmli (1970). Staining of gels by reduction of silver ions was carried out according to Oakley *et al.* (1980). The following proteins served as mol. wt. markers (in daltons): aldolase (40 000; Ald), ovalbumin (45 000; Ova), catalase (60 000; Cat), bovine serum albumin (66 200; BSA), phosphorylase B (92 500; Phosph), β -galactosidase (116 250; β -Gal) and myosin (200 000; Myo). For fluorography, tritium-labeled proteins were visualized with EN-³HANCE (New England Nuclear, Dreieich, FRG). Protein determinations were carried out according to Lowry *et al.* (1951) or with a micromethod according to Neuhoff *et al.* (1979). BSA (Sigma) served as standard.

Proteins separated by SDS-PAGE were transferred onto nitrocellulose filters (0.45 μm pore size, type HA, Millipore, Neu-Isenburg, FRG) by electrophoresis (9 V/cm, 16 h) according to Towbin *et al.* (1979). The nitrocellulose filter was cut into strips, and incubated with a protein-containing buffer to saturate all free binding sites [3% (w/v) BSA, 10% (v/v) horse serum, 50 mM Tris, 150 mM NaCl, pH 7.4]. IgG fractions of polyclonal L1 rabbit antibodies were then added to the strips at concentrations of 8–50 $\mu\text{g}/\text{ml}$ in the saturation buffer for 16 h at 4°C. Washing procedures, incubation with HRP-coupled goat anti-rat IgG (Miles) fraction and visualization of enzyme activity with the substrate 5-chloronaphthol are described in

detail for the ELISA immunobinding test (Hawkes *et al.*, 1982).

Biosynthetic labeling of glycoproteins in cell culture

Cultures of cerebellar cells ($1.4-3.8 \times 10^7$ cells/10 ml culture medium in a Petri dish, 10 cm in diameter) and neuroblastoma clone N2A were grown in culture medium for 48 h in the presence of $10 \mu\text{Ci}$ L-[6- ^3H]fucose (27 Ci/mmol, Amersham, Braunschweig, FRG) per ml. For isolation of L1 antigen, immunoaffinity chromatography with monoclonal L1 antibody was carried out as described above for isolation of L1 antigen from brain tissue. Pasteur pipettes filled with $\sim 200 \mu\text{l}$ of gel matrix material were used. In some cases, immunoaffinity chromatography was carried out in solubilisation buffer to which $50 \mu\text{M}$ pepstatin, $50 \mu\text{M}$ leupeptin (Sigma, St. Louis, USA) and $500 \mu\text{M}$ PMSF (Serva, Heidelberg, FRG) had been added.

Cells and cell culture

Monolayer cultures of trypsin-dissociated cerebella from embryonic and early post-natal mice were prepared and maintained as described in detail by Schnitzer and Schachner (1981).

The following neural tumor cell lines were obtained and maintained *in vitro*: neuroblastoma C1300 clone N2A, and clone NB41A3 were a gift from Dr. K. Reuss (Merck, Darmstadt, FRG). Clone N2A was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum. Clone NB41A3 was maintained in DMEM supplemented with 15% horse serum and 2.5% fetal calf serum. The murine medullo-epithelioma UAL-1 (Mukai *et al.*, 1974) was a gift of Dr. W. Doerfler (University of Cologne, Cologne, FRG) and maintained in the same culture medium as clone NB41A3. The neuroblastoma-glioma hybrid cell line 108-CC15 was a gift of Dr. B. Hamprecht (University of Würzburg, Würzburg, FRG) and maintained in DMEM supplemented with 10% fetal calf serum and addition of HAT medium selective components (Hamprecht, 1977). The rat neuroblastoma clones B103 and B104 were obtained from Dr. W. Seifert (Max-Planck-Institut für Biophysikalische Chemie, Göttingen, FRG) and maintained in DMEM supplemented with 10% fetal calf serum.

Immunocytological procedures

Indirect immunofluorescence on viable and paraformaldehyde-acetone treated cell cultures was performed as described by Schnitzer and Schachner (1981). Cryostat sections from fresh frozen tissue were stained by indirect immunofluorescence according to Goridis *et al.* (1978a). Double immunofluorescence procedures were carried out (Schnitzer and Schachner, 1981) using rhodamine- and fluorescein-conjugated goat anti-rat immunoglobulins (Cappel, from Dynatech, Denkendorf, and Paesel, Frankfurt, FRG). Directly tetramethylrhodamine-conjugated monoclonal antibody to O4 antigen (Sommer and Schachner, 1982) was a gift from I. Sommer. Tetanus toxin was a gift from Dr. E. Habermann (University of Giessen, FRG). A rabbit antiserum to glial fibrillary acidic protein was obtained from Dr. E. Bock (University of Copenhagen, Copenhagen, Denmark). Fluorescein- and rhodamine-conjugated goat anti-rabbit immunoglobulins were obtained from Miles (Frankfurt, FRG).

Re-aggregation of dissociated neural cells in vitro

Re-aggregation of single cells in suspension was performed according to Fischer and Schachner (1982) with some modifications. Monolayer cultures of cerebella from 6-day-old C57BL/6J mice were maintained *in vitro* for 18–24 h (Schnitzer and Schachner, 1981). For investigation of the Ca^{2+} -independent aggregation mechanism, monolayer cultures were washed twice in calcium-magnesium-free Hank's balanced salt solution (CMF-HBSS) and detached from the poly-L-lysine coated plastic substrate with trypsin ($20 \mu\text{g}/\text{ml}$, Worthington, code TRL, via Flow Laboratories, Bonn, FRG) and DNase I ($20 \mu\text{g}/\text{ml}$, Worthington, code DP) in CMF-HBSS containing 1 mM EDTA for 15 min at room temperature. Trypsin was inactivated by soybean trypsin inhibitor (Serva, Heidelberg, FRG) at a final concentration of $40 \mu\text{g}/\text{ml}$. Cells were detached from the culture plate by pipetting. The cell suspension was washed twice and resuspended in CMF-HBSS. Cells were then resuspended at a concentration of $2.5 \times 10^5/\text{ml}$ in 1 ml CMF-HBSS containing 10 mM Hepes (Serva, Heidelberg, FRG) and 0.002% DNase I and rotated in small Erlenmeyer flasks (Bellco, Hüttenberg, FRG), at 85 r.p.m. at 37°C in a shaking water bath (Infors HT, Basel, Switzerland or New Brunswick Co., USA). Particle number was counted in a Coulter Counter (Model TA II or Model ZF with a $200 \mu\text{m}$ capillary, Coulter electronics GmbH, Krefeld, FRG). Neuroblastoma clone N2A was detached from culture flasks by trypsin as described for cerebellar cells. Aggregation of N2A cells was carried out at a concentration of 8×10^5 cells/ml and a rotation speed of 65 r.p.m. Aggregation in the presence of Fab fragments was carried out at various concentrations. Before aggregation, cells were pre-incubated with Fab fragments for 20 min on ice.

Acknowledgements

The authors are grateful to M. Schleicher for technical assistance, to Drs. E. Bock, W. Doerfler, E. Habermann, B. Hamprecht, K. Reuss, W. Seifert and I. Sommer for gifts of antibodies, toxin and cells, and to Drs. A. Faissner, G. Fischer, C. Lagenauer and R. Mailhammer for their comments on the manuscript. This work was supported by Deutsche Forschungsgemeinschaft (Scha 185/6-4) and Studienstiftung des Deutschen Volkes.

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Received on 11 March 1983; revised on 5 October 1983