A Monoclonal Antibody against Meningococcus Group B Polysaccharides Distinguishes Embryonic from Adult N-CAM

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Abstract. The neural cell adhesion molecules (N-CAM) occur chiefly in two molecular forms that are selectively expressed at various stages of development. Highly sialylated forms prevalent in embryonic and neonatal brain are gradually replaced by less sialylated forms as development proceeds. Here we describe a monoclonal antibody raised against the capsular polysaccharides of meningococcus group B (Men B) which specifically distinguishes embryonic N-CAM from adult N-CAM. This antibody recognizes α2-8-linked N-acetylneuraminic acid units (NeuAcα2-8).

Immunoblot together with immunoprecipitation experiments with cell lines or tissue extracts showed that N-CAM are the major glycoproteins bearing such polysialosyl units. Moreover we could not detect any sialoglycolipid reactive with this antibody in mouse brain or in the neural cell lines examined.

By indirect immunofluorescence staining this anti-Men B antibody decorated cells such as AtT20 (D16/16), which expressed the embryonic forms of N-CAM, but not cells that expressed the adult forms. In primary cultures this antibody allowed us to follow the embryonic-to-adult conversion in individual cells.

In addition, the existence of cross-reactive polysialosyl structures on Men B and N-CAM in embryonic brain calls for caution in efforts to develop immunotherapy against neonatal meningitis.

THE neural cell adhesion molecules (N-CAM)[1] mediate Ca^{++}-independent aggregation of neural cells in vitro and have been shown to play an important role during neuroontogenesis (for reviews see references 4, 13, and 25). They exist in several molecular forms that differ both in their protein structure and carbohydrate content. Isolation of N-CAM from mouse brain at different stages of development shows that the embryonic form possesses an unusually large amount of sialic acid (30% by weight) in a polymeric form with unusual (α2-8) linkages (5). Interestingly, antigenic similarities between brain glycopeptides bearing such polysialosyl units and capsular polysaccharides from bacteria causing meningitis have been noticed (6, 7). The N-CAM undergo a transition perinatally from these highly negatively charged embryonic forms to several adult forms that contained only one-third the amount of sialic acid. Complete removal of sialic acids by neuraminidase treatment generates polypeptide chains closely similar for the adult and embryonic forms of N-CAM purified from avian brain (22). In rodents, the 120,000-D form seems to be prominent in adults (14). The embryonic-to-adult conversion results in altered binding properties of the molecules (15, 27). To demonstrate the functional significance of the different forms of the N-CAM and to understand the molecular mechanisms underlying their interconversions, it is necessary to design models and to generate specific probes that would allow these processes to be monitored in individual cells. Here we report (*a*) a monoclonal antibody raised against the capsule of meningococcus group B (Men B) (NeuAcα2-8)n, which specifically cross-reacts with the embryonic form of N-CAM, (*b*) the potential of this antibody for biochemical and immunohistochemical analysis, and (*c*) the existence of a tumor cell line (AtT20 D16/16) that expresses the embryonic form of N-CAM.

1. *Abbreviations used in this paper*: Men B, meningococcus group B; N-CAM, neural cell adhesion molecules; NeuAc, acetylneuraminic acid; RT, room temperature.

Materials and Methods

Tissues and Cells

Tissues from the nervous system were dissected from 17-d mouse embryos or adult mice for immunoblot experiments and for N-CAM immunopurification. Tissue cultures were made from 13-d mouse embryos and postnatal day 6 rats, respectively.

AtT20 D16/16 cells (26), HT29 (9) and N18, NG108, C6 obtained from Dr. M. Niremberg (National Institutes of Health, Bethesda), were grown in plastic tissue culture flasks (Corning Glass Works, Corning, NY) and passaged weekly. Cells were fed three times a week with Dulbecco's modi-

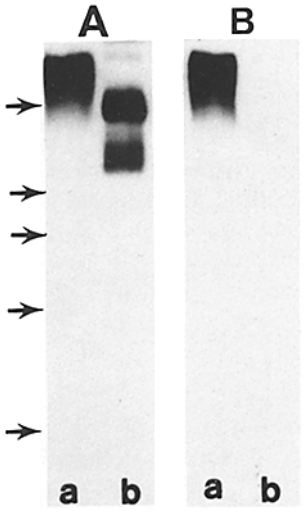

Figure 1. Immunoblot analysis of N-CAM reactivity with P61 and anti-Men B monoclonal antibodies. 10 μg of purified N-CAM from embryonic (lanes *a*) and adult mouse brains (lanes *b*) were run on 7% polyacrylamide gel and transferred to nitrocellulose paper. They were then reacted either with P61 monoclonal antibody (*A*) or anti-Men B antibody (*B*). P61 revealed adult forms at 180,000 and 140,000 D (*A*, lane *b*) that did not react with anti-Men B (*B*, lane *b*). By contrast embryonic N-CAM reacted with both P61 and anti-Men B (lanes *a*) in a molecular range >180,000 D.

fied Eagle's medium (DME; high glucose) supplemented with 10% heat inactivated fetal calf serum and HAT for NG108 hybrid. Media and serum were from Gibco (Grand Island, NY).

Antibodies and Purified N-CAM

The preparation and the specificity of two polyclonal rabbit antisera directed against the NH_2-terminal domain and purified adult mouse N-CAM, respectively, have been described elsewhere (11, 24).

P61, a rat monoclonal antibody recognizing a cytoplasmic determinant expressed by the 180,000- and 140,000-mol-wt polypeptides of adult N-CAM and by embryonic N-CAM, was obtained by fusion of rat spleen cells immunized with embryonic N-CAM and Y3 rat myeloma cells (11).

The anti-Men B antibody was prepared by fusion of spleen cells from a mouse immunized with viable Men B (strain P355) and X63Ag8.653 myeloma cells. Hybridoma clones secreting specific polysaccharide antibodies were identified using a solid phase radioimmunoassay (29). All monoclonal antibodies anti-Men B specific were of the IgM class. In these studies we used the clone termed 2-2B. Thermodynamic binding characteristics and specificity to meningococcal strains have been described elsewhere (18). Ascites fluid containing 5-10 mg of specific antibody/ml were prepared in pristane-primed mice. N-CAM were prepared by immunoaffinity purification from brain membranes as described (23, 24).

