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Inhibition of *in vitro* Peripheral Myelin Formation by Monoclonal Anti-Galactocerebroside

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This work investigates the role of galactocerebroside (GalC) in peripheral myelin formation. A monoclonal antibody against GalC was introduced into a myelinating culture system consisting of rat sensory neurons and Schwann cells, without other cell types. At levels that saturated Schwann cell surface GalC, anti-GalC IgG prevented by more than 99% the appearance of myelin sheaths. Ensheathment and basal lamina deposition were unaffected and many Schwann cells were in the 1:1 relationship that typically develops between Schwann cells and axons prior to myelination. Thus, the anti-GalC antibody did not interfere with the formation of the mesaxon but prevented its elongation. When experimentally restrained from myelination, Schwann cells did not accumulate the myelin proteins PO and basic protein; only low levels were expressed. The proposed mechanism of inhibition is the removal of GalC from Schwann cell surfaces by internalization of the GalC–anti-GalC antigen–antibody complex. This apparently prevented the interaction of adjacent cell surfaces during the elongation of Schwann cell membranes that constitute the myelin lamellae.

Fast electrical conductance along peripheral nerve fibers of the vertebrate nervous system is achieved by the provision of myelin segments. Each segment contains a lipid-rich, multilamellar membrane scroll, formed by one Schwann cell (Peters and Muir, 1959). In developing peripheral nerves, the formation of myelin depends on specific cellular interactions between neurons and Schwann cells. Early in development, Schwann cells insert processes between the fibers of nerve bundles and multiply upon contact with the axon membranes (Wood and Bunge, 1975). Clusters of Schwann cells within a common basal lamina then begin to engulf groups of neurites. The large axons are segregated by individual Schwann cells, which establish a 1:1 relationship with single axon segments within a basal lamina. Schwann cells will myelinate only those axon segments with which they are in a 1:1 relationship. Myelin lamellae form by the extension of the mesaxon in a spiral configuration around the axon. Finally, the myelin lamellae compact into the mature sheaths (Webster and Favilla, 1984). In contrast, nonmyelinating Schwann cells ensheathe several axons, each in a separate trough of their plasma membrane, and do not single out individual fibers.

The molecules that are thought to be involved in the process of myelin formation include the known myelin components, myelin-associated glycoprotein (Quarles et al., 1973; Sternberger et al., 1979; Figlewica et al., 1981), the myelin proteins PO and basic protein (Greenfield et al., 1973; Trapp et al., 1981, 1984), and the glycolipids GalC and sulfatide (Yao, 1984). GalC, a simple galactosphingolipid, is the major glycolipid of myelin (Yao, 1984). During the development of the nervous system, before myelin sheaths are formed, GalC is expressed as the first specific myelin component to appear on the surface of myelin-forming cells (Mirsky et al., 1980; Ranscht et al., 1982). We have investigated the role of this glycosphingolipid in the formation of peripheral myelin.

For these studies we used a culture system that reproduces *in vitro* the interaction between Schwann cells and neurons that occurs *in vivo* during myelination. The culture system also allows perturbation of the developmental sequence by means of the culture conditions chosen. In serum- and ascorbate-free culture medium, Schwann cells proliferate in association with axons, but do not ensheathe and myelinate. In a complex serum-containing culture medium, basal lamina formation, ensheathment, and myelination are initiated. In experiments intended to define the role of GalC in the process of peripheral myelination, the cultures were exposed to monoclonal anti-GalC immunoglobulin from the time myelination was initiated until mature myelin was formed in control cultures. In this study, we examined the effect of anti-GalC antibody in myelination, basal lamina formation, and the expression of the myelin proteins PO and basic protein.

Materials and Methods

Cell cultures. Disassociated rat dorsal root ganglion (DRG) neurons were established in culture, free of non-neuronal cells (Bunge et al., 1983), and later repopulated with a pure population of Schwann cells. For the preparation of neuronal cultures, the DRGs from 8–10 rat embryos (15 d after gestation) were dissected into Leibovitz (L15) medium and trypsinized [0.25% trypsin in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (HBSS)] for 30 min at 35°C before dissociation. The cell suspension was washed with L15 medium containing 10% human placental serum (HPS) and plated in medium A (see below) onto collagen-coated glass (Bornstein, 1958) (A. Thomas Co., Philadelphia) or Aclar film (Allied Chemical Co.) coverslips (22 mm diameter in each case) at a density between 5000 and 10,000 cells/coverslip. Medium A consisted of Eagle's minimum essential medium (MEM) with 10% HPS, 350 mg % glucose, and partially purified NGF at 50 U/ml. The elimination of non-neuronal cells was achieved by 3 successive 48 hr treatments with fluorodeox-

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yuridine (FUdR; 10 μM) in medium A, beginning 1 d after plating. Each treatment was followed by a 48 hr recovery period in medium A without FUdR. After the last antimitotic treatment, the cultures were kept in medium A for 8–10 d before Schwann cells were added.

Schwann cells were derived from explant cultures of 15-d-old rat embryo DRGs cultured essentially free of fibroblasts, as described by M. Bunge et al. (1983). Two days prior to harvesting the Schwann cells, the neuronal cell bodies were excised, leaving only the Schwann cell population in the culture dish. A single-cell suspension of Schwann cells was obtained by digesting the culture substrate with 0.05% collagenase in Earle's BSS and treating the resulting Schwann cell aggregates with trypsin (0.25% in HBSS). Both enzyme incubations were for 30 min at 35°C. The cells were washed in L15 medium with 10% HPS and seeded in medium A, at a density of 10,000 cells, onto the neuronal network previously prepared.

After the Schwann cells had attached to the neurites, the culture medium was replaced with chemically defined N2 medium (Bottenstein and Sato, 1979) supplemented with NGF. The cultures were kept in this medium for a period of 3–4 weeks, during which Schwann cells populated the neurites. Myelin formation was not observed under this culture condition (Moya et al., 1980). Ensheathment of neurites, basal lamina formation, and myelination were induced by culture medium containing HPS (15%) and either chicken embryo extract (5–10%) or ascorbic acid (50 μg/ml). Typically, the first myelin segments could be visualized by the seventh day following the change to myelinating medium; additional myelin formed during the following weeks. During this time, if not indicated otherwise, the culture medium was renewed every 3–4 d. In antibody-blocking experiments, the culture medium was replaced every 24 hr in both control and experimental cultures. All sera used during the treatment were decomplemented at 56°C for 30 min.

Antibodies. Monoclonal anti-GalC IgG3 (Ranscht et al., 1982) was purified from ascites fluid produced in pristane-primed Balb/c mice or from serum-free hybridoma culture supernatants (Murakami et al., 1982). Ascites fluid was precipitated with 50% ammonium sulfate and dialyzed against phosphate-buffered saline, pH 7.2 (PBS). The retentate was cycled over a 5 ml protein A–Sepharose column for several hours to achieve complete binding. The column was washed with 2–3 column volumes of PBS and eluted stepwise with 0.1 M citrate–citric acid at pH 6 and then at pH 3 (Watanabe et al., 1981). Fractions of 1 ml were collected and immediately neutralized with 1 M Tris, pH 8.8. Protein peak fractions were combined, concentrated by ultrafiltration, and assayed for (1) activity by indirect immunofluorescence, (2) purity by SDS acrylamide gel electrophoresis (Laemmli, 1970), and (3) protein content (Bramhall et al., 1969). Fractions eluted at pH 6 did not usually contain anti-GalC activity and were discarded. Serum-free hybridoma culture supernatants in batches of 500–1000 ml were cycled for 24–48 hr over a protein A–Sepharose column (5 ml) without prior precipitation and eluted with 0.1 M citrate–citric acid at pH 3. Fractions of 1 ml were collected, neutralized, concentrated, and assayed as described for ascites fluid. Because of the high degree of precipitation in the IgG fraction, yields were relatively low. Typically, 2–3 mg IgG3 were derived from 50 ml ascites fluid and 1–2 mg from 1 liter of serum-free culture supernatant. IgG from culture supernatants was used in blocking experiments in a concentration range of 12–50 μg/ml, IgG from ascites fluid at 100–200 μg/ml.

