Oligodendrocyte Cell Adhesion Molecules Are Related to Neural Cell Adhesion Molecule (N-CAM)

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The glycoproteins responsible for calcium-dependent oligodendrocyte aggregation were purified and characterized. Using detergent extraction, lentil-lectin-Sepharose 4B affinity chromatography, and preparative gel electrophoresis, 3 proteins were purified to apparent homogeneity, with relative M_r s of 120,000, 140,000, and 180,000. The aggregation assay showed that all 3 proteins had the ability to block antibody-mediated inhibition of oligodendrocyte aggregation. The 120,000 protein was the most active of the three. Antisera were raised in rabbits to these 3 individual proteins. Western blot analyses showed that all three antisera recognized 120,000, 140,000, and 180,000 proteins, which indicated that the proteins were related. Western blot analyses of cultured oligodendrocytes and purified rat myelin showed only the 120,000 protein. Immunoprecipitation of iodinated membrane proteins of cultured oligodendrocytes also indicated the presence of only the 120,000 M_r protein. Deglycosylation of the 120,000 protein by *N*-glycanase resulted in a 110,000 protein. The immunoblot pattern suggested some similarities between oligodendrocyte adhesion molecules and the neural cell adhesion molecule (N-CAM). Therefore, the 120,000, 140,000, and 180,000 M_r proteins were compared to N-CAM by Western-blot analysis, immunofluorescence staining, and by immunoprecipitation. The results suggest that oligodendrocytes contain a 120,000 membrane glycoprotein that is related to N-CAM.

The glycoproteins responsible for adhesion between neurons have been studied independently under the names N-CAM (neural cell adhesion molecules) (Edelman et al., 1983; Rutishauser, 1983), D2-CAM (Jorgensen et al., 1980), and BSP-2 (Goridis et al., 1983; Hirn et al., 1983). It has been suggested that neuronal-glia adhesion involves another type of adhesion molecule—NgCAM (Edelman, 1984; Grumet et al., 1983, 1984). It was reported earlier that glia do not express N-CAM/D2-CAM *in vitro* (Grumet et al., 1983, 1984; Hirn et al., 1981). However, recently, Noble et al. (1985) and Keilhauer et al. (1985) have shown that glial cells express N-CAM/D2-CAM *in vitro*. We have reported that oligodendrocytes (OL) have cell adhesion molecules, which are responsible for OL aggregation (Bhat and Silberberg, 1985). Here we report on the purification and characterization of an oligodendrocyte cell adhesion molecule (OL-CAM), which is closely related to one of the N-CAM family of glycoproteins.

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Materials and Methods

OL aggregation assay

The immunological assay of OL aggregation was as described previously (Bhat and Silberberg, 1985). OL (2×10^5 cells) in 10 μ l PBS were added to tubes that contained 40 μ l of cold PBS, plus a solution of antibody or a mixture of antibody and the protein fractions to be assayed, which had previously been incubated together for 20 min at room temperature. After 30 min at 4°C, the cells were diluted to 100 μ l with cold Dulbecco's modified Eagle's medium (DME) containing 20 μ g/ml DNase, 1% fetal bovine serum, and 10 mM HEPES, mixed vigorously, placed in a capped tube, and rotated in a shaker at 100 rpm at 37°C. After 0 and 30 min of incubation, aliquots were removed and unaggregated single cells were counted, using a hemocytometer (Bhat and Silberberg, 1985).

Preparation of membrane extracts of rat brain and cultured OL

Rat brains or cultured OL were homogenized in PBS containing 0.3 mM phenylmethyl sulfonyl fluoride (PMSF; Sigma) and centrifuged at $1000 \times g$ for 5 min to remove the nuclei and cell debris. The supernatant was spun at $15,000 \times g$ for 20 min. The pellet was suspended in PBS containing 0.3 mM PMSF, 0.5% NP40 (NP40 buffer), and incubated overnight at 4°C. The suspension was centrifuged at $100,000 \times g$ for 90 min. This supernatant is NP40 extract.