Ganglioside Extraction and Immunodetection

Total gangliosides were isolated from tissues after extraction of lipids as described (3). Briefly, ~500 mg (wet wt) of tissues or cells was homogenized in a Dounce homogenizer with 1.5 ml of H_2O at 4°C. The homogenate was added to 5 ml of methanol with constant stirring. 2.5 ml chloroform was added and the mixture was stirred at room temperature (RT) for 90 min. The homogenate was centrifuged at 1,500 *g* for 15 min. The pellet was rehomogenized, reextracted, and the mixture centrifuged as before. The supernatant solutions were combined, evaporated to dryness, and submitted to a Folch partition. The upper phase containing gangliosidic molecules was passed through a column of Sephadex G 25 (Pharmacia, Uppsala, Sweden) to separate lipids from salts and other nonlipid contaminants as described (3). Total gangliosidic extracts from 5 mg of wet weight tissue were chromatographed on high performance thin layer chromatography plates (silica gel 60; E. Merck, Darmstadt, FRG) in chloroform/methanol/0.25% KCl in H_2O (5:4:1). To compare the chemical and immunostaining, two identical series of gangliosidic extracts were chromatographed on the same plate. Gangliosides were either visualized with resorcinol reagent or immunostained with anti-Men B as described previously (17). Briefly, the dried chromatograph was soaked for 30 s in polyisobutyl methacrylate (Polysciences, Inc., Warrington, PA) in hexane, dried, and sprayed with buffer A (0.05 M Tris, 0.15 M NaCl pH 7.8 with 1% bovine serum albumin and 0.1% sodium azide). The plate was then overlaid with the anti-Men B antibody diluted with buffer A (60 μl/cm^2) and incubated for 3 h at RT. The chromatograph was washed by dipping in cold phosphate-buffered saline (PBS) and overlaid with buffer A containing 2 × 10^6 cpm/ml of ^{125}I-labeled goat anti-mouse IgM (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). After 1 h at RT, the chromatograph was washed in cold PBS, dried, and exposed to XAR-5 x-ray film (Eastman Kodak Co., Rochester, NY).

Immunofluorescence Staining

Cells were seeded on poly-L-lysine (5 μg/ml H_2O)-coated glass coverslips and usually examined for staining after 2 d in culture for cell lines or after 6, 14, and 22 d for primary cultures. Live cultures were washed in Hepes-buffered balanced salt solution supplemented with 1% fetal calf serum. Cultures were incubated with the first antibody for 30-60 min at RT at the appropriate dilution in DME plus 10% fetal calf serum (1:5,000 of the ascites fluid for anti-Men B, or 1:1,000 for rabbit antisera). These cultures were washed three times and incubated in appropriate fluorochrome-conjugated second antibodies for 30 min at RT. For double labeling experiments the antibodies were applied sequentially. At the end of the staining procedures cultures were washed three times, fixed at 4°C for 5 min with acetic acid/alcohol (5:95), washed again, mounted in PBS/glycerol (50:50), and examined under a Zeiss fluorescence microscope. IgM subclass anti-Men B antibody was visualized with rhodamine-labeled goat anti-μ chain antibody (1:75 dilution) and rabbit antibodies with fluoresceinated goat anti-rabbit antibody (1:50 dilution; Cappel Laboratories, Cochranville, PA).

Primary Tissue Cultures

Primary cultures of dissociated cells were prepared on poly-L-lysine-coated glass coverslips or plastic tissue culture dishes as described (12, 20). Briefly, structures were dissected out, chopped with fine scissors, and incubated with 0.25% trypsin (Gibco) at 37°C for 10-20 min. Cells were dissociated by trituration through a 21 G needle, harvested by centrifugation, and resuspended in DME with 10% heat inactivated horse serum for spinal cord culture or 10% fetal calf serum and 5% horse serum for cerebral cortex cultures.

Immunoblot

Tissue or cells were extracted in 2% Nonidet P-40 and 20 mM Tris-HCl buffer (pH 7.2) containing protease and neuraminidase inhibitors as described (23). Extracts were made 10 mg/ml protein in the above buffer and boiled for 3 min with an equal volume of electrophoresis sample buffer containing 5.6% SDS and 10% β-mercaptoethanol. They were subjected to electrophoresis in SDS-containing 7 or 10% polyacrylamide gels. The proteins in the gels were blotted onto cellulose nitrate sheets (Schleicher & Schuell, Inc., Keene, NH). Molecular weight marker proteins were blotted along with extracted proteins. The markers were myosin (200,000), galactosidase (120,000), phosphorylase b (92,000), serum albumin (68,000), and ovalbumin (45,000) . Antibody staining was performed as described (19) and bound monoclonal antibody detected by reaction with ^{125}I-labeled rabbit anti-rat IgG or goat anti-mouse IgM (specific for μ chain).

Labeling Procedure and Immunoprecipitation

AtT20 cell monolayers were washed in methionine-free DME and then incubated in this medium supplemented with 10% dialyzed fetal calf serum containing 0.25 mCi/ml [^{35}S]methionine (800 Ci/mmol; Amersham International, Amersham, UK) for 2 h. Then chase medium (DME with 2 mM methionine) was added and the incubation continued for 30 min. The cells were detached with 1 mM EDTA, collected by centrifugation, and lysed in 2% Nonidet P-40 as described for immunoblot procedures. The lysate was clarified by centrifugation (30 min in an airfuge at 20 psi).

Nonspecifically precipitating material was removed before adding specific antiserum by incubating the lysate with normal rabbit serum and protein A coupled to Sepharose beads (Pharmacia). Radiolabeled N-CAM were isolated by immunoprecipitation. Two successive runs were conducted with anti-N-CAM polyclonal antibody (5 μl each). Antigen-antibody complexes were bound to protein A coupled to Sepharose beads. Then, the resulting supernatant was reacted with a preformed complex made of 30 μl goat anti-mouse IgM antibody and 2 μl of anti-Men B ascites fluid. The immunoprecipitates were washed, solubilized, and fractionated on 7% polyacrylamide gels as described (11). The gel was impregnated with EN^{3}HANCE (New England Nuclear, Boston, MA), dried, and exposed for 5 d to RX films with a sensitizing screen.