The monoclonal antibody against the Schwann cell surface component 217C (Peng et al., 1982) was obtained from Dr. J. DeVellis. Anti-217C IgG2 was purified from 500 ml serum-free hybridoma culture supernatant and used as a control in the blocking experiments. Monoclonal IgG3 (a gift from Dr. P. Burrows) was used as a control in one experiment. This antibody was purified from ascites fluid, as described for anti-GalC IgG3.

Antibodies against the extracellular matrix components laminin and collagen type IV were provided by Dr. R. Timpl, and the monoclonal antibody to heparan sulfate proteoglycan by Dr. C. Cornbrooks. Antibodies against the myelin proteins PO and myelin basic protein were obtained from Drs. J. Brockes and J. Trotter, respectively.

Immunohistochemistry. For visualization of cell surface and matrix components, living cultures were incubated with either primary antibody against GalC [ascites fluid (1:500) or undiluted hybridoma culture supernatant], 217C antigen (Peng et al., 1982) (hybridoma supernatant 1:25), laminin (Timpl et al., 1979) (rabbit serum 1:100), type IV collagen (rabbit serum 1:100), or heparan sulfate proteoglycan (Eldridge et al., 1986) (mouse monoclonal ascites fluid 1:50). The binding of these antibodies was monitored using fluorescein (Fl)- or rhodamine (Rd)-conjugated goat anti-mouse (GAM) or goat anti-rabbit (GAR) immunoglobulins (Cappel; 1:100). Before the antibody applications, the cultures were incubated for at least 15 min at room temperature with L15 medium supplemented with 10% heat-inactivated horse serum. The cultures were postfixed with either acidic ethanol (95% ethanol/5% acetic acid) at −20°C for 15 min, or with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 (buffer A) for 20 min at room temperature. After fixation, the cultures were washed with buffer A for 20 min at room temperature, rinsed with buffer A, and mounted for visualization with gelvatol (Rodriguez and Deinhard, 1960) or phosphate-buffered glycerol.

For double-staining of external and internal antigens, living cultures were incubated sequentially with the antibodies to surface antigens and the appropriate fluorochrome conjugate. The cultures were then fixed to expose internal antigens by 10 min treatments with 4% paraformaldehyde in buffer A, followed by 4% paraformaldehyde plus 0.2% Triton X-100 in buffer A. Fixed cultures were dehydrated and rehydrated by successive 2 min washes in 50, 100, and 50% acetone and buffer A at 4°C. Finally, the cultures were incubated sequentially with antibodies to internal antigens and the appropriate fluorochrome conjugate. All the antibody incubations were in L15 supplemented with 10% heat-inactivated horse serum for 30 min at room temperature, and were followed, as was each fixation step, by successive washes in L15 with horse serum without antibody.

Histology. Myelin segments were visualized by staining with Sudan black. The cultures were fixed at 4°C with either 4% paraformaldehyde or 2.5% glutaraldehyde in buffer A overnight and subsequently for 60 min with 0.1% osmium tetroxide. Following an alcohol dehydration series to 70% ethanol, the cultures were stained with 0.5% Sudan black in 70% ethanol for 1 hr. After rehydration, the cultures were washed with buffer A and mounted with gelvatol or glycerin jelly.

Electron microscopy. Cultures for electron-microscopic analysis were fixed, first with 0.46% glutaraldehyde in buffer A containing 0.1 M sucrose overnight, and then, after washing with buffer A, with 2% osmium tetroxide for 1 hr. Removal of the fixative was followed by washes in buffer A and dehydration of the cultures to 100% ethanol. Embedding was through propylene oxide (5 min), propylene oxide–Durcopan (1:1; 2–4 hr), and in Durcopan (overnight). Polymerization of Durcopan was complete after several days at 65°C. Silver sections were cut on an LKB Huxley ultramicrotome and examined under a Philips 200 electron microscope.

Results

Expression of GalC in neuron/Schwann cell cultures

The cultures used consisted of 2 cell populations, neurons and Schwann cells. The absence of fibroblasts from the cultures is an important aspect of the antibody-blocking experiments described here. Fibroblasts can form a perineurium-like sheath that is an effective barrier to the penetration of antibodies to their cellular binding sites (Mithen et al., 1982). The degree of neuron–Schwann cell interaction depended on the culture conditions. Schwann cells attached to neurites and proliferated in chemically defined, serum-free culture medium, but did not form a basal lamina and did not ensheathe or myelinate axons (Moya et al., 1980). The introduction of a richer culture medium containing HPS and either chicken embryo extract or ascorbic acid initiated basal lamina deposition, ensheathment, and extensive myelination. Myelin segments became visible after 8–10 d in this culture condition and gradually increased in number over the following weeks.

As a first step, we defined immunohistochemically the expression of GalC in the 2 different culture conditions. The results of these experiments are shown in Figure 1. In serum-free culture medium, the Schwann cells were not elongated, but showed a rounded morphology (Fig. 1*a*). In this culture condition, GalC was expressed on the surfaces of the majority of Schwann cells (Fig. 1*b*). This observation shows that Schwann cells in contact with neurites express GalC on their surface before they begin