Purification of OL-CAM

Lentil lectin Sepharose 4B (Sigma) was equilibrated with NP40 buffer and the gel (50 ml) was mixed with 50 ml of NP40 extract for 2 hr at 4°C. The supernatant was removed and the gel was packed in a column. The column was washed with 500 ml NP40 buffer and eluted with 0.5 M α -methylmannoside (Sigma) in NP40 buffer. The eluted material was dialyzed against PBS and concentrated by Diaflo-concentrating system (Amicon) using 50,000 M_r cut-off membranes. The concentrated glycoproteins were then precipitated by acetone. The concentrated material was incubated with 6 vol of acetone at -20°C for 16 hr. After centrifugation at $8000 \times g$ for 30 min, the pellet was dissolved in sample buffer for preparative gel electrophoresis. For aggregation assay, the precipitates were washed 2 times with PBS to remove traces of NP40 and SDS. For preparative SDS-PAGE, 3-mm-thick, 7.5% separating and 5% stacking gels were used (Laemmli, 1970). After electrophoresis (10 mA, 16 hr), the gel was cut into thin (3 mm) slices in the region of 95,000–200,000 M_r , minced, and eluted with 3 ml of NP40 buffer for 3 d at 4°C. The eluted proteins were tested for activity by immunoblot (see below) and aggregation assay. The purity of the fractions was checked by SDS-PAGE. If more than 1 protein band was observed, the eluted protein was re-electrophoresed using 1.5 mm SDS-PAGE, and re-eluted as above. The proteins exhibiting neutralizing activity in the above aggregation assay were the oligodendrocyte cell adhesion molecules (OL-CAM).

Antibody preparation

Production of antibodies to OL-conditioned medium was as described (Bhat and Silberberg, 1985). In order to raise antibodies to individual polypeptides, the 120,000, 140,000, and 180,000 polypeptides were eluted from the preparative gels and precipitated by acetone. About 50 μ g of acetone-precipitated proteins were suspended in 250 μ l PBS, mixed with 250 μ l Freund's complete adjuvant, and injected into rabbits 3 times at 2 week intervals, and boosted with 100 μ g protein in PBS alone.

Figure 1. Western-blot analysis of OL-CAM. Lane 1, 120,000 (5 μ g); lane 2, 140,000 (5 μ g); lane 3, 180,000 (5 μ g); lane 4, lentil lectin elute (50 μ g); lane 5, NP40 extract (100 μ g). Proteins were resolved by SDS-PAGE and then electrophoretically transferred to nitrocellulose paper. Proteins were immunologically stained using anti-conditioned medium (1:5000 dilution) and HRP-conjugated IgG (HRP-IgG) (1:3000 dilution).

One week after the booster injections, the rabbits were bled and sera were separated. The preparation of immunoglobulins and Fab fragments was as previously described (Bhat and Silberberg, 1985).

Cell culture and OL isolation

Preparation and maintenance of both primary mixed cultures of fetal rat brain and rat OL were as described previously (Bhat and Silberberg, 1985).

SDS-PAGE and immunoblot

SDS-PAGE (Laemmli, 1970) was carried out with 1.5-mm-thick, 7.5% separating and 5% stacking gels. After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose paper (Bio-rad) (Towbin et al., 1979). Immunostaining was performed using appropriate antisera and an immunoblot kit (Bio-rad) as suggested by the supplier.

Table 1. Purification of OL-CAM from 150 3-week-old rat brain membranes

Fraction	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (n-fold)
NP40 extract	750	12,750	17	100	1
Lentil lectin elute	45	10,710	238	84	14
120,000	0.24	1380	5750		
140,000	0.36	785	2175		
180,000	0.48	990	2063		
120,000, 140,000, and 180,000 combined	1.08	3155	2921	25	172

The amount of OL-CAM activity in a sample was estimated by its ability to neutralize the aggregation-blocking ability of Fab, prepared from the sera of rabbits immunized with OL-conditioned medium. One unit of OL-CAM activity is defined as the amount of OL-CAM required to reduce the aggregation by 25%. OL-CAM was purified from 3-week-old rat brain membranes by NP40 extraction, followed by lentil-lectin affinity chromatography and preparative SDS-PAGE (see Materials and Methods for details).

