Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 6181–6185, August 1989
Cell Biology

Reversible inhibition of oligodendrocyte progenitor differentiation by a monoclonal antibody against surface galactolipids

(myelin basic protein/galactosylcerebroside/sulfatide/myelinogenesis)

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Communicated by Mary J. Osborn, May 22, 1989

ABSTRACT **We have hypothesized that oligodendrocyte (OL) surface glycolipids, specifically galactocerebroside and sulfatide, play a role in the regulation of OL development by acting as sensors/transmitters of environment information. In support of this hypothesis we report here a reversible inhibition of OL progenitor cell differentiation by a monoclonal antibody [Ranscht mAb (R-mAb); Ranscht, B., Clapshaw, P. A. & Seifert, W. (1982) *Proc. Natl. Acad. Sci. USA* 79, 2709–2713] that reacts with these glycolipids. When isolated OL progenitors or mixed primary cultures are grown in the presence of the antibody, myelinogenic development is blocked in a dose-dependent manner at concentrations as low as 2 μg of IgG per ml. The inhibited cells express the OL progenitor markers O4 and vimentin but are negative for galactosylcerebroside, sulfatide, 2′,3′-cyclic nucleotide 3′-phosphohydrolase, myelin basic protein, and myelin basic protein RNA expression. In contrast, the levels of total cellular protein and the expression of astrocytic glial fibrillary acidic protein in mixed cultures are not affected. Antibody-blocked cells have a distinctive morphology in which long, sparsely branched processes emanate from round cell bodies. Upon removing the perturbing antibody, the cells rapidly resume differentiation. Reverted mixed primary cultures, in which OL progenitors of several sequential developmental stages are present at the time of plating, differentiate more rapidly than control cultures, suggesting that the antibody-induced block results in a synchronization of developmental progression along the OL lineage by accumulating cells at the inhibition point. However, the normal temporal sequence of marker expression is maintained. Control studies with several other antibodies recognizing OL cell surface antigens, including HNK-1, neural cellular adhesion molecule (N-CAM), 1A9, anticholesterol, and O1, did not inhibit development. Since the inhibition occurs in highly enriched populations of OL progenitors, the inhibition does not involve cell–cell interactions between OLs and other cell types but concerns interactions of OLs with themselves, soluble factors, or OL extracellular matrix molecules and adhesion factors that provide essential environmental signals required for normal myelinogenic development.**

The differentiation of oligodendrocytes (OLs) is a critical event during the course of brain development leading to the elaboration of the myelin sheath. Disruption of myelinogenesis leads to serious neurological deficits (1).

Oligodendrocytes are derived from immature neuroectodermal cells of the subventricular zone of the forebrain (e.g., refs. 2 and 3). In rodents the majority of OLs are generated postnatally and pass through a series of phenotypic stages from immature to mature myelin-forming cells (4–6). This sequential differentiation of the OL lineage can be reproduced in culture (7), even in the absence of neurons (8, 9). In the rat, several stages of development have been identified. The O-2A bipotential progenitor is a bipolar, proliferative, migratory cell that is labeled with antibody A2B5 (10, 11). It can differentiate into either a type II astrocyte or an OL, depending on environmental signals (12). Platelet-derived growth factor acts as a mitogen, extending the proliferative phase of O-2A cells (13–15).

Further progression along the OL-specific lineage is characterized by the appearance of the O4 antigen, a stage that may retain some bipotentiality (reviewed in ref. 9). The cells are not yet authentic OLs insofar as they do not express the early OL marker galactocerebroside (GalC) (16). The differentiation of $O4^+GalC^-$ progenitors into OLs is responsive to environmental cues, such as insulin-like growth factor I (17). Subsequently, additional myelin-specific markers appear in an ordered temporal sequence, including first GalC, sulfatide, and 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP), followed by proteolipid protein, and finally myelin basic protein (MBP) (3, 9). The recognition of this ordered sequence of steps, identified by a panel of immunological and molecular probes (18), allows one to identify the developmental state of these cells in experimental situations.

As a working hypothesis, we have proposed that galactolipids such as GalC and sulfatide have functional roles in the regulation of OL differentiation and myelination (19). Here we report that growing OL progenitor cells in the presence of a monoclonal antibody (mAb) that recognizes GalC (20) and sulfatide (unpublished observations) reversibly inhibits OL development at a characteristic stage in the lineage. Preliminary reports of this work have been presented in abstract form (21, 22).