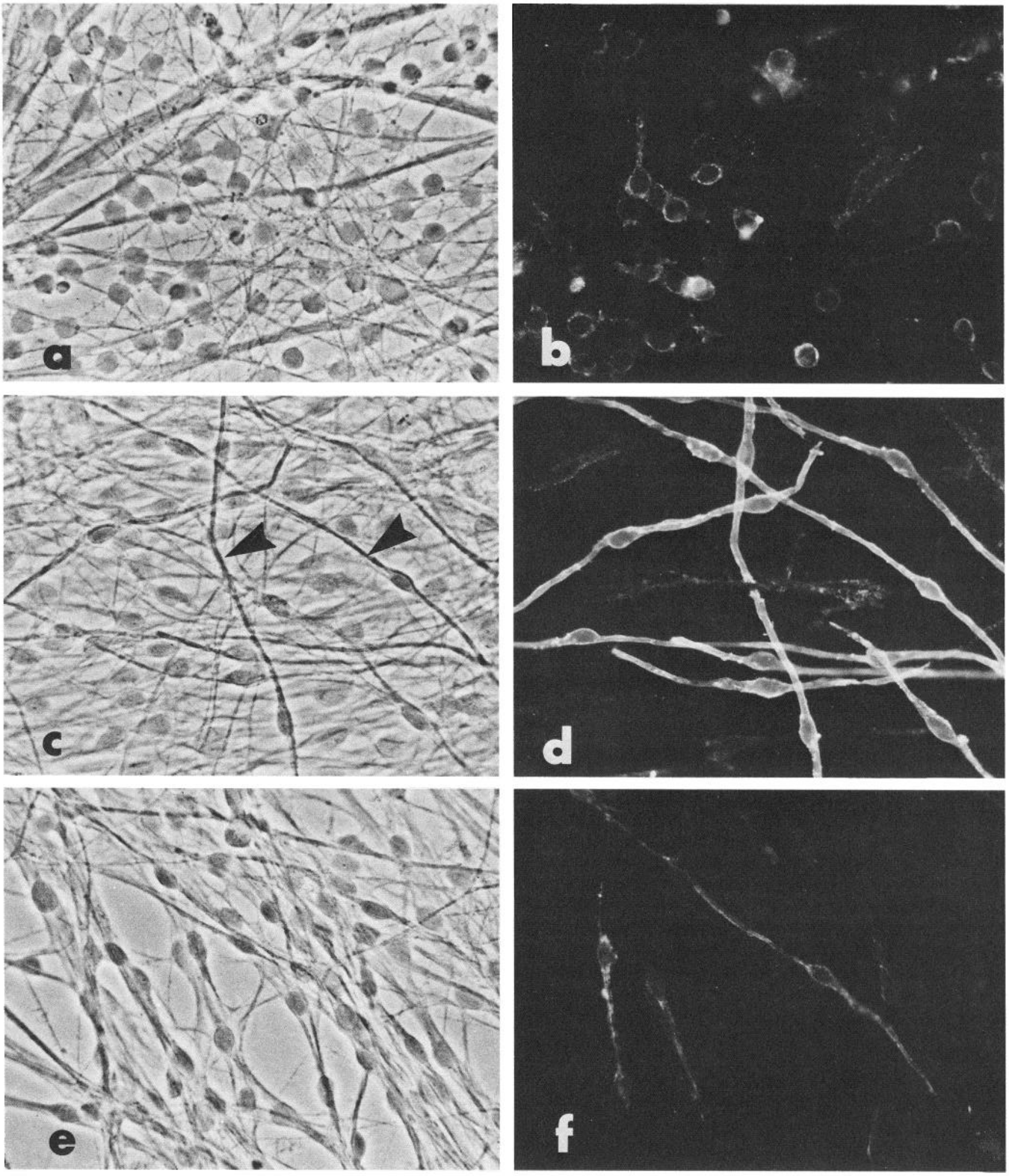

Figure 1. Galactocerebroside expression in neuron plus Schwann cell cultures in defined medium (*a, b*), in myelinating medium (*c, d*), and in myelinating medium containing anti-GalC antibody (*e, f*). Axons of the outgrowth zone (some distance from the aggregated neuronal somas) traverse these fields. *a* and *b,* The rounded configuration characteristic of Schwann cells interacting with axons in defined medium. The phase-contrast image in *a* illustrates Schwann cells with distinct phase-dark, nearly spherical shapes related to the neurites crossing the field. *b,* A fluorescent image of the field in *a* stained to show the presence of GalC on the Schwann cell surface. A majority of Schwann cells are GalC-positive. Only a few cells exhibit processes that extend along axons. In myelinating medium (*c, d*), the Schwann cells elongate along axons and myelin sheaths are formed (*arrowheads*). Many nonmyelinating Schwann cells also contain surface GalC, but at lower levels than in myelinating cells. The culture shown in *e* and *f* was stained after 14 d in myelinating medium in the presence of anti-GalC antibody (12 μg/ml). Myelination is inhibited and the level of surface GalC is decreased. Irregular strands of faint staining shown in *f* indicate the presence of residual bound antibody. The cultures

to ensheathe axons. When basal lamina deposition, ensheathment, and myelination were initiated by the introduction of the rich culture medium, Schwann cells elongated and aligned to neurites (Fig. 1*c*). In this culture condition, GalC was also expressed on most Schwann cells. Myelinating Schwann cells, however, expressed much higher levels of cell surface GalC than did Schwann cells not engaged in myelin formation (Fig. 1*d*).

In order to define the working concentration of anti-GalC for the blocking experiments, we determined the saturation level of anti-GalC binding to Schwann cells in myelin-promoting culture medium. This was done by making visual comparisons of cultures stained with different concentrations of anti-GalC solutions for 30 min at room temperature. The saturation level was defined as the lowest level giving maximal brightness of fluorescence and was determined visually; it was approximately 5 μg/ml when the antibody was purified from hybridoma culture supernatant and 100 μg/ml when the IgG fraction was isolated from ascites fluid. One explanation for these differences is that the IgG fraction from ascites is likely to contain immunoglobulins other than those against GalC. While this method did not yield quantitative data, it provided a useful means of comparing different batches of anti-GalC IgG and served as a rough basis for determining the concentration of active antibody in the IgG fractions. In the antibody-blocking experiments, we used the anti-GalC antibody at concentrations exceeding the saturation level of Schwann cell surface GalC, e.g., 12–50 μg/ml when the antibody was purified from hybridoma culture supernatant and 100–200 μg/ml when the antibody was isolated from ascites fluid.

Experiments were performed to determine the fate of Schwann cell surface GalC in the continued presence of suprasaturating levels of anti-GalC IgG. Anti-GalC was added to the neuron–Schwann cell cultures in myelin-promoting culture medium. After several hours, anti-GalC bound to Schwann cell surfaces was visualized with fluorochrome-conjugated GAM-IgG. In comparison to untreated control cultures stained for Schwann cell surface GalC, the intensity of fluorescent staining in the GalC-treated cultures was always dramatically reduced, in some cases even beyond detection. The staining intensity could not be increased by incubating the cultures with fresh primary anti-GalC antibody before staining with the fluorochrome conjugate. This indicated that the amount of free or unreacted GalC on the Schwann cell surface had fallen to low or undetectable levels in the continued presence of anti-GalC.

After administration of a brief, 30 min pulse of suprasaturating concentrations of anti-GalC, the antibody bound during the pulse was rapidly cleared from the Schwann cell surfaces. Twelve and 24 hr after the pulse, bound anti-GalC could not be detected on Schwann cell surfaces (in one experiment) or had fallen to low levels (in a second experiment). Newly synthesized GalC started to reappear on Schwann cell surfaces 36 hr after the pulse administration. Taken together, these experiments suggest that GalC was internalized as an antigen–antibody complex. In fact, granules of fluorescent material could be visualized inside Schwann cells after permeabilization and staining with fluorescent secondary antibody.

These experiments show that treatment with anti-GalC antibody causes GalC to be removed from Schwann cell surfaces. To study the role of GalC in myelin formation, the expression of Schwann cell surface GalC was suppressed for the duration of the experiments by feeding the cultures with fresh antibody at 24 hr intervals.

Table 1. Inhibition of myelin formation by monoclonal anti-GalC IgG

	Myelin (Untreated controls, %)	Inhibition (%)
Anti-GalC		
Exp. 1 (50 μg/ml)	0.4 ± 0.1	99.6
Exp. 2 (12 μg/ml)	0.4 ± 0.1	99.6
Exp. 3 (24 μg/ml)	0.0 ± 0.0	100.0
Exp. 4 (100 μg/ml)	0.7 ± 0.2	99.3
Anti-217C		
50 μg/ml	96.6 ± 2.4	3.4

Neuron–Schwann cell cultures were treated with anti-GalC antibody at concentrations exceeding the saturation level of Schwann cell surface GalC (e.g., 12–50 μg/ml when the antibody was purified from hybridoma culture supernatant, 100–200 μg/ml when purified from ascites fluid). After 14–16 d, when myelin was light-microscopically visible in untreated control cultures, the cultures were fixed and stained with Sudan black. Myelin segments were quantitated in cultures from 4 experiments. For each condition, duplicate cultures were analyzed. Fifty microscopic fields over the assumed *x*- and *y*-axis were counted from each culture. Since the absolute number of myelin segments per 50 microscopic fields in untreated control cultures varied between experimental groups from 800 to 4700 segments, myelination is presented as the percentage value of untreated control cultures. Myelination was almost completely blocked in the presence of suprasaturating levels of anti-GALc. At a comparable concentration (50 μg/ml purified from hybridoma culture supernatant), anti-217C did not alter the number of myelin segments beyond the experimental variation of untreated control cultures.