Iodination and immunoprecipitation

Iodination of the NP40-extracted proteins (250 μ g) was performed using 25 μ Ci 125 I Bolton-Hunter reagent (ICN Biochemicals) as described (Bolton and Hunter, 1973). Immunoprecipitation was as described by Dobberstein et al. (1979). After immunoprecipitation, the slurry was boiled for 10 min with sample buffer. After electrophoresis, the gel was autoradiographed using Kodak X-O-Mat film.

Myelin

Isolation of myelin fractions from adult rat brains was as described by Norton and Poduslo (1973), using sucrose density gradient centrifugation.

N-Glycanase cleavage of OL-CAM

Membranes (100 μ g protein) from enriched cultures of OL were solubilized with 0.5% SDS and 0.1 M β -mercaptoethanol and boiled for 3 min. The sample was diluted with sodium phosphate buffer (pH 8.6), EDTA and NP40. N-Glycanase (peptide: N-glycosidase F) (Genzyme) was then added and the reaction mixture incubated overnight at 30°C. The final concentrations were 0.17% SDS, 0.02 M β -mercaptoethanol, 0.2 M sodium phosphate, 5 mM EDTA, 1.25% NP40, and 10 U/ml N-glycanase, in a total volume of 30 μ l. Controls were treated similarly, except for the N-glycanase. The reaction was terminated by boiling for 3 min, and analyzed by Western blot.

Protein estimation was done by the method of Lowry et al. (1951).

Results

Purification of OL-CAM

Earlier, we reported that rat OL have cell adhesion molecules (Bhat and Silberberg, 1985). Antibodies raised to OL-conditioned medium inhibited the aggregation of OL, which was used to quantitate the OL-CAM. Immunoblot analysis of OL-conditioned medium showed many protein bands. When OL membranes were analyzed by immunoblot, about 6 bands were observed (data not shown) (later, it was found that most of these bands were serum components). However, the NP40 extract of 3-week-old rat brain membranes showed only 3 bands, with apparent M_s of 180,000, 140,000, and 120,000. Lentil lectin Sepharose 4B was used to purify the molecules responsible for OL aggregation. As is shown in Table 1, the molecules bound to lentil lectin had OL-CAM activity. Analysis with SDS-PAGE showed many bands, but Western-blot analysis showed only 3 bands. The aggregation assay showed that, at this stage, there was a 14-fold purification of OL-CAM activity. The lentil-lectin-bound glycoproteins were used to purify OL-CAM by means

Figure 2. SDS-PAGE of OL-CAM. Lane 1, Lentil lectin elute (100 μ g); lane 2, 180,000 (15 μ g); lane 4, 140,000 (20 μ g); lane 6, 120,000 (20 μ g); lanes 3 and 5, fractionated proteins (15 μ g) that did not have OL-CAM activity. Proteins were resolved by 7.5% separating gel and staining with Coomassie blue.

of preparative SDS-PAGE. The eluted proteins were analyzed by aggregation assay (Table 1) and by Western blot (Fig. 1). Of the 10 fractions in the region of 95,000–200,000, only 3 had OL-CAM activity, with apparent M_r s of 120,000, 140,000, and 180,000. On SDS-PAGE analysis, these 3 fractions showed only 1 band each with Coomassie blue or silver stain (Fig. 2). The aggregation assay showed about 172-fold purification. It is of interest to note that the 120,000 protein had the highest OL-CAM activity, as compared to those of 140,000 and 180,000.

Immunoblot and immunoprecipitation analysis of rat brain membranes, myelin, and membranes from cultured OL using anti-OL-CAM

Membranes from rat brain and cultured OL (about 90% enriched) were solubilized by 1.0% NP40 and analyzed by immunoblot (Fig. 3) and SDS-PAGE after 125 I iodination and immunoprecipitation (Fig. 4). As is shown in Figure 3, antibodies raised to individual bands reacted with all 3 proteins, which is comparable with anti-OL-conditioned medium and anti-N-CAM (see below). However, cultured OL had only 1 band, with an apparent M_r of 120,000. Highly purified rat myelin had only a 120,000 band (Fig. 3).