MATERIALS AND METHODS

Culture Systems. *Mixed primary cultures.* Mixed primary cultures of 19- to 21-day fetal rat brain telencephala were prepared and grown as described (23). The culture medium consisted of a modification of N2 medium supplemented with heat-inactivated 1% fetal calf and 1% horse sera.

$O4^+GalC^-$ progenitors. $O4^+GalC^-$ progenitors were prepared from P5 rat telencephala as described elsewhere (9). This relatively synchronized subpopulation differentiates rapidly into OLs. Highly purified $O4^+GalC^-$ progenitors survive poorly in the defined medium, but addition of 25% (vol/vol) meninges-conditioned medium enhances survival (9). Although the total number of $O4^+$ cells decreased over 15 days in culture (DIC) to ≈30% of the initial attached population, the surviving cells carried out normal myelinogenic development and were considered representative of the population as a whole.

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Abbreviations: CNP, 2′,3′-cyclic nucleotide 3′-phosphohydrolase; DIC, days in culture; GalC, galactocerebroside; mAb, monoclonal antibody; MBP, myelin basic protein; OL, oligodendrocyte; R-mAb, Ranscht mAb described by Ranscht *et al.* (20); N-CAM, neural cellular adhesion molecule.

Secondary cultures of mixed progenitor and OL populations. Secondary cultures of mixed progenitor and OL populations were isolated from primary cultures at 12 DIC as described elsewhere (24) and grown in the same media used for primary cultures.

Antibodies. Hybridoma cells producing the IgG3 mAb Ranscht mAb (R-mAb) (ref. 20; received from M. Noble, London) were grown in 5% heat-inactivated fetal calf serum in Dulbecco's modified Eagle's medium. The culture medium was concentrated by ammonium sulfate precipitation followed by dialysis, titered for anti-OL activity by immunofluorescence microscopy on primary cultures and for IgG3 content by dot blot assay (19) against purified mouse myeloma IgG3 standard (FLOPC 21/κ, J606/κ, Miles Scientific), aliquoted, snap frozen in liquid nitrogen, and stored at −80°C. Aliquots were thawed and stored for short periods at 4°C. The IgM mAbs O1 and O4 (25) were concentrated from hybridoma cell culture supernatants as described (19).

We have recently characterized these three antibodies in some detail (unpublished data): O4 reacts among glycolipids with sulfatide (26, 27) and seminolipid; however, it also binds to a component on the cell surfaces of $O4^+GalC^-$ OL progenitor cells (28) and R-mAb-inhibited cells (this manuscript), neither of which incorporates detectable amounts of $^{35}SO_4$ or [^{3}H]galactose into sulfatide. O1 reacts with GalC and monogalactosyldiglyceride (26, 27). R-mAb, nominally an anti-GalC (20), in fact recognizes sulfatide and GalC but is not equivalent to either O4 or O1 in its reactions with live cells; thus the possibility of cross-reactions with other uncharacterized OL cell surface molecules is not ruled out. Rabbit anti-CNP and anti-MBP have been described (19, 29). Monoclonal anti-vimentin was purchased from Boehringer Mannheim. Anti-neural cellular adhesion molecule (N-CAM) (30), HNK-1 (Becton Dickinson Immunocytometry Systems), anti-cholesterol (31), and 1A9 (32) were obtained from S. Bhat (Univ. of Pennsylvania) and G. Maxwell, S. Patel, and A. L. Gard (Univ. of Connecticut), respectively.

Antibody Perturbation Experiments. Antibody in fresh culture medium was added at the times indicated and readded along with fresh medium every 2–3 days for the duration of antibody treatment (see figure legends). To study reversibility of antibody-mediated effects, the antibody-containing medium was removed and the cultures were washed once with fresh culture medium before the addition of fresh antibody-free growth medium.

Immunofluorescence Microscopy. Cells were labeled for immunofluorescence microscopy as described (19) using the following antibody titers: R-mAb, 1:20; O4 and O1, 1:50; anti-CNP and anti-MBP, 1:100; antivimentin, 1:5.