Control antibody

In order to determine whether there were nonspecific effects of anti-GalC in the process of myelin formation, we used as a control a monoclonal antibody, 217C (Peng et al., 1982), against a protein component of the Schwann cell surface (J. DeVellis, personal communication). In many aspects, 217C resembles the antibody to the rat neural antigen Ran-1 (Brockes et al., 1977; Fields and Dammermann, 1985). The 217C antigen was expressed on all Schwann cells in defined culture medium. In myelinating cultures, 217C antigen was present on the Schwann cell surfaces during ensheathment and mesaxon formation, but appeared to be down-regulated as the myelin sheath matured. In myelinated cultures, 217C antigen was seen on all nonmyelinating Schwann cells, but only a few, if any, of the myelinated segments showed detectable levels of this antigen (data not shown).

In the continued presence of anti-217C at concentrations exceeding the saturation level (e.g., 50 μg/ml), Schwann cells never internalized 217C to completion. When compared immunohistochemically to untreated control cultures, the Schwann cell surface level of 217C was somewhat reduced, but less than that of GalC in the continued presence of anti-GalC. One explanation for this observation is that the 217C protein antigen, in the presence of its specific antibody, may be internalized at a slower rate than GalC.

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illustrated in *a–d* were stained in the living state with mouse monoclonal anti-GalC antibody and rhodamine-conjugated goat anti-mouse IgG; the culture in *e, f* was stained in the living state with only the secondary antibody. ×440.

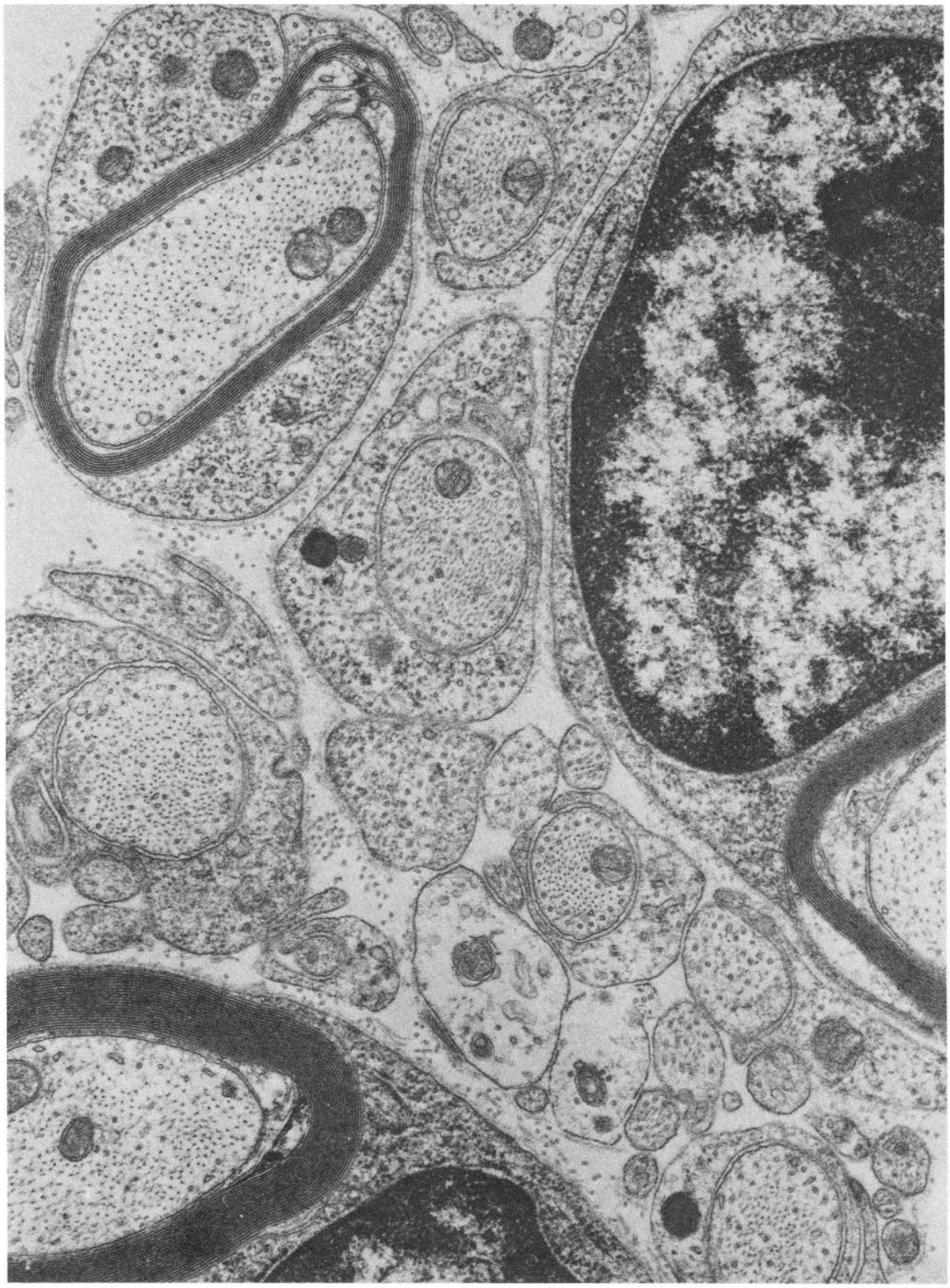

Figure 2. Schwann cell–axon interaction in control cultures. This electron micrograph shows that axonal ensheathment, segregation of larger axons, myelination, and extracellular matrix deposition have occurred during the 14 d period in myelinating medium. Extracellular components include the basal lamina sheaths around each axon–Schwann cell unit and extracellular collagen fibrils. ×25,650.

Monoclonal anti-GalC prevents the formation of myelin

To examine the effect of monoclonal anti-GalC on myelin formation, neuronal cultures were fully populated with Schwann cells in defined medium. The cultures were then switched to the myelin-promoting culture medium containing monoclonal anti-GalC IgG at concentrations exceeding the saturation level of Schwann cell surface GalC. By this experimental regimen, Schwann cell surfaces were exposed to the antibody before the initiation of basal lamina formation, ensheathment, and mye-

lination. To suppress the expression of GalC on Schwann cell surfaces, the culture medium of experimental anti-GalC-treated cultures was replaced every 24 hr. An identical feeding schedule was applied to control cultures. Control cultures were fed with the myelin-promoting culture medium alone or containing antibody to the Schwann cell surface component 217C. In one experiment, IgG3 myeloma protein was used as a control. The batch of anti-GalC immunoglobulin, whether purified from ascites fluid or prepared from serum-free hybridoma culture supernatant, was kept constant within one experimental group. After 12–14 d, myelin formation was assessed by staining the cultures with Sudan black. By this time, a large number of myelin segments were visible by light microscopy in untreated control cultures (Fig. 1*c*). Myelin segments in each condition were counted. The results are shown in Table 1.

The results from these experiments show that anti-GalC consistently prevented the appearance of myelin figures (Table 1 and Fig. 1*e*). Myelination was inhibited almost completely at anti-GalC levels as low as 12 μg/ml when the antibody was purified from culture supernatant, and 100 μg/ml when the antibody was purified from ascites fluid. The few and short myelin segments (<0.7% of the number in control cultures) that were occasionally observed were located exclusively in central culture areas between neuronal cell bodies. In these thicker culture areas there are sometimes local diffusion barriers that may hinder the penetration of the antibody. When we stained the anti-GalC-treated cultures at the end of the experiment, after 14 d, with fluorochrome-conjugated GAM-IgG, we could detect, in some experiments, residual GalC on a few Schwann cell surfaces (Fig. 1*f*). The level of GalC, however, was very low in comparison to that in untreated control cultures. No effect on myelination was observed with control antibody 217C at comparable concentrations (50 μg/ml from culture supernatant) or with mouse IgG3 from ascites fluid at concentrations between 50 and 200 μg/ml (data not shown).