N-Glycanase cleavage of OL-CAM

Membranes from enriched cultures of OL were treated with N-glycanase. N-Glycanase hydrolyzes N-asparagine-linked ol-

Figure 3. Western-blot analysis of rat brain, myelin and cultured OL membranes using anti-N-CAM and anti-OL-CAM sera. NP40 extract (lanes 1, 4, 8, 9; 100 μ g), myelin (lanes 3, 6; 100 μ g) and OL membranes (lanes 2, 5, 7, 10; 50 μ g) were resolved by SDS-PAGE. The proteins were then electrophoretically transferred to nitrocellulose paper, followed by immunostaining using anti-120,000 (1:2000; lanes 1–3) anti-N-CAM-IgG (0.05 mg/ml; lanes 4–6), anti-180,000 (1:2000; lanes 7, 8), and anti-140,000 (1:2000; lanes 9, 10).

igosaccharides from glycoproteins, yielding free oligosaccharide and a protein containing aspartic acid at the glycosylation site (Elder and Alexander, 1982). As is shown in Figure 5, treatment with N-glycanase gave a protein with an apparent molecular weight of 110,000.

N-CAM and OL-CAM are identical or closely related proteins

In the adult rat or mouse brain, the N-CAM reportedly has 3 separate but related proteins, with apparent M_r s of 120,000, 140,000, and 180,000 (Edelman and Choung, 1982; Hirn et al., 1981; Rasmussen et al., 1982). Therefore, we wanted to compare N-CAM with OL-CAM by using antibodies raised to purified N-CAMs. As is shown in Figures 6 and 3, both anti-N-CAM and anti-conditioned medium stained identical bands in both the NP40 extract and purified proteins. Immunoprecipitation using anti-N-CAM and anti-conditioned medium showed identical bands (Fig. 4). Western-blot analysis of cultured OL membrane proteins with anti-N-CAM showed only a 120,000 protein (Fig. 3).

Immunofluorescence analysis of cultured OL using anti-N-CAM and anti-OL-CAM

Four days after isolation and culturing, OL were stained by various antisera (Fig. 7 and Table 2). OL were identified using antibodies to cell-specific surface markers such as "04," "01," and galactosyl-cerebroside (GalC). Anti-GalC (Raff et al., 1978) and "01" (Singh and Pfeiffer, 1985; Sommer and Schachner, 1981) antibodies stain well-differentiated OL, whereas "04" stains developing OL (Sommer and Schachner, 1982). In some experiments, we used monoclonal anti-GalC, while in others the monoclonal "01" antibody was used. In both cases, the results were similar. We report here only the experiments using

Figure 4. Immunoprecipitation of OL-CAM from rat brain and cultured OL membranes. NP40 extracts of rat brain (lanes 2, 4, 6, 8) and OL (lanes 1, 3, 5, 7) were radio-iodinated. ^{125}I NP40 extract proteins (30 μg) were immunoprecipitated with anti-120,000 (4 μl ; lanes 1, 2), anti-140,000 (4 μl ; lanes 3, 4), anti-180,000 (4 μl ; lanes 5, 6), and anti-N-CAM-IgG (40 μg ; lanes 7, 8). The immunoprecipitates were dissolved in sampler buffer, boiled for 10 min, and resolved by SDS-PAGE. Gels were dried and fixed for autoradiography on Kodak X-O-mat film at $\sim 70^\circ\text{C}$.

"01" antibody. Nearly all of the "04"-positive cells were positive for either anti-N-CAM or anti-OL-CAM (Table 2, Fig. 7). More than 99% of the "01"-positive cells were positive for N-CAM, and more than 80% of the "01" cells were positive for anti-OL-CAM (Fig. 7 and Table 2).

Ca²⁺ dependency of OL aggregation

OL were assayed for aggregation in the presence and absence of Ca^{2+} . As is shown in Table 3, the aggregation of OL is dependent on the presence of Ca^{2+} . This is in contrast to neuronal aggregation brought about by N-CAM (Edelman et al., 1983).