Biochemical Assays. Assays for GalC and sulfatide synthesis, CNP activity, steady-state levels of MBP RNA, amounts of MBP and glial fibrillary acidic protein, and total protein have been described (19, 33).

RESULTS

The effect of R-mAb on OL progenitor differentiation was tested on three different culture systems that have complementary properties. (*i*) Mixed primary cultures most readily provide material for biochemical analyses and allow studies of effects on a range of stages of the OL lineage (23). (*ii*) Isolated $O4^+GalC^-$ progenitor populations afford an assessment of direct effects of the antibody on the cells, inhibition reversibility, and OL viability (9). (*iii*) Secondary cultures of mixed OL progenitors (24) and OLs permit comparisons with experiments in the literature that have used this system.

Antibody Inhibition and Reversibility in Mixed Primary Cultures. Mixed primary cultures were divided into two groups. One group was given fresh medium and served as the untreated control (Fig. 1, circles). The second group was given medium containing R-mAb at a 1:40 dilution 4 days after plating (Fig. 1, squares). The number of $O4^+$ cells in both sets of cultures increased over ≈8 DIC to reach a plateau—i.e., this increase was not affected by R-mAb treatment (not shown; see below, Fig. 6). This indicates that the differentiation and entry of O-2A precursors to the $O4^+$ OL-specific pathway and $O4^+$ cell viability were not affected by R-mAb treatment. In contrast, the appearance of cells immunolabeled with antibodies R-mAb and anti-MBP (Fig. 1, squares) and with O1 and anti-CNP (not shown) was blocked. Antibody-blocked cells stained with mAb O4 and had a distinctive morphology in which long processes with sparse branching, instead of the usual highly branched process network of mature OLs, emanated from the cell bodies (Fig. 2).

The antibody-induced block of differentiation observed by immunofluorescence microscopy was confirmed and extended by biochemical assays (Fig. 3) carried out after 20 DIC (i.e., 16 days of treatment with R-mAb). R-mAb treatment caused dramatic reductions in [^{3}H]galactose incorporation into GalC and sulfatide, CNP activity, MBP RNA levels, and MBP protein content compared to untreated control cultures (Fig. 3; Fig. 4 *A* and *B*). Therefore the block of MBP expression appears to be primarily at the level of MBP RNA synthesis or stability. Persisting low levels of activity of early markers in blocked cells (GalC and sulfatide synthesis, and CNP activity, Fig. 3) are attributed to a baseline population of $GalC^+$ cells and the presence of cells weakly stained with R-mAb and O1. In contrast, the levels of total protein and astrocytic glial fibrillary acidic protein in antibody-treated cultures were nearly normal.

The capacity of cells blocked by R-mAb to reinitiate differentiation was tested by replacing the R-mAb-containing medium with control medium at 20 DIC. $R\text{-}mAb^+$ cells began to reappear within 1 day (Fig. 1, open triangles), whereas MBP^+ cells (Fig. 1, closed triangles), MBP (Fig. 4*A*), and MBP RNA (Fig. 4*B*) all began to reappear within 3 days. These cultures reached control levels of differentiated expression within 4 days with respect to R-mAb antigen expression and within 7–9 days for MBP-related expression

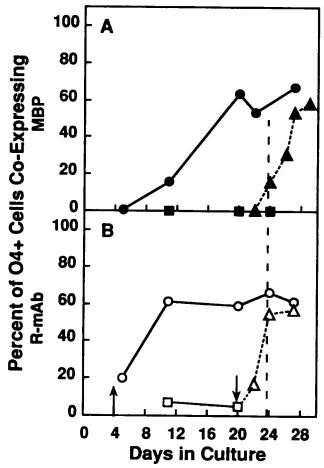

FIG. 1. R-mAb-induced inhibition and reinitiation of differentiation in mixed primary cultures from 19- to 21-day fetal rat telencephala. R-mAb (1:40) was added to one set of cultures at 4 days after plating (up arrow) and removed at 20 days (down arrow). A representative experiment out of four complete studies is shown. Data are presented as the percent of $O4^+$ cells that were also stained in double-label immunofluorescence microscopy with anti-MBP (*A*) or R-mAb (*B*). Circles, untreated control cultures; squares, cultures treated with R-mAb; triangles and dotted lines, cultures treated with R-mAb and then grown further in antibody-free medium. The dashed vertical line is included to facilitate a comparison between the times of onset of R-mAb and anti-MBP immunoreactivity.