GalC is involved in the elaboration of myelin lamellae

The ultrastructure of cultures treated with anti-GalC IgG was examined by electron microscopy. The experiments were conducted as previously described, and the cultures were fixed after 14 d. Samples from representative areas in experimental and control cultures were processed for electron-microscopic examination. In Figures 2 and 3, the relation of Schwann cells to neurites in control and anti-GalC-treated cultures is shown.

Untreated control cultures (Fig. 2) developed compacted myelin segments. Many Schwann cells were in a 1:1 relationship with axons and had commenced to form a myelin sheath. In a number of instances, the myelin sheaths were not completed, since the cultures were not fully matured when they were analyzed. Profiles of nonmyelinating Schwann cells ensheathing smaller axons were frequently observed. A small number of collagen fibrils and a patchy, often discontinuous, basal lamina was deposited around the neuron–Schwann cell units. Again, this indicates that the cells were still in a state of transition, rather than fully matured (Fig. 2).

In the presence of anti-GalC at a concentration of 50 μg/ml, the formation of myelin sheaths was prevented (Fig. 3). Many profiles of Schwann cell–axon units in a 1:1 relationship were observed, but multiple lamellae did not form. At the same time, the basal lamina around these Schwann cells was comparable to that in controls. Several examples of the nonmyelinating type of ensheathment were detected. These observations suggest that the anti-GalC antibody interferes with the elongation of the mesaxon rather than with an earlier axon–Schwann cell recognition.

The possibility that GalC might play some role in axon–Schwann cell interactions involving the ensheathment of small unmyelinated axons was suggested by recent observations of the localization of GalC on nonmyelinating Schwann cells in the sympathetic trunk (Jessen et al., 1985). We have not been able to demonstrate an effect on this type of Schwann cell ensheathment using anti-GalC that was purified from hybridoma supernatants at the highest concentration tested (50 μg/ml). This concentration was 4 times the level at which a striking reduction in myelination was noted. We did observe an effect of high levels (200 μg/ml) of anti-GalC on this type of ensheathment when the antibody was purified from ascites fluid. At this concentration, the Schwann cells left the axons bare and unensheathed and appeared rounded by light-microscopic analysis. Basal lamina deposition was substantially reduced, compared to controls, and consisted of thin, discontinuous patches of material at the cell surface. The nuclei of many of the Schwann cells appeared abnormal, with a nearly complete absence of heterochromatin. Our interpretation of these results is that high levels of anti-GalC might have had a generalized or nonspecific effect on Schwann cell function. This leaves open the possibility that GalC has some role in ensheathment.

Schwann cells treated with anti-GalC retain their capability to deposit basal lamina

Electron micrographs of Schwann cells prevented from forming myelin lamellae by monoclonal anti-GalC suggested that such Schwann cells retained their capability to secrete a basal lamina (Fig. 3). The molecular composition of the basal lamina in antibody-treated and control cultures (prepared and treated as previously described) was examined in indirect immunofluorescence with antibodies against laminin, heparan sulfate proteoglycan, and the noncollagenous domain of collagen type IV. The results are shown in Figures 4 and 5.

In untreated control cultures (Figs. 4, *a, b*; 5, *a, b*), essentially all Schwann cells expressed laminin and type IV collagen. Around myelin segments, the matrix components were organized in a columnar way. Comparable results were obtained in cultures treated with monoclonal 217C antibody (50 μg/ml) (data not shown).

In cultures treated with monoclonal anti-GalC IgG at a concentration of 50 μg/ml, no alteration in the deposition of collagen type IV (Fig. 4, *c, d*) and laminin (Fig. 5, *c, d*) was noted. Similarly, the secretion of heparan sulfate proteoglycan appeared unaltered (data not shown). These experiments demonstrate that Schwann cells, even when prevented from forming myelin by the anti-GalC antibody, retain other functions essential for myelin formation, i.e., the secretion and deposition of basal lamina components.

Schwann cells arrested in myelination do no accumulate myelin proteins

In developing peripheral nerves, the myelin proteins PO and basic protein can first be detected several days after the appearance of GalC (Mirsky et al., 1980; Ranscht et al., 1982), and are manifest only on GalC-positive Schwann cells. Therefore, it was of interest to determine whether Schwann cells express these proteins even when experimentally restrained from myelination by treatment with anti-GalC antibody. Neuron cul-