Results

A Monoclonal Antibody Raised against Men B Specifically Distinguishes Embryonic N-CAM from Adult N-CAM

Embryonic N-CAM exhibit peripheral carbohydrate units containing α2-8-linked NeuAc residues (6) that are similar to polysialosyl chains characterized in capsular polysaccharides of *E. coli* KI (21) or Men B (1). These results prompted us to search for a cross-reactivity of monoclonal antibodies raised against Men B with embryonic N-CAM.

Here we report the reactivity of a monoclonal antibody of IgM subclass obtained by a fusion of spleen cells from a mouse immunized with viable group B meningococci and Ag8-653 nonsecreting myeloma cells. Hybridoma clones secreting anti-polysaccharides antibodies were identified using a solid phase radioimmunoassay. The clones positive on group B and negative on group C carbohydrates were retained. The antibody used in this study is the clone 2-2B, and its thermodynamic and binding characteristics have been reported elsewhere (18).

When reacted with immunopurified N-CAM in immunoblot experiments this antibody bound only the embryonic forms (Fig. 1 *B*); Fig. 1 *A* shows the same preparations reacted with the monoclonal P61 recognizing an intracytoplasmic domain of the 180,000- and 140,000-mol-wt chains and the embryonic forms.

N-CAM Are the Major Glycoproteins Bearing (α2-8)n Polysialic Units in AtT20 Cells

Cell lines of neural origin were screened for their expression of N-CAM using the monoclonal P61. Fig. 2 *A* shows an immunoblot obtained with cell extracts. Cell lines of neural origin such as the neuroblastomas N18 and NG108 and the astrocytoma C6 expressed typical forms of adult N-CAM (lanes *c–e*, respectively) with polypeptides of 180,000- and 140,000-mol-wt. Interestingly, the cell line AtT20 (D16/16) (24) derived from a mouse anterior pituitary tumor, expressed a form that migrated as the embryonic form of the mouse brain (compare lanes *f* and *g*). As expected N-CAM molecules were not expressed by the cell line HT29, a colon adenocarcinoma (lane *a*).

When the same cell extracts were reacted with the anti-Men B monoclonal antibody only embryonic mouse brain and AtT20 cells showed a positive reaction (Fig. 2 *B*, lanes *f* and *g*) in a molecular weight range expected for embryonic N-CAM. No other bands were detected on the gel outside of the embryonic N-CAM zone, either in cell lines or brain extracts. This indicates that embryonic N-CAM might be the major, if not the only, glycoproteins bearing (NeuAcα2-8)n structures. However, formally other glycoproteins containing polysialosyl units may migrate in the same region of the gel as N-CAM. We tested this hypothesis on metabolically labeled AtT20 cells and sequential immunoprecipitation experiments with polyclonal anti-N-CAM antibody and anti-Men B antibody.

Precleared antigen preparations from AtT20 cells labeled with [^{35}S]methionine were treated twice with the polyclonal anti-N-CAM antiserum. Fig. 3, lane *b* showed the analysis

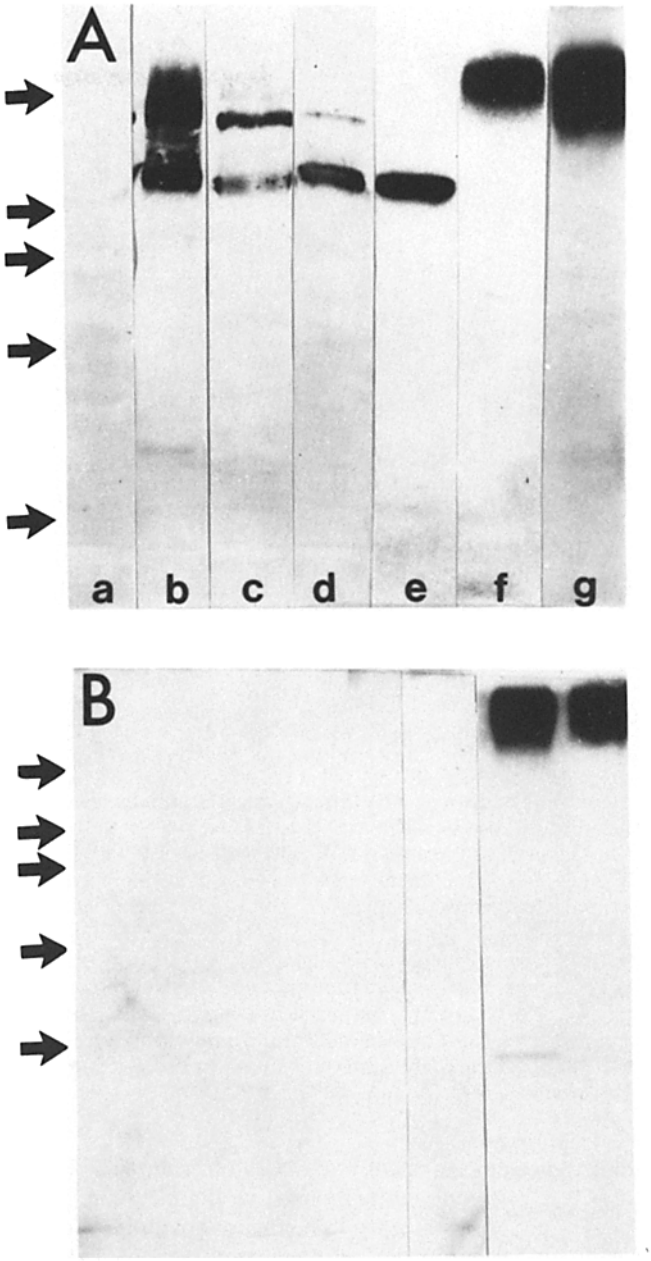

Figure 2. Immunoblot analysis of N-CAM expressed by different tissues or cell lines reacted with rat monoclonal P61 (*A*) and mouse monoclonal anti-Men B (*B*). Tissue or cell lines were extracted with 2% Nonidet P-40 in Tris-HCl buffer containing protease and neuraminidase inhibitors. They were subjected to electrophoresis in 7% (*A*) or 10% (*B*) SDS polyacrylamide gels. Samples contained 70 μg protein per lane. Lane *a*, HT29, human colon adenocarcinoma which did not express N-CAM; lane *b*, mouse adult brain cortex; lane *c*, N18; lane *d*, NG 108 mouse neuroblastoma; and lane *e*, C6 (rat × mouse glioma) expressed adult forms of N-CAM. Lane *f*, AtT20 (D16/16) mouse anterior pituitary tumor cell line and lane *g*, mouse embryonic brain expressed the embryonic forms. Molecular weight markers positions are indicated by arrows.