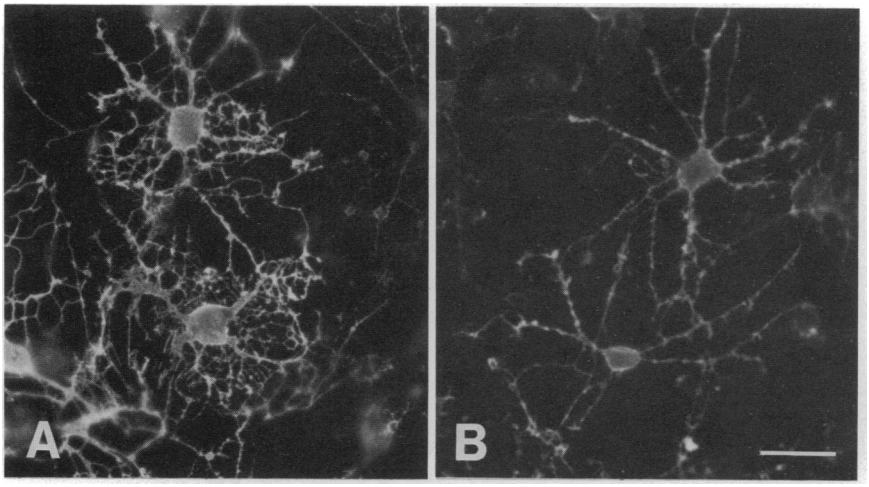

FIG. 2. Immunofluorescence photomicrograph of $O4^+$ cells in mixed primary cultures after 12 DIC in untreated control cultures (*A*) or cultures treated with R-mAb from 3 to 12 DIC (*B*). (Bar = 25 μm.)

(Figs. 1, 3, and 4). The lag in MBP expression, relative to the appearance of R-mAb$^+$ cells, was maintained. [^{3}H]Galactose incorporation into GalC and sulfatide, and CNP activity, also reappeared efficiently after removal of the antibody (Fig. 3). Reverted mixed primary cultures differentiated more rapidly than control cultures, suggesting that the R-mAb block resulted in a synchronization of developmental progression along the OL lineage by accumulating progenitors at the inhibition point.

Antibody Inhibition and Reversibility in Cultures of Isolated OL Progenitors. OL progenitors were introduced into culture and one set was treated with R-mAb (1:40) from 1 to 8 DIC. In control cultures, the progenitor cells rapidly developed into GalC$^+$MBP$^+$ OL (Fig. 5, circles). In the presence of R-mAb, however, only a base level of weakly stained R-mAb$^+$ cells present in the starting population was maintained (Fig. 5, open squares). MBP$^+$ cells did not appear in the treated cultures even after 17 days (Fig. 5, closed squares). The cells remained attached to the substratum, stained intensely with O4, and 87% also stained with anti-vimentin. Upon removal of the antibody, the cells rapidly resumed differentiation (Fig. 5, triangles); the characteristic lag between R-mAb antigen and MBP expression was again maintained.

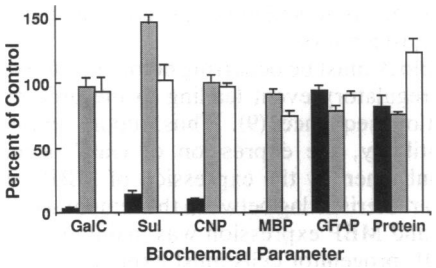

FIG. 3. Biochemical analyses of mixed primary cultures treated with R-mAb. Cultures initiated from 19- to 21-day fetal rat telencephalon were grown in (*i*) normal medium and analyzed at either 20 DIC (set at 100%) or 29 DIC (open columns), (*ii*) medium supplemented with R-mAb at 1:40 dilution from 4 to 20 DIC and analyzed at 20 DIC (solid black columns), or (*iii*) antibody-supplemented medium from 4 to 20 DIC and then grown for an additional 9 days in fresh medium lacking R-mAb to study the reversibility of the antibody-induced block and analyzed at 29 DIC (shaded columns). Harvested cultures were analyzed for various markers of myelinogenesis and compared to the levels in untreated, 20 DIC control cultures set at 100%. In experiments with less frequent time points than were taken in the experiments shown in Figs. 1, 4, and 5, thereby precluding accurate timing, the reappearances of GalC and sulfatide synthesis, and CNP activity, were nevertheless complete by ≤9 days after removal of the antibody. Biochemical parameters: GalC, GalC synthesis assayed by incorporation of [^{3}H]galactose into GalC identified by TLC; Sul, sulfatide synthesis assayed by incorporation of [^{3}H]galactose into sulfatide identified by TLC; CNP, enzymatic activity; MBP, amount of MBP determined by Western dot blot; GFAP, amount of glial fibrillary acidic protein determined by Western dot blot; Protein, total cellular protein. Bars, standard errors of the mean, $n = 3$.