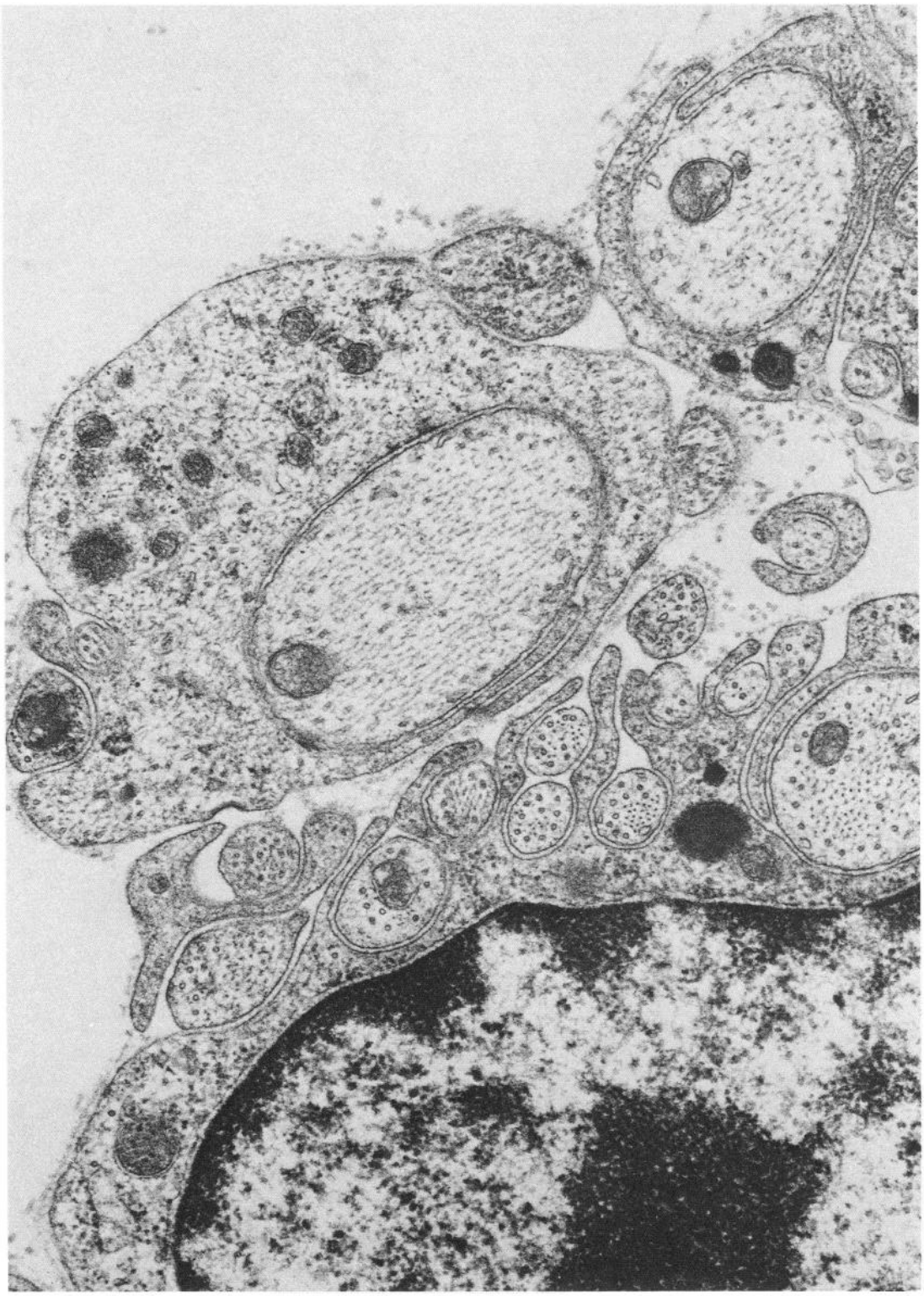

Figure 3. Schwann cell–axon interactions in antibody-treated cultures. Note that small axons have been ensheathed and large axons have been segregated by the Schwann cells to form the 1:1 relationship prerequisite for myelination. Myelin sheaths with more than a few turns are only rarely observed in these cultures. Extracellular matrix deposition (of basal lamina and collagen fibrils) is comparable to that observed in control cultures. ×41,100.

tures were populated with Schwann cells in serum-free culture medium and then grown for 14–16 d in myelin-promoting culture conditions in the presence of anti-GalC. PO and basic protein expression was examined on cultures fixed and permeabilized for immunohistochemical staining, as described in Materials and Methods.

In defined culture medium (i.e., nonmyelinating conditions), the majority of Schwann cells expressed GalC on their surface.

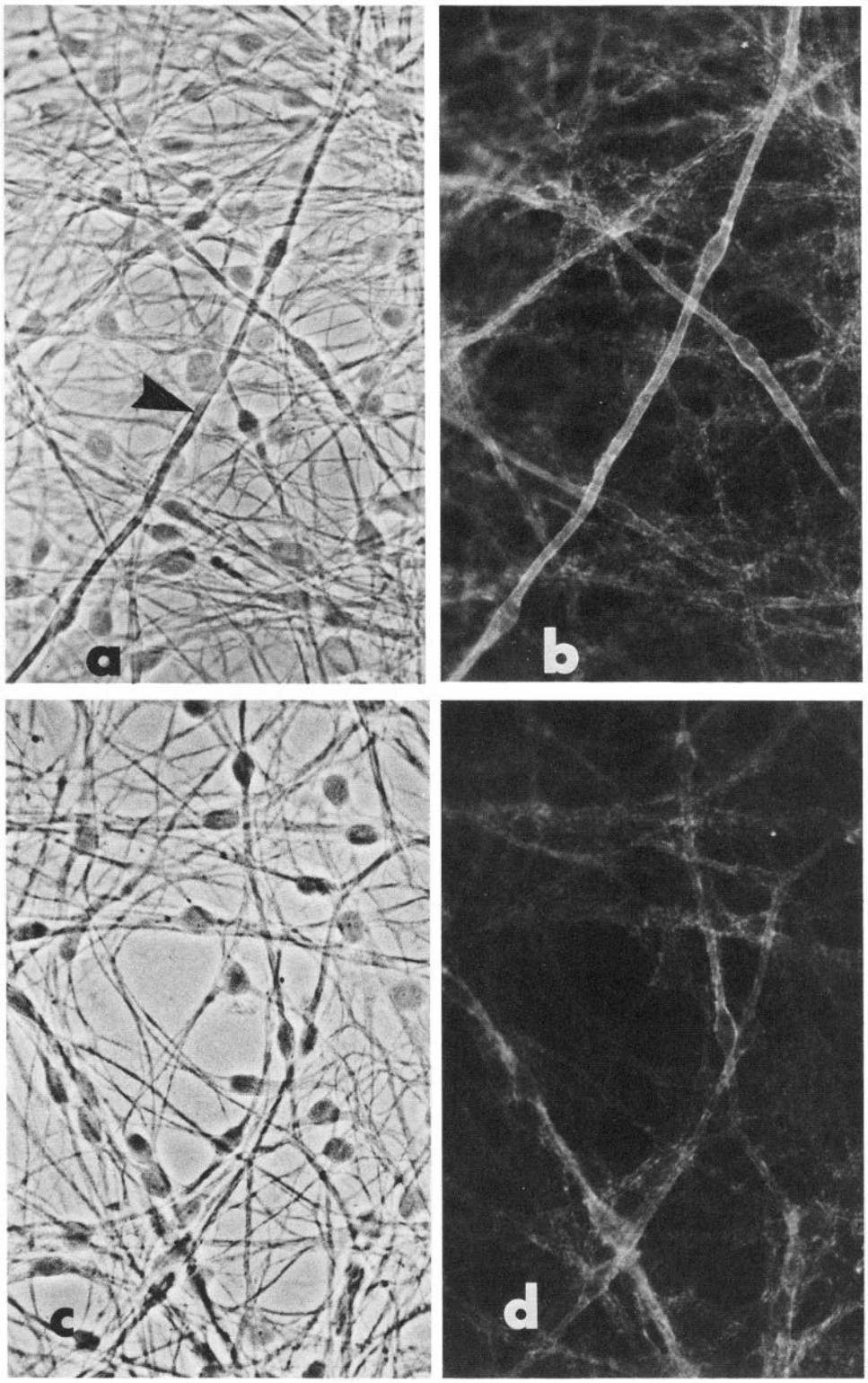

Figure 4. Type IV collagen deposition in control and antibody-treated cultures. *a* and *b,* Control cultures. *c* and *d,* Cultures treated with anti-GalC antibody for 14 d. The phase-contrast image in *a* shows compacted myelin segments traversing the field (example at *arrow*). In *b* the fluorescence due to bound rabbit anti-type IV collagen antibody and fluorescein-conjugated goat anti-rabbit IgG is shown. Note that the myelin segments appear to be more brightly stained than nonmyelinating Schwann cells. In the antibody-treated culture in *c,* the Schwann cells appear to be elongated and aligned along axons, but compact myelin has not formed. In *d,* the fluorescent image of the field in *c* illustrates that type IV collagen deposition is essentially normal in the anti-GalC-treated culture. ×440.

A small number (<5%) of these cells contained PO glycoprotein and basic protein at levels considerably lower than did myelinating Schwann cells (data not shown). After the cultures were shifted to myelinating conditions, both PO (Fig. 6, *a, b*) and myelin basic protein (data not shown) were readily demonstrated in regions where Schwann cells compacted a myelin sheath. In cultures treated with anti-GalC antibody, a small population of Schwann cells (less than 5%) contained myelin proteins. This population included the Schwann cells that had occasionally formed myelin in dense central culture areas (less than 0.7% of the total Schwann cell population; see Table 1) and expressed the myelin proteins at normal levels (compare Fig. 6*b*). In addition, in the presence of the anti-GalC antibody, a subpopulation of Schwann cells not engaged in myelination contained