of the first run. The final supernatant was then split in half; one half was treated with the anti-Men B antibody (lane *c*) and the other half with the same polyclonal anti-N-CAM (lane *d*). Lane *e* showed as a positive control an immunopre-

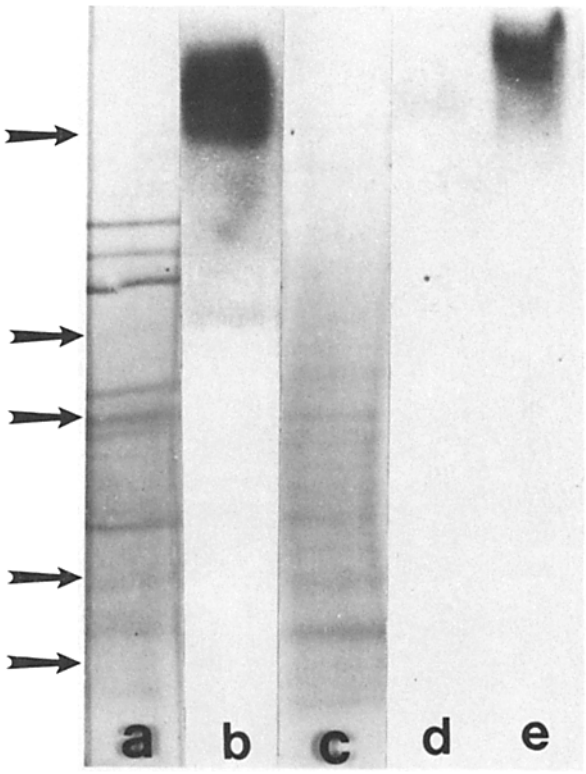

Figure 3. Comparison of [^{35}S]methionine-labeled molecules immunoprecipitated by rabbit anti-N-CAM and anti-Men B antibodies. Monolayer cultures of AtT20 cells were labeled with [^{35}S]methionine, lysed, and immunoprecipitated, and were prepared with control rabbit antiserum (lane *a*), anti-N-CAM rabbit antiserum (lane *b*), or anti-Men B antibody (lane *e*). Lanes *c* and *d* were the bands immunoprecipitated by anti-Men B antibody and anti-N-CAM polyclonal antiserum, respectively, from supernatants after two runs with polyclonal antiserum anti-N-CAM. Molecular weight marker positions are indicated by arrows and were myosin (200,000), galactosidase (120,000), phosphorylase D (92,000), serum albumin (68,000), and ovalbumin (45,000).

cipitation using anti-Men B antibody on an antigen preparation that had not been prereacted with anti-N-CAM antibody. Lane *a* was a control precipitation with an irrelevant rabbit antiserum. This experiment confirmed that at least in the AtT20 cell line the majority of molecules bearing the Men B epitope also reacted with our polyclonal anti-N-CAM antibody, since no immunoreactive molecules were detectable after two consecutive runs with anti-N-CAM polyclonal, with the exception of a band at ~47,000 D. This band might represent N-CAM degradation products, since it was not consistently seen in all experiments.

Anti-Men B Monoclonal Antibody Does Not React with Polysialoglycolipids in Mouse Brain

To discover whether these polysialosyl structures could be borne by molecules other than glycoproteins, such as gangliosides, we extracted the lipidic fraction from cell lines or tissues previously examined in Fig. 2 and analyzed the extracts by thin layer chromatography (Fig. 4). The plates were stained with resorcinol (*A*) to reveal major components or immunostained with the anti-Men B monoclonal antibody (*B*) as described above. Anti-Men B positive bands were never observed in any of the extract examined. However, when the antibody was used at a 1:500 dilution (1:5,000 in immunoblot) immunofluorescence staining or cross-reactivity can be seen with the polysialoglycolipid GT3 (NeuαAc2-8)3 (Fig. 4, lane *b*) as well as larger gangliosides with undetermined structures purified from 10 d-old embryonic chicken retina (3) (not shown). Note that neither the ganglioside GT3 nor other gangliosides were detected in mouse brain (Fig. 4 *B*, lanes *h* and *i*), presumably because they are not expressed in this species. Staining of the same preparations with an IgM antibody (18B8) that recognizes a similar carbohydrate structure (3) gave a different reactivity pattern (not shown). By contrast, reaction with other irrelevant IgM antibodies produced in ascites fluids or second antibody alone did not give any positive bands.

Anti-Men B Monoclonal Antibody Stains Cell Lines That Express Embryonic But Not Adult N-CAM

Indirect immunofluorescence labeling of live AtT20 cells revealed a strong immunoreactivity (Fig. 5, *A* and *B*) that was completely abolished by preincubation of the anti-Men B antibody with an excess of colominic acid (NeuAcα2-8)n (Fig. 5 *C*). Double immunofluorescence labeling using a rabbit antiserum against the NH_2-terminal part of N-CAM (24) revealed that cells such as C6 that express adult N-CAM were not labeled by the anti-Men B monoclonal (Fig. 5, *D–F*). This is in accordance with biochemical data described earlier in Figs. 2 and 4. When cells were permeabilized before the application of the anti-Men B antibody (not shown) the pattern of staining did not change, indicating that the majority of reactive epitopes were localized on the cell surface.

Immunofluorescence on Neural Primary Culture

We also used our antibody to study the N-CAM expressed in neural primary cultures. Fig. 6, *A* and *B* shows the staining of a neuronal primary culture prepared from embryonic mouse spinal cord. Neuronal cell bodies as well as neurites were brightly stained with the anti-Men B antibody, whereas the underlying flat fibroblast-like cells were unstained, indicating that these cells do not express (NeuAcα2-8)n units.