Discussion

Our earlier studies have indicated the presence of cell adhesion molecules in oligodendrocytes (Bhat and Silberberg, 1985). The glycoproteins responsible for Ca^{2+} -dependent OL aggregation were purified from rat brain membranes by using detergent extraction, lentil-lectin affinity chromatography, and preparative SDS-PAGE. Three glycoproteins (120,000, 140,000, and 180,000) had OL-CAM activity. Only the 120,000 protein of myelin and cultured OL had OL-CAM activity, which suggests that the 120,000 glycoprotein is responsible for OL aggregation. Preliminary studies indicated some similarities between N-CAM and OL-CAM. Therefore, OL-CAM was compared with N-CAM.

Table 2. Percentage of 04+ or 01+ cells that are positive for N-CAM or OL-CAM 4 d after OL isolation

Antisera	Positive cells (%)
04/N-CAM	100
01/N-CAM	99
04/120	98
01/120	92
04/140	97
01/140	80
04/180	97
01/180	82

After isolation, OL were plated on glass coverslips. Four days after, cells were stained using various antisera. After immunostaining, labeled cells were counted using an epifluorescence microscope Carl Zeiss with a 63 $\times$ objective lens. Positive cells in 10 fields were counted and the percentage of 04+/N-CAM+ or OL-CAM+, and 01+/N-CAM+ or OL-CAM+ were calculated. Values are means from 4 separate experiments.

The results indicate that OL-CAM is closely related to one of the (120,000) N-CAM family of glycoproteins.

Treatment of OL membranes with *N*-glycanase, which hydrolyzes *N*-asparagine-linked oligosaccharides from glycoproteins, gave a 110,000 protein (Fig. 5). This is in agreement with the primary translation products D2-CAM mRNAs, one of which has an apparent M_r of 112,000 (Hansen et al., 1985).

N-CAM/D2-CAM is expressed in multiple forms that differ in their carbohydrate composition (Edelman et al., 1983; Goridis et al., 1983) and in the size of cytoplasmic domains (Gennarini et al., 1984), but are thought to be otherwise identical (Gennarini et al., 1984; Hoffman et al., 1982). Each of these molecular-weight classes may consist of several closely similar, but distinct, polypeptides. This possibility gains some support from the finding that N-CAMs from different brain regions can be distinguished serologically (Chuong and Edelman, 1984) and that each of these polypeptides may be derived from individual mRNAs (Hansen et al., 1985). In postembryonic brain, the 3 dominant forms of these molecules have relative M_r s of 120,000, 140,000, and 180,000 (Edelman and Choung, 1982; Hirn et al., 1981; Rasmussen et al., 1982). Glial cells reportedly do not express N-CAM/D2-CAM *in vitro* (Grumet et al., 1983, 1984; Hirn et al., 1981). However, recently Noble et al. (1985) and Keilhauer et al. (1985) have shown that all classes of macroglia express N-CAM/D2-CAM-like polypeptides *in vitro*. In addition to neurons, the presence of N-CAM in glial cells *in vivo* has been reported (Silver and Rutishauser, 1984).

With these studies as background, we compared N-CAM with OL-CAM. Three types of assays were used to compare OL-CAM with N-CAM: (1) Western-blot analysis, in which antibodies react with the denatured protein; (2) immunoprecipita-

Table 3. Effect of Ca^{2+} on OL aggregation

Additions	Cells aggregated at 30 min (%)
(a) EDTA	48 $\pm$ 4
(b) EDTA + Ca^{2+}	88 $\pm$ 1

OL were isolated from 10-d-old primary mixed cultures of fetal rat brain. Cells were washed 2 times with Ca^{2+} - Mg^{2+} free Hanks' buffer and incubated in Hanks' balanced salt solution containing 20 μg DNase/ml, 1% fetal bovine serum, and 10 mM HEPES. (a) 1 mM EDTA and (b) 1 mM EDTA and 2 mM CaCl_2 were added to incubation medium before assaying for aggregation. Values are mean $\pm$ SD from 4 experiments.