Dose–Response. Mixed primary cultures were treated from 5 to 20 DIC with varying concentrations of R-mAb and the number of $O4^+$, R-mAb$^+$, and MBP$^+$ cells was determined by immunofluorescence labeling. A significant reduction in the appearance of R-mAb$^+$ and MBP$^+$ cells occurred at antibody dilutions as low as 1:100, corresponding to ≈2 μg of total IgG3 per ml, and inhibition was nearly complete at 1:20 (Fig. 6*B*). In contrast, the number of $O4^+$ cells that appeared per culture was not affected at any of the concentrations tested (Fig. 6*A*).

Specificity of the Block for R-mAb: Treatment with Other Antisera. Several other antibodies that bind to surface antigens of OLs and/or their progenitors were tested to ascertain the degree of specificity of the inhibition by the R-mAb. The antibodies were added to OL progenitor cultures at 2 DIC and assayed for MBP immunoreactivity at 6 DIC. Antibody concentrations were chosen that gave more intense immuno-

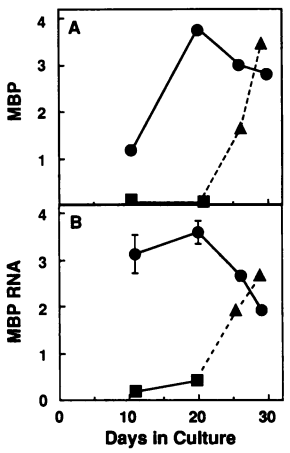

FIG. 4. MBP (*A*) and MBP RNA (*B*) expression in untreated control mixed primary cultures (circles), cultures treated with R-mAb at 1:40 dilution from 4 to 20 DIC (squares), or cultures treated with R-mAb from 4 to 20 DIC followed by growth in antibody-free medium from 20 to 29 DIC (triangles and dotted lines). Data are expressed as arbitrary units from scanning densitometer analyses of stained Western or Northern dot blots. Bars, standard errors of the means ($n = 3$) or ranges ($n = 2$). Where no bars appear, the error or range was less than the symbol size.

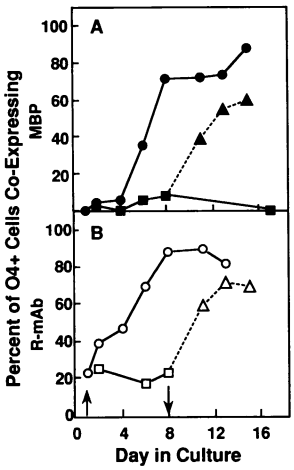

FIG. 5. R-mAb induced inhibition and reinitiation of differentiation in cultures of OL progenitors isolated from P5 rat pup telencephala. R-mAb (1:40) was added to one set of cultures 1 day after plating (up arrow) and removed at 8 days (down arrow). A representative experiment out of two complete studies is shown. Data are presented as the percentage of $O4^+$ cells that were also stained in double-label immunofluorescence microscopy with anti-MBP (*A*) or R-mAb (*B*). Circles, untreated control cultures; squares, cultures treated with R-mAb; triangles and dotted lines, cultures treated with R-mAb and then grown further in antibody-free medium.