In double immunofluorescence labeling of cultured cells from brains of young rats we could clearly discern cells that reacted with the rabbit anti-N-CAM antibody but did not fix the anti-Men B antibody. An example is shown in Fig. 6, *C*, *D*, and *F* where cells, probably astrocytes type II as judged by their morphology (19), expressed the adult form of N-CAM. Similar situations can also be seen with neurones. In every case cells that reacted positively with anti-Men B were also stained by the rabbit anti-N-CAM antibody.

Anti-Men B Monoclonal Antibody As a Marker of the Embryonic-to-adult Conversion

Primary cultures prepared from embryonic day 13 mouse spinal cord were examined at different times in cultures for their expression of N-CAM and their reactivity towards anti-Men B monoclonal antibody. After 1 wk in our culture conditions, all neurones and their neurites stained with both anti-N-CAM polyclonal antibody and anti-Men B as shown in Fig. 7, *A–C*. When sister cultures were examined after 3 wk in vitro, neurones still stained with anti-N-CAM polyclonal but most no longer reacted with anti-Men B (Fig. 7,

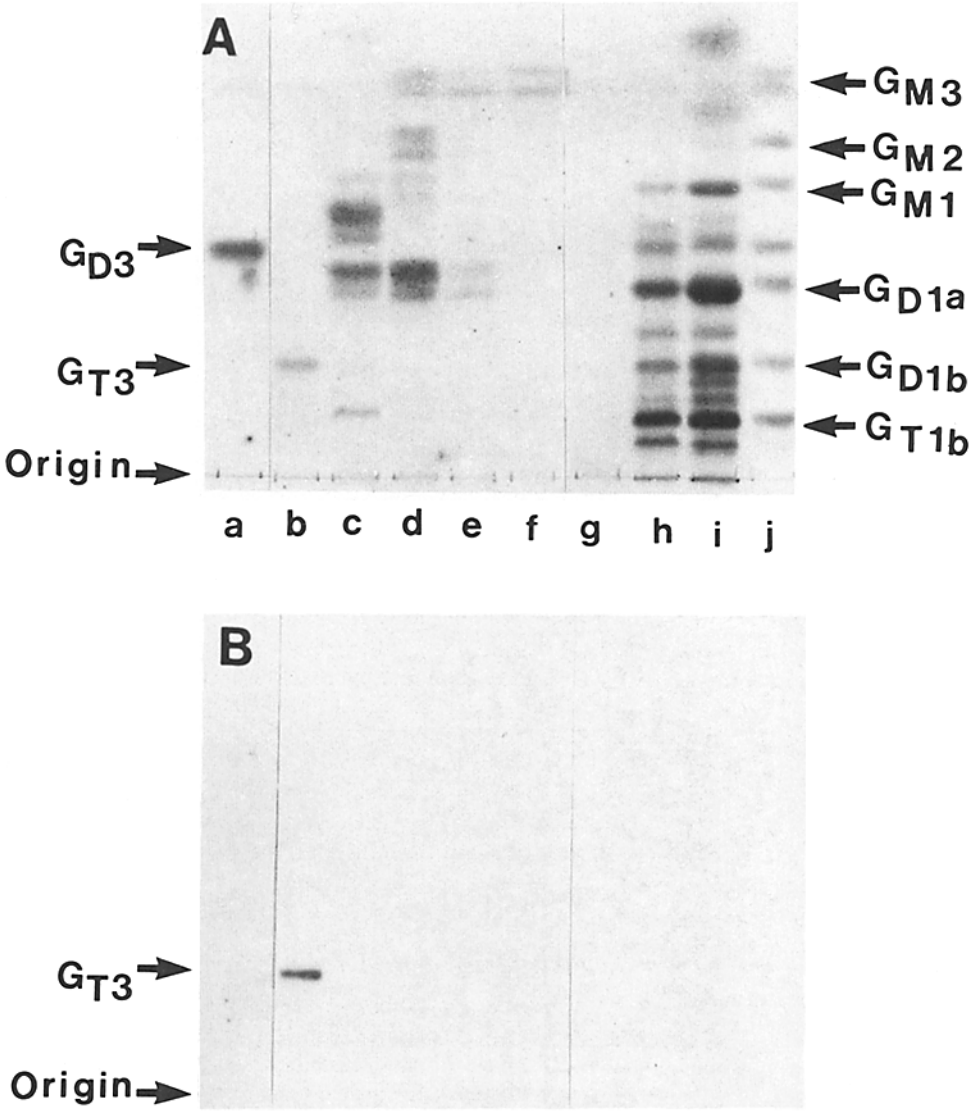

Figure 4. Thin layer chromatography of sialoglycolipids expressed by tissues or cell lines reacted with resorcinol reagent (*A*) or immunostained with mouse monoclonal anti-Men B (*B*). Resorcinol visualized major ganglioside components (*A*). Reference gangliosides are spotted in lanes *a*, *b*, and *j*. GD3 (lane *a*) (NeuNAcα2-8 NeuNAc2-3β1-4Glcβ1-1Cer) and GT3 (lane *b*) (NeuNAcα2-8 NeuNAcα2-8-NeuNAcα2-3GaLβ1-4Blcβ1-1Cer) are indicated in the left margin, and standard gangliosides GT1b, GD1b, GD2a, GM1, GM2, and GM3 (lane *j*) in the right margin. Lanes *c–g* are the total gangliosides isolated from cell lines AtT20, NG108, N18, C6, and HT29, respectively. Lanes *h* and *i* are embryonic and adult mouse brain, respectively. *B* shows the autoradiograph of the glycolipids reacted with the anti-Men B antibody. Only the ganglioside GT3 showed a reactivity, at a 1:500 dilution of the ascites fluid.

D–F) except for some bundles of fibers that remained positive. This indicates that the embryonic-to-adult conversion occurs in vitro and affects the majority of cells that express N-CAM.