Figure 5. N-Glycanase cleavage of OL-CAM. Western-blot analysis of N-glycanase-treated OL membranes using anti-120,000 (1:3000 dilution). See Materials and Methods for detailed procedure. 1, N-glycanase-treated; 2, control.

tion analysis, in which antibodies react with the native protein; and (3) immunofluorescence staining of cultured cells, in which antibodies react with accessible surface molecules. Western-blot analysis of NP40 extract of rat brain membranes and purified OL-CAM from rat brain showed identical patterns with anti-N-CAM- and anti-OL-conditioned medium, and anti-120,000, anti-140,000, or anti-180,000 polypeptides. Immunoprecipitation using anti-N-CAM or anti-OL-CAM showed identical bands. Immunofluorescence analysis of the cultured OL also gave comparable results. These results suggest that N-CAM and OL-CAM are identical or related proteins. However, there is one major difference: OL-CAM requires Ca^{2+} for OL aggregation, while N-CAM does not require Ca^{2+} for neuronal aggregation.

Intercellular adhesion might occur via 2 distinct mechanisms, distinguished by *in vitro* aggregation assay: Ca^{2+} -dependent and -independent (see review by Damsky et al., 1984). Takeichi et al. (1981) have observed Ca^{2+} -dependent and -independent aggregation with a multipotent EC cell line, AT805, and with nullipotent F9EC cells. Thomas and Steinberg (1981) demonstrated the existence of Ca^{2+} -dependent and -independent adhesion mechanisms in neural retinal cells. The studies done on the involvement of Ca^{2+} in N-CAM used chicken N-CAMs (Brackenbury et al., 1981). It is believed that rodent N-CAM also exhibits Ca^{2+} -independent adhesion mechanisms. Moreover, adult chicken N-CAM has very little 120,000 protein. Therefore, it is possible that N-CAM may exhibit both Ca^{2+} -dependent and -independent adhesion mechanisms. Also, it is possible that N-CAMs in different cell types may exhibit different requirements for Ca^{2+} . Further studies are needed to substantiate this hypothesis.

The following findings suggest that the aggregation of OL is brought about by the 120,000 protein of the N-CAM family:

Figure 6. Western-blot analysis of OL-CAM using anti-N-CAM. Lanes 1, 2, $M_r = 120,000$ (5 μg); lanes 3, 4, 140,000 (8 μg); lanes 5, 6, 180,000 (5 μg). Proteins in the nitrocellulose paper were stained by using anti-N-CAM-IgG (0.05 mg/ml) and HRP-IgG (1:3000 dilution).

(1) 120,000 has the highest OL-CAM activity (Table 1); (2) Western-blot analysis and immunoprecipitation of iodinated OL membranes showed only 120,000 protein (Figs. 3 and 4); (3) highly purified myelin membranes also showed only 120,000 protein (Fig. 3); (4) Keilhauer et al. (1985) have shown that cultured neurons do not express 120,000, and pulse-chase studies, in cultured rat neuronal cells, of D2-CAMs showed no 120,000 polypeptides (Lyles et al., 1984); (5) the studies of Chuong and Edelman (1984) showed that neuron-rich regions of mouse brain, e.g., hippocampus and olfactory bulb, had very little protein of 120,000 M_r , while, conversely, OL-rich regions such as cerebellum, cerebral cortex, and spinal cord had high levels of 120,000; (6) in explant cultures, 180,000, 140,000, and 120,000 are synthesized *de novo*, and not by modification of the existing high-molecular-weight N-CAMs (Friedlander et al., 1985).

To summarize: We have isolated OL-CAM, which is closely related to one of the N-CAM family of proteins. It will be of

Figure 7. Immunofluorescence staining of cultured OL by anti-N-CAM and anti-OL-CAM. OL were cultured on glass coverslips after isolation. Four days after isolation and culture, the cells were stained by anti-N-CAM-IgG (0.05 mg/ml), anti-120,000 (1:50), "04" (1:100) and "01" (1:100). Rhodamine-conjugated goat anti-rabbit IgG (1:100) and fluorescein-conjugated goat anti-mouse IgG (1:50) were used as second antibodies. *1a* and *3a*, "04"; *2a* and *4a*, "01"; *1b* and *2b*, anti-N-CAM; *3b* and *4b*, anti-120,000. *a*, Fluorescein (428 $\times$); *b*, rhodamine (428 $\times$); and *c*, phase contrast (428 $\times$).

interest to see whether OL-CAM serves as a means of communication between OL and neurons, and OL and astrocytes.

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