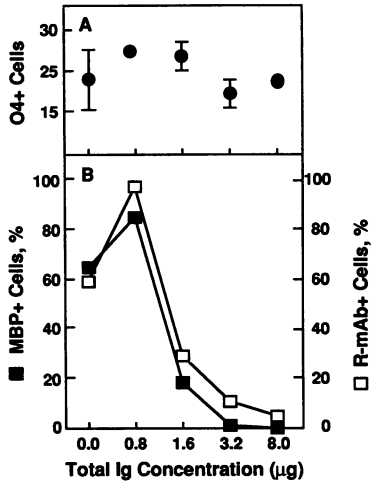

FIG. 6. Titration of the R-mAb inhibition of myelinogenesis in mixed primary cultures. R-mAb was added to cultures at 5 DIC at the total IgG3 concentration shown and harvested for assay by immunofluorescence microscopy at 20 DIC. A representative experiment is shown. (*A*) The average number of $O4^+$ cells per 6.25×10^{-4} cm^2 grid (duplicates with ranges) is shown for the cultures in *B*. (*B*) Data are presented as the percentage of $O4^+$ cells also labeled with an antibody against MBP (closed squares) or also labeled when restained with R-mAb (open squares).

fluorescent staining than did R-mAb at the concentrations used for inhibition (1:40). Anti-N-CAM (1:100), HNK-1 (1:200), O1 (1:20), 1A9 (1:100), and anticholesterol (1:100) were all ineffective at blocking differentiation (not shown) as were nonspecific purified mouse hybridoma control IgG3 (8 μg/ml) and IgG and proteins from fresh hybridoma culture medium concentrated as for R-mAb. Moreover, R-mAb lost its capacity to block differentiation when preadsorbed with liposomes containing GalC or sulfatide (but not glucocerebroside) before being added to the cultures.

DISCUSSION

We have shown that the normal progression along the OL lineage from $O4^+GalC^-$ progenitor to terminally differentiated OLs is blocked when progenitors are grown in the presence of the R-mAb. The continued presence of the R-mAb is required to maintain the developmental block. Removal of the perturbing antibody leads to rapid resumption of lineage progression. Several conclusions can be drawn from these data.

(*i*) The point in the OL lineage at which the inhibition occurs must be restricted to the period after the appearance of the $O4^+GalC^-$ progenitor (9) through its differentiation into $GalC^+$ cells. The reasoning for this is as follows. OL progenitor populations in brain develop asynchronously, progressing along a spatial and temporal gradient within a given region (34). This asynchrony is maintained in mixed primary cultures. During a prolonged treatment of mixed primary cultures with the blocking antibody, the number of $O4^+$ cells increased normally. Therefore, cells developmentally younger than those at the block point continued to progress, began to express the R-mAb antigen, bound R-mAb, and became developmentally inhibited. The blocked cells continued to express vimentin and retained a relatively simple morphology, characteristic of $O4^+GalC^-$ progenitors (9, 28), but apparently accumulated at or just beyond the $GalC^-/GalC^+$ interface. Thus, upon release the population of previously blocked cells differentiated into OLs more synchronously than control cultures.

(*ii*) R-mAb must act directly on the OL progenitors themselves, not indirectly via interactions of progenitors with other cell types. This is concluded in view of the specificity in mixed cell-type cultures of the R-mAb antibody for the OL lineage (e.g., the expression of glial fibrillary action protein by astrocytes was not affected) and the fact that inhibition could be effected in highly enriched populations of OL progenitors.

(*iii*) The reversion must have been due to recovery of the blocked cells themselves rather than repopulation by more immature progenitors still flowing into the developing population. This is concluded because earlier stages of the OL lineage had been removed in the experiments using enriched $O4^+GalC^-$ progenitors.

(*iv*) The block must be occurring before the activation of an important regulatory event leading to the ordered terminal differentiation sequence (9). Thus, upon removal of the R-mAb antibody, the expression of GalC occurred first, followed only then by the expression of MBP—that is, the normal, characteristic lag between the temporal development of R-mAb and MBP expression was maintained.

Target OL progenitor cells must express the R-mAb antigen on their surfaces in order to react with the antibody, yet inhibited cells had very little R-mAb antigen on their surfaces as monitored by immunofluorescence microscopy. In addition, the level of glycolipid synthesis in R-mAb-blocked cells, assessed by metabolic labeling with $^{35}SO_4$ and [^{3}H]galactose, was very low or absent. Therefore, either recycling of surface antigen must have occurred, as demonstrated previously for anti-GalC (35), or a low level of new antigen synthesis and transport sufficed to keep the cells "positive."