Discussion

A cross-reaction between capsular polysaccharides from Men B but not group A (ManNAc-P-)n or C(NeuAcα2-9)n in human and rat brain was first observed by Finne (6, 7) using a polyclonal antiserum raised in horse against Men B. Independent investigators (28) described high molecular weight proteins from embryonic neuronal membranes that reacted with similar antisera. However, these reagents, because of their low titer in specific antibodies, can only be used at very low dilutions (1:25 to 1:50), which probably prevents their use in immunohistochemical detection of the reactive antigens. We confirm here the previous observations using a monoclonal antibody raised against Men B. The specificity of this antibody for (NeuAcα2-8)n was assessed by its cross-reactivity with embryonic N-CAM molecules reported to express such structures (5) and by the observation that preincubation of the antibody with colominic acid (NeuAcα2-8)n completely abolishes immunostaining. Our immunoblot experiments together with sequential immunoprecipitation data on AtT20 cells suggested that the embryonic N-CAM are the major, if not the only, molecules of the mouse brain that express such polysialosyl units.

It is striking that such a dramatic difference in immunoreactivity exists between the embryonic and adult forms of N-CAM towards the anti-Men B monoclonal antibody, since the adult forms of N-CAM still contain a significant amount of sialic acids (5). For example, a calculation of the average length of the polysialosyl chains in N-CAM purified from adult mouse brain gave values of about three residues per chain (13). That observation is consistent with the reported substrate specificity of the bacteriophage-associated endosialidase (8) which is active only on polysialic chains longer than eight residues. The authors consequently proposed a unique molecular conformation of the polysialosyl chain borne by N-CAM molecules in embryonic brain. Experiments are in progress to define precisely the structure of the epitope rec-

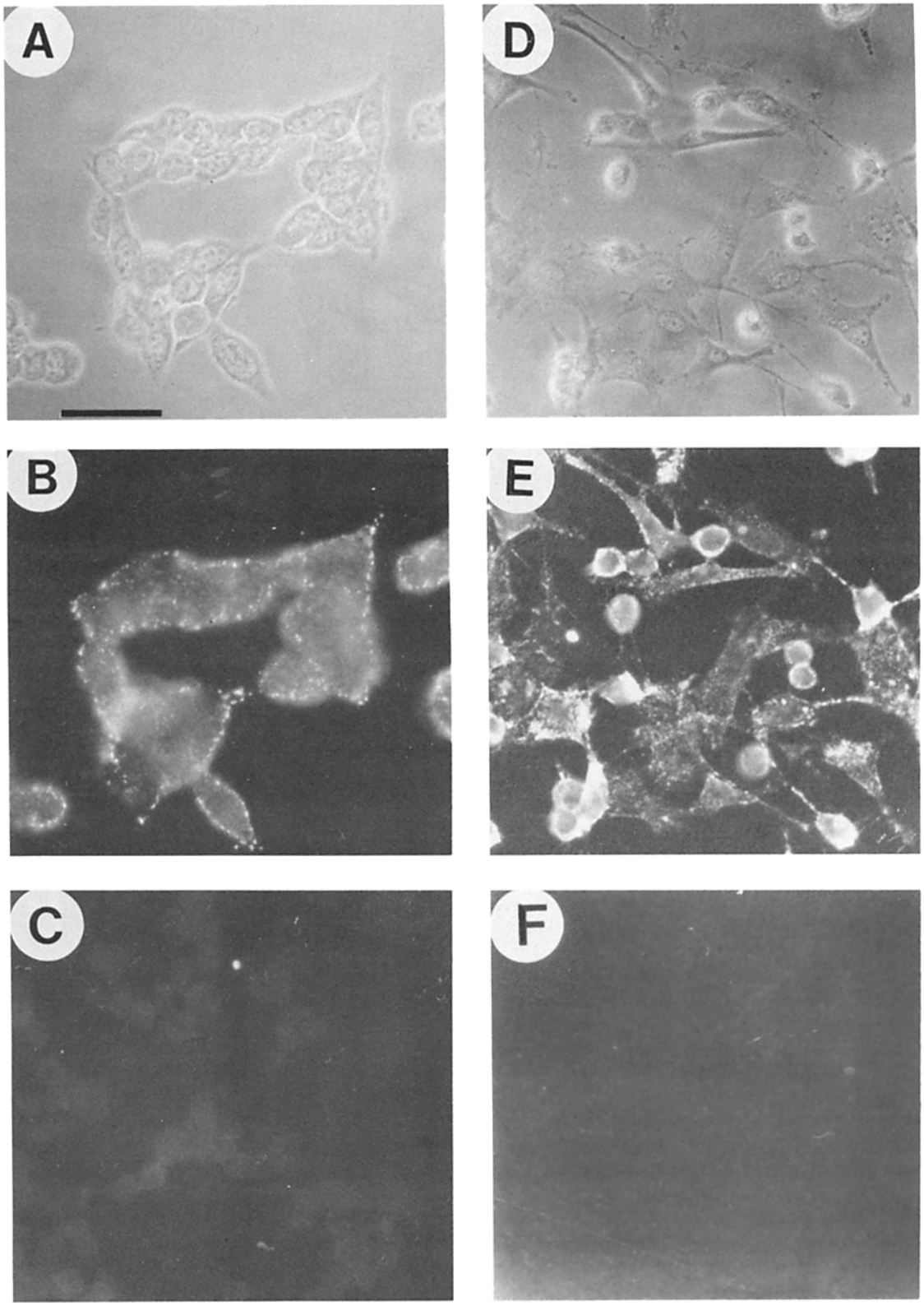

Figure 5. Immunofluorescence staining of cell lines with anti-Men B and rabbit anti-N-CAM antibodies. (*A*) is the phase-contrast micrograph of a live AtT20 cell culture that has been stained with anti-Men B antibody (*B*). *C* represents the staining of a sister culture with anti-Men B antibody that has been preadsorbed with colominic acid (phase-contrast picture not shown). Note that this treatment completely abolished the fluorescence. *E* and *F* represent live C6 astrocytoma cells that had been stained with rabbit anti-N-CAM antibody revealed by fluorescein-labeled second antibody and with anti-Men B antibody revealed by a rhodamine-labeled second antibody, respectively. *D* shows the same filed viewed with phase-contrast optics. This cell line, which expressed an adult form of N-CAM (major polypeptide at 140,000 D), did not stain with anti-Men B antibody (*F*). Bar, 50 μm.