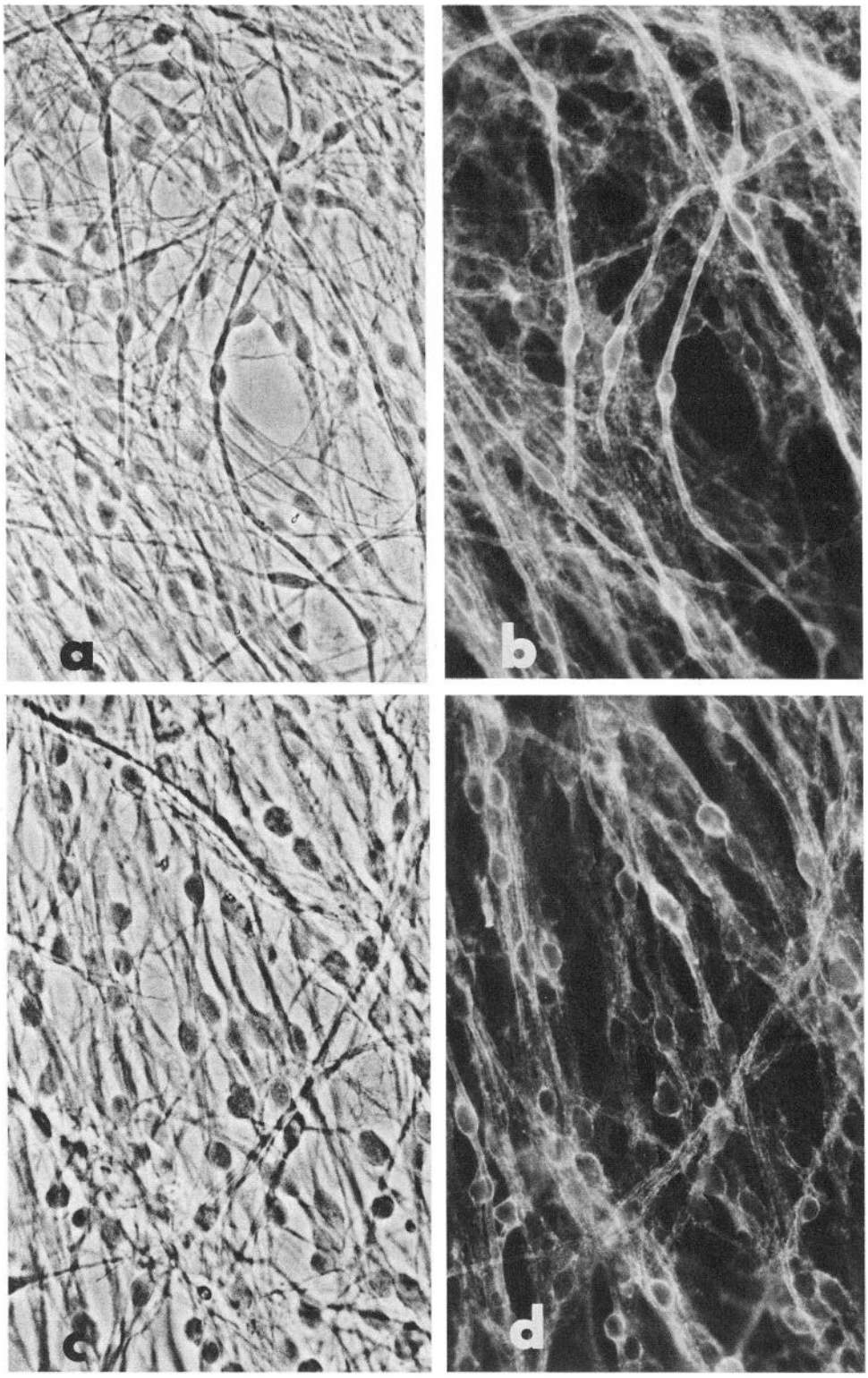

Figure 5. Laminin deposition in control and antibody-treated cultures. *a* and *b*, Control cultures. *c* and *d*, Cultures treated with anti-GalC antibody for 14 d. The cultures were stained with rabbit anti-laminin and fluoresceinated goat anti-rabbit IgG. The intensity of fluorescence due to laminin staining is essentially the same in control and treated cultures. ×440.

low but definite levels of the myelin proteins (Fig. 6, *c, d*). These Schwann cells also showed low levels of noninternalized surface GalC. In conclusion, a small population of Schwann cells in axonal contact expressed myelin proteins at low levels even when experimentally restrained from myelin formation either by defined culture medium or the anti-GalC antibody.

The effect of anti-GalC on myelin formation is reversible

If Schwann cells that were treated with anti-GalC retained their ability to form myelin, then removing the antibody from the culture medium should allow myelination to proceed. To test this hypothesis, neuron–Schwann cell cultures were treated with