The identity of the antigen responsible for the perturbation is not firmly established. R-mAb was originally described as having a primary specificity for GalC, with a 16-fold lower cross-reactivity with sulfatide (20). Although it does react with GalC and the structurally related galactosyldiacylglycerol, recent experiments in our laboratory have demonstrated that under a variety of conditions the cross-reaction with sulfatide is much stronger than previously reported, generally equaling the reaction with GalC (unpublished data). O1 mAb, which reacts with GalC, did not inhibit OL differentiation. These data suggest that the target molecule could be sulfatide.

Several studies in the literature support the hypothesis that GalC and sulfatide may have functional importance in myelinogenesis and neurological disease (reviewed in ref. 19). Recently, Ranscht *et al.* (36) used R-mAb to reversibly block myelination of sensory axons in culture by Schwann cells. Although ensheathment of small axons and basal lamina formation were unaffected, elongation and immunofluorescently detectable expression of P0 glycoprotein and MBP were inhibited. The authors proposed that removal of GalC from the cell surface by internalization as antigen–antibody complexes prevented normal interactions between opposing Schwann cell membranes required for the formation of myelin lamellae. Since OLs in culture can differentiate in the absence of neuronal contact and the formation of multiple myelin lamellae, the mechanism for the inhibition reported here is apparently a different one. Dyer and Benjamins (35, 37) demonstrated that mature OLs in culture respond to anti-GalC treatment by rapidly internalizing the membrane-bound antibodies and reorganizing surface GalC into patches. Long-term exposure of OLs to anti-GalC caused extensive contraction of membrane sheets with accompanying reorganization of the underlying cytoskeleton. They did not observe inhibition of GalC metabolism.

Unresolved at this point is the question of the cellular mechanism by which the R-mAb exerts its reversible inhibition of myelinogenesis. Two principal models seem reasonable. First, antibody binding could alter the function of a growth factor receptor. Specifically, this model predicts that galactolipids are associated with receptors involved in receptor-mediated transduction across the plasma membrane of extrinsic signals for myelinogenesis. In favor of this model, insulin-like growth factor 1 has been implicated in this stage of OL development (17).

Second, antibody binding could alter specific OL–substrate interactions—that is, the perturbation involves adhesion molecules and extracellular matrix interactions in which galactolipids are important. In favor of this model, immature myoblast differentiation has been reversibly inhibited by an antibody against the extracellular matrix protein integrin (38); O4 antibody greatly enhanced OL aggregation, leading to the stimulation of myelinogenesis (19); cell-substrate adhesion is critical for the development of lamb OLs in culture (39, 40); the adhesion glycoprotein laminin and the platelet glycoprotein thrombospondin (41) have distinct functional domains for sulfatide binding, suggesting a possible role for sulfatide in cellular adhesion; myelinogenic expression can be blocked by a tripeptide sequence common to a number of cell binding molecules (42). In the latter case, the inhibition they observed appeared later in myelinogenesis than reported here for R-mAb and was irreversible when the tripeptide was presented to the cells during optimal myelinogenic expression. Therefore, the peptide inhibition is apparently proceeding by a different mechanism than R-mAb inhibition. Although in the present experiments R-mAb-treated cells remained firmly attached to the substrata, morphologically subtle but mechanistically critical alterations in homotypic adhesions among the OL progenitors, or in the attachment of OLs with the substratum, cannot be completely ruled out. The absence of inhibition by antibody HNK-1 makes it unlikely that molecules with the HNK-1 epitope, such as myelin-associated glycoprotein, fibronectin receptor, cytotactin, or sulfoglucuronylglycolipid (43–45), are the target antigens.

In summary, the observed reversible antibody-induced inhibition of myelinogenesis at a specific progenitor stage of development offers interesting experimental possibilities for studying the regulation of OL development. In addition, it has important implications for demyelinating diseases such as multiple sclerosis (46), in which a similar inhibition would impair remyelination by populations of developmentally quiescent progenitor cells (9) that persist into adulthood (47).

We thank our colleagues A. L. Gard, G. Gonye, A. Warrington, D. Avossa, M. J. Osborn, J. H. Carson, and E. Barbarese for helpful comments during the course of the work and preparation of the manuscript and Dr. M. J. Osborn for important contributions before and during the sponsorship of the manuscript. This work was supported by National Institutes of Health Grant NS10861.

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