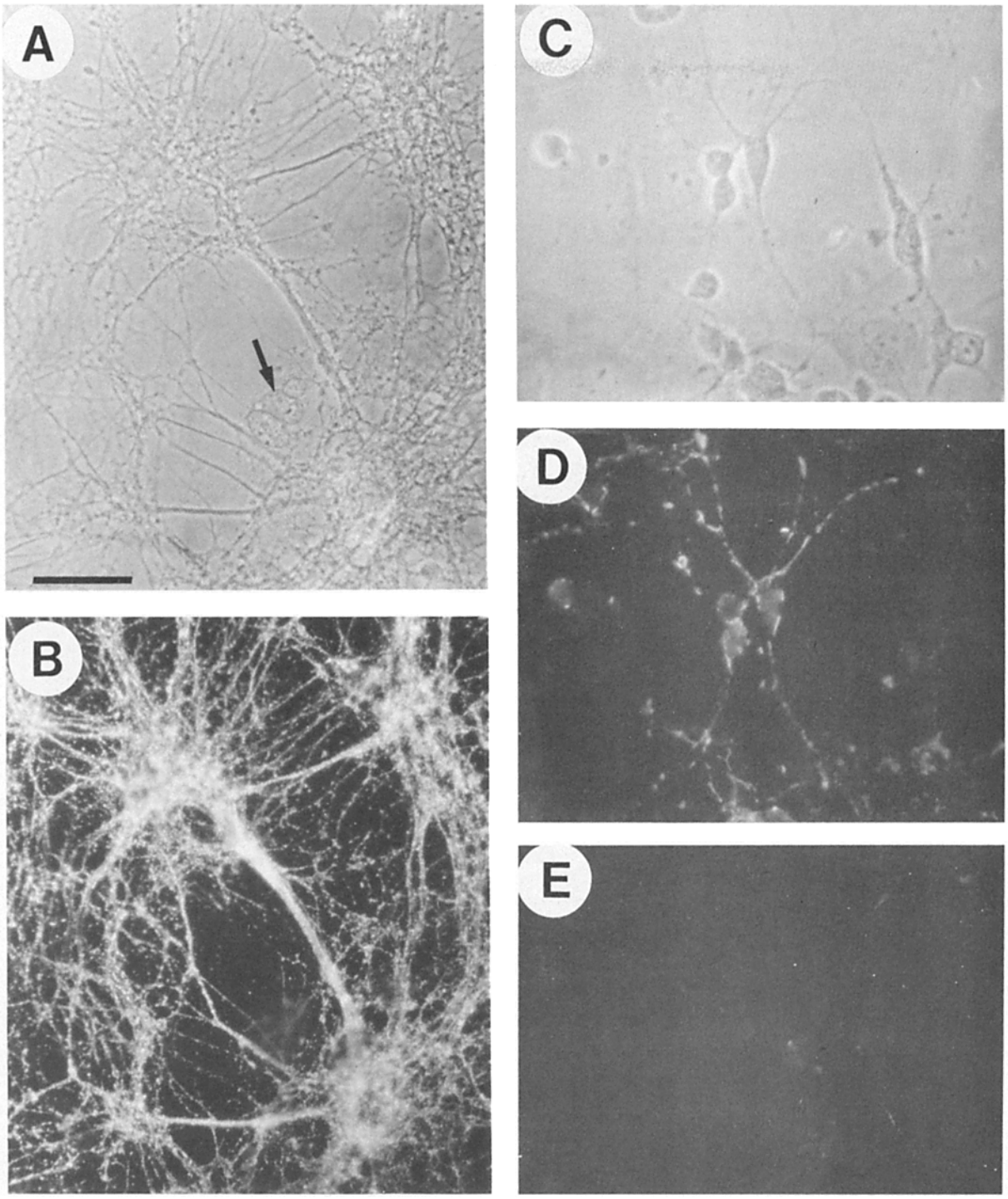

Figure 6. Immunofluorescence staining of primary cultures of cells from embryonic and neonatal brain. *B* shows the staining with the anti-Men B antibody of a mixed spinal cord–dorsal root ganglia culture from 13-d mouse embryos maintained for 15 d in culture. Note that some flat cells in the background, nuclei indicated by arrows, were negative. In contrast, processes and cell bodies of the neurones were strongly and uniformly stained, leading to the conclusion that they expressed the embryonic form of N-CAM. (*A*) is the corresponding phase-contrast micrograph. (*C*) is the phase-contrast image of a primary culture from cerebral cortex of a 2-d-old rat maintained for 8 d in culture. The same field double-stained with polyclonal anti-N-CAM antibody revealed by a fluorescein-labeled second antibody and with anti-Men B antibody revealed by rhodamine-labeled second antibody is shown in *D* and *F*, respectively. Bar, 50 μm. In such cultures some cells stained both for polyclonal and anti-Men B (not shown), whereas some stained only with the polyclonal (compare *D* and *E*), presumably because they expressed only the adult form of the N-CAM. It should be stressed that the binding of one antibody does not prevent the binding of the other because they recognize different epitopes of the same molecule.