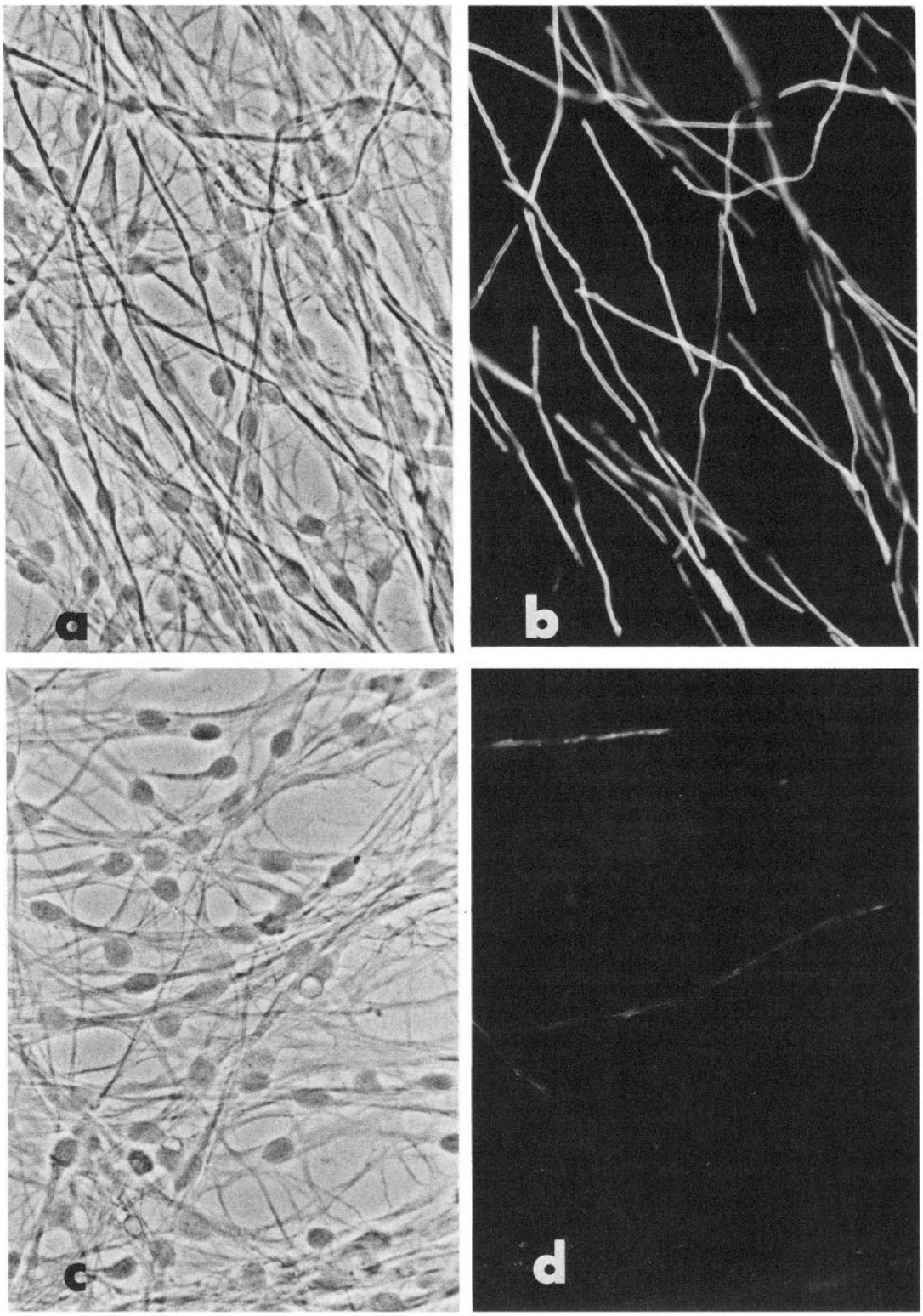

Figure 6. PO expression in Schwann cells interacting with axons in the presence of anti-GalC antibody. *a,* Phase-contrast image of a myelinated control culture. *b,* Fluorescent image of the same field, illustrating staining with rabbit anti-PO antiserum and fluoresceinated goat anti-rabbit IgG after fixation and permeabilization with acid alcohol (see Materials and Methods). Note the correspondence of bright PO staining with the phase image of compact myelin. *c,* Phase-contrast image of a culture treated with anti-GalC antibody for 2 weeks. Compact myelin images are seldom observed in these cultures. The corresponding fluorescent staining of PO is shown in *d.* Some Schwann cells contain detectable amounts of PO, but the intensity of fluorescence is markedly less than that in myelinating Schwann cells. The fields illustrated in *b* and *d* were photographed and printed identically so that a comparison of fluorescence intensity could be made. ×440.

anti-GalC IgG purified from ascites fluid at a concentration of 150 μg/ml for 16 d. By this time, myelin segments had formed in control, but not in antibody-treated cultures. Shortly after switching to the antibody-free culture medium, Schwann cells expressed GalC at normal levels. By 16 d, extensive myelination was monitored using Sudan black staining. This rapid recovery demonstrates that Schwann cells retained their capability to form normal myelin despite their previous treatment with anti-GalC.

Discussion

In both the developing PNS and CNS, GalC is the first myelin component to appear on myelinating cells (Mirsky et al., 1980; Ranscht et al., 1982) and has been suggested as playing some role in myelination (Bornstein and Raine, 1970; Dubois-Dalcq et al., 1970; Fry et al., 1973; Hruby and Seil, 1977; Dorfman et al., 1979; Saida et al., 1979). The present study was undertaken to define the role of GalC in neuron–Schwann cell interactions during peripheral myelin formation. In this analysis we examined the effects of monoclonal anti-GalC IgG on Schwann cell function in a culture system consisting of neurons and Schwann cells.

In these studies, anti-GalC IgG consistently prevented myelination. Low levels of antibody (12 μg/ml) were sufficient to block myelin formation by more than 99%. The anti-GalC antibody specifically blocked the formation of myelin lamellae by preventing the elongation of the mesaxon. Concomitantly, the accumulation of myelin proteins that accompanies the compaction of myelin was prevented. The ensheathment of small axons and the deposition of extracellular matrix were unaffected. In the presence of anti-GalC antibody, the segregation of the large axons and the formation of the mesaxon also proceeded normally.

The proposed mechanism for the inhibition of myelin formation by anti-GalC is the removal of GalC from Schwann cell surfaces by internalization as antigen–antibody complexes. Through the reapplication of fresh anti-GalC to the cultures at 24 hr intervals, the levels of GalC on the Schwann cell surface were kept low, in some cases beyond detection, for the duration of the experiments. A possible explanation of our results, therefore, is that an insufficient number of GalC molecules were able to interact with a putative receptor on the opposing Schwann cell membrane, and thus prevented the spiral movements of the mesaxon that forms the myelin lamellae.

The internalization of the GalC antigen–antibody complex from the Schwann cell surface did not alter the expression of 217C antigen or affect the deposition of a number of extracellular matrix proteins. Therefore, Schwann cells treated with anti-GalC retain an essential function that is prerequisite for myelin formation. Schwann cells do not ensheathe and myelinate axons in the absence of a basal lamina (Moya et al., 1980; Carey et al., 1985). Even though a Schwann cell surface protein and several extracellular matrix components were not internalized in the continued presence of the anti-GalC antibody, the possibility that molecules not accessible to examination were internalized together with the GalC antigen–antibody complex, and contributed to the observed effects, cannot be excluded.

Anti-GalC cross-reacts with its sulfate ester, sulfatide, but to a 16-fold lesser extent than with GalC (Ranscht et al., 1982). At the concentrations of anti-GalC used in these experiments, it is unlikely that all the sulfatide would be bound by the antibody; thus some free sulfatide would be left on the Schwann cell surface. If sulfatide and not GalC mediated myelination, we should have obtained a much smaller inhibitory effect on myelination than the one observed. Thus, while our study cannot rule out a possible role for sulfatide in myelination, we believe that the best interpretation of our results is that myelination was blocked through a direct effect of the antibody on GalC.

It was not possible to confirm the above results by the use of monovalent anti-GalC Fab′ fragments. The affinity of the monovalent antibody was too low to yield any data. At concentrations of 1 mg/ml anti-GalC Fab′, no binding to Schwann cell surfaces could be monitored in indirect immunofluorescence. This concentration already exceeds the saturation level of divalent antibody by a factor of 200. A further complication in these studies was the relatively low yield of anti-GalC IgG after purification. Anti-GalC IgG tended to form large precipitates that were insoluble under physiological conditions.

The specificity of the effect of anti-GalC on myelination was controlled with the monoclonal antibody against the Schwann cell surface component 217C. Anti-217C did not affect the normal process of myelination at the same doses at which anti-GalC blocked the formation of myelin completely. This suggests that the prevention of myelin lamellae formation is a specific effect of the anti-GalC antibody. In contrast to GalC, the 217C–anti-217C complex was never internalized beyond detection. This possibly reflects a different rate of internalization and replacement of this protein antigen on the Schwann cell surface from that of the glycolipid GalC. Therefore, the 217C control can only show that the binding of IgG to the Schwann cell surface does not, in itself, prevent myelination. Ideally, as a control for these experiments, one would have liked to use a monoclonal antibody against another Schwann cell surface glycolipid. Such an antibody, however, is not currently available.

The issue has been raised, but not resolved (see Results), of the role of GalC in the ensheathment of small unmyelinated fibers. Clearly, anti-GalC, at concentrations sufficient to completely block myelination, did not dramatically alter ensheathment, while higher levels of antibody appeared to have effects on myelination, ensheathment, extracellular matrix deposition, and nuclear morphology, which we interpreted as being nonspecific. It should be pointed out that the major criterium for the analysis and interpretation of our experiments was normal Schwann cell morphology. Furthermore, the culture system used in the present experiments has been optimized for the study of myelination rather than ensheathment. In this system the ensheathment of small axons is incomplete compared to myelination, within the time frame of the experiment, and a valid method of sampling to quantitate ensheathment has not been firmly established (M. Bunge, personal communication). It is therefore likely that a small effect of anti-GalC on ensheathment would not have been detected by our analysis. Thus, the role of GalC in ensheathment remains to be established.

Antibody to GalC, at levels that blocked myelination completely, did not prevent the larger axons from being segregated from other axons and being surrounded by a single Schwann cell. The process of mesaxon formation was initiated, but extensive mesaxon elongation and compaction, required for myelin formation, did not occur. In a separate study, evidence is presented that Schwann cell nuclear movements in the early phases of myelin deposition indicate an active progression of the inner lip of the Schwann cell spiral over the axonal surface (R. Bunge et al., 1986). The mechanism of mesaxonal elongation

appears to require that the inner lip slips by the next Schwann cell surface layer. Removal of GalC from this interface may prevent the necessary membrane slippage and "freeze" the process of spiral growth. GalC may not be the only, but certainly seems to be, one of the key components involved in the construction of the remarkable spiral structure that forms each myelin internode.

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