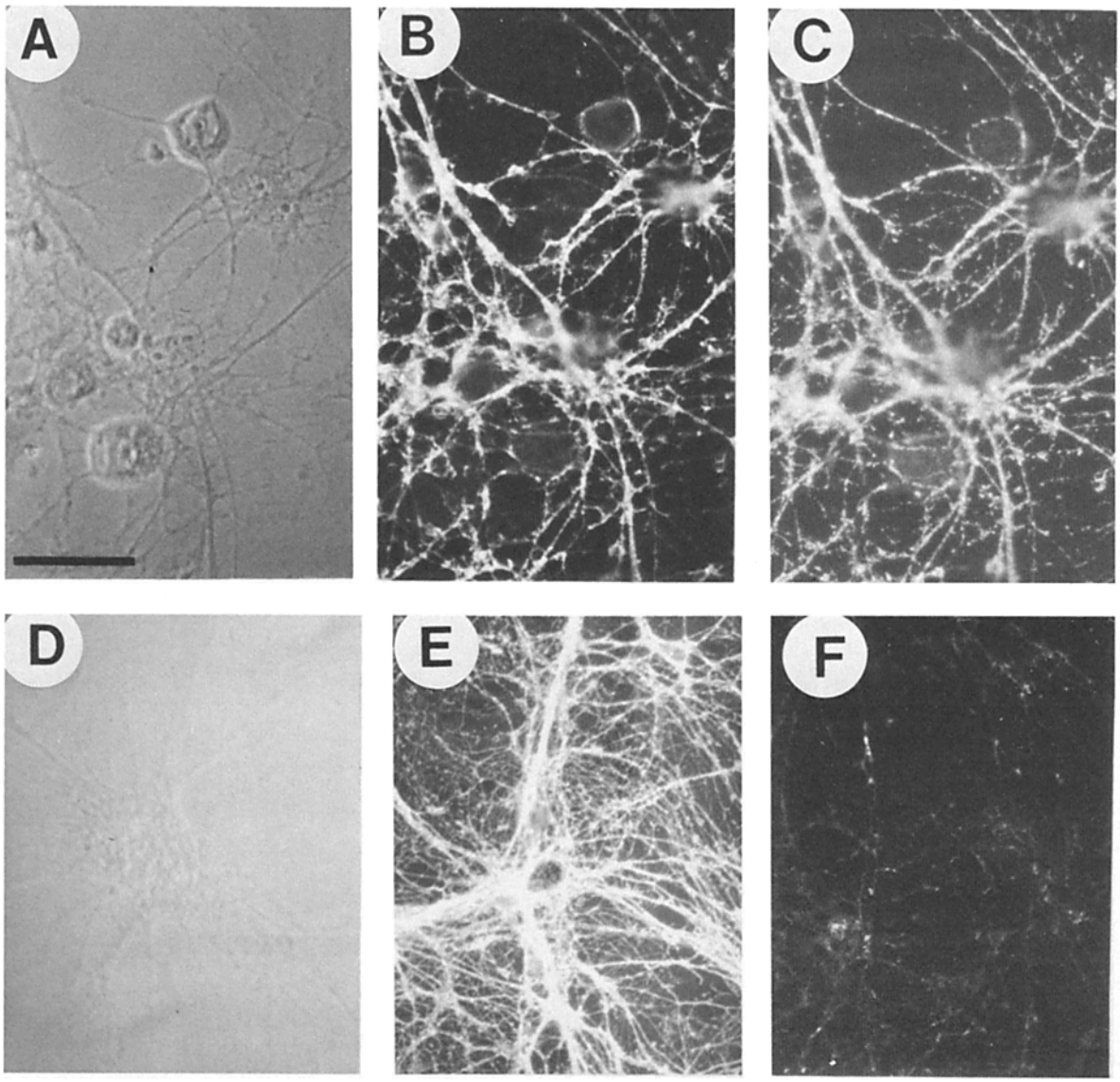

Figure 7. Observation of the embryonic-to-adult conversion in primary culture from embryonic mouse spinal cord. Cultures were prepared as described in Materials and Methods from 13-d-old mouse embryos and maintained for 1 wk (*A–C*) or 3 wk (*D–F*) in vitro. Then they were double-labeled with polyclonal rabbit anti-N-CAM (*B* and *E*) and with anti-Men B monoclonal antibody (*C* and *F*). While the majority of the neurones were positive for both anti-Men B and anti-N-CAM in 1-wk cultures (compare *B* and *C*), they had become negative for anti-Men B after 3 wk (compare *E* and *F*). This is in good agreement with a maturation process of the N-CAM molecules expressed by neurones. *A* and *D* are the corresponding phase-contrast micrographs for *B* and *C*, and *E* and *F*, respectively. Bar, 50 μm.

ognized by our monoclonal anti-Men B. Experiments with the glycolipids indicate that at least three residues are necessary for recognition. For example, the antibody does not recognize GD3 (Fig. 4, lane *a*). We cannot exclude that under the conditions of the thin layer chromatography experiments the conformation of the three (α2-8) linked polymeric residues expressed by glycolipids is different from the conformation of such a structure borne by glycoproteins. In other words, our monoclonal antibody might be able to recognize three residues only in a particular conformation. We clearly showed that adult N-CAM were not immunoreactive with anti-Men B either in immunoblot or in immunofluorescence studies, using the experimental conditions described in the present study.

Previous attempts to study embryonic forms of N-CAM have relied upon either examination of heterotypic organs (10) or biochemical analysis of primary cultures of central nervous tissues (16). Such studies cannot resolve whether individual cells can synthetize embryonic N-CAM in the absence of any intercellular communication. The description of the mouse AtT20 (D16/16) cell line expressing embryonic N-CAM will be of general use for structural and biosynthetic studies concerning this molecule. In particular, it will be a useful system to search for endogenous enzymes participating in the synthesis of these unusual polysialosyl polymers.

Double immunofluorescence staining on primary cultures indicates that using these antibodies we can detect the expression of polysialosyl structures by individual cells. To our knowledge, this is the first report of a monoclonal antibody recognizing polysialic acid–dependent determinants on

N-CAM that is useful for both biochemical studies and histological analysis of individual cells. We clearly showed that the embryonic-to-adult conversion correlates with a complete loss of immunoreactive polysialosyl units from all N-CAM molecules once conversion is completed for a given cell. For example immunofluorescence studies on spinal cord primary cultures demonstrated an absence of Men B immunopositive cells after 3 wk in vitro, whereas N-CAM immunoreactivity remained unchanged over the same time period. This antibody should allow us to study the embryonic-to-adult conversion, to discover factors that control the conversion and, more specifically, to describe its spatiotemporal occurrence in normal as well as perturbated developing mouse brain, thereby elucidating the role of these molecules in neural ontogenesis.

In addition, the cross-reactivity of the group B and K1 polysaccharides with embryonic N-CAM could explain difficulties in preparing vaccines from these two substances (2). Since *E. coli* meningitis frequently occurs in the central nervous system of new born infants, there is the intriguing possibility that it may be related to the expression of identical polysialosyl structures by both embryonic N-CAM and infecting bacteria. It would thus be of interest to identify the neural receptors for these structures and to assess their role in cell recognition.

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