

Galactocerebroside Is Expressed by Non-Myelin-forming Schwann Cells In Situ

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ABSTRACT Interest in the glycosphingolipid galactocerebroside (GC) is based on the consensus that in the nervous system it is expressed only by myelin-forming Schwann cells and oligodendrocytes, and that it has a specific role in the elaboration of myelin sheaths.

We have investigated GC distribution in two rat nerves—the sciatic, containing a mixture of myelinated and non-myelinated axons, and the cervical sympathetic trunk, in which > 99% of axons are non-myelinated. Immunohistochemical experiments using mono- and polyclonal GC antibodies were carried out on teased nerves and cultured Schwann cells, and GC synthesis was assayed biochemically. Unexpectedly, we found that mature non-myelin-forming Schwann cells in situ and in short-term cultures express unambiguous GC immunoreactivity, comparable in intensity to that of myelinated fibers or myelin-forming cells in short-term cultures. GC synthesis was also detected in both sympathetic trunks and sciatic nerves. In the developing sympathetic trunk, GC was first seen at day 19 in utero, the number of GC-positive cells rising to ~95% at postnatal day 10. In contrast, the time course of GC appearance in the sciatic nerve shows two separate phases of increase, between day 18 in utero and postnatal day 1, and between postnatal days 20 and 35, at which stage ~94% of the cells express GC. These time courses suggest that Schwann cells, irrespective of subsequent differentiation pathway, start expressing GC at about the same time as cell division stops.

We suggest that GC is a ubiquitous component of mature Schwann cell membranes in situ. Therefore, the role of GC needs to be reevaluated, since its function is clearly not restricted to events involved in myelination.

Galactocerebroside (GC)¹ is a simple glycosphingolipid that, because it is the major glycolipid of the myelin sheath, has been the subject of numerous studies. It has generally been assumed that in the nervous system, only oligodendrocytes and myelin-forming Schwann cells express this molecule, and that it is absent even from closely related cells such as non-myelin-forming Schwann cells. GC has therefore assumed a specific importance in studies on myelin and myelin formation (16, 18, 20–22). Our recent studies reported below suggest that this view is incorrect. Unexpectedly, we find that GC is expressed by non-myelin-forming Schwann cells in adult rat peripheral nerves, and is therefore not restricted to myelin-forming cells.

¹ Abbreviations used in this paper: GC, galactocerebroside; GFAP, glial fibrillary acidic protein.

The possibility that GC is a universal component of Schwann cell plasma membranes appears not to have been seriously investigated before. The association of this molecule with oligodendrocytes and myelin-forming Schwann cells is, however, well documented. GC comprises ~15% of the myelin lipid in the rat central nervous system (8) and can be localized immunohistochemically over myelin sheaths in tissue sections from both the central and peripheral nervous systems (25).

We have shown previously that even in the absence of axonal contact, oligodendrocytes express GC for many weeks in culture, and the molecule can therefore be used as a specific surface marker for these cells in dissociated cell cultures from the central nervous system (16, 21). GC can also be demonstrated immunohistochemically on the surface of myelin-

forming Schwann cells after they have been removed from axonal contact and plated out in tissue culture. Under these conditions, however, Schwann cells, unlike oligodendrocytes, gradually stop expressing GC during the first few days *in vitro* (16). In development, both oligodendrocytes and Schwann cells start expressing GC significantly before other myelin components, such as sulfatide and basic protein, can be detected (22, 28).

On the basis of the present results, the role of GC in the nervous system needs to be reevaluated. We suggest that, rather than being uniquely associated with myelin and myelin-forming cells, GC is in fact a ubiquitous component of mature Schwann cell membranes *in situ*. Clearly, therefore, the function of this glycolipid is not restricted to events involved in myelination.

MATERIALS AND METHODS

Teased Nerve Preparations

Cervical sympathetic trunk and sciatic nerves from Sprague Dawley rats of various ages were excised, placed in a small amount of phosphate-buffered saline (PBS), and partially teased into nerve bundles and individual fibers under the dissecting microscope, using fine syringe needles. In routine experiments, samples were allowed to air dry before immunofluorescent staining. In two experiments, the results obtained on dried nerves using GC antibodies (see below) were confirmed using wet, partially teased sympathetic trunks.

Cell Cultures

Cervical sympathetic trunk and sciatic nerves were removed from Sprague Dawley rats of various ages, ranging from embryonic day 15 to postnatal day 35. The tissues were dissociated in trypsin alone, or trypsin and collagenase, and were cultured on poly-L-lysine-coated glass coverslips essentially as described previously for sciatic nerves (5). The nerves from 15- and 16-d embryos were digested in 0.05% trypsin in Eagle's minimum essential medium plus 0.02 M HEPES, pH 7.4, for 25 min at 37°C before dissociation. Nerves from older embryos and postnatal rats were digested for times ranging from 5 min to 1 h in 0.15% collagenase (type II, Flow Laboratories Ltd., Irvine, UK) in Eagle's minimum essential medium plus 0.02 M HEPES followed by addition of 0.05% trypsin in the same medium for a further 15 min before dissociation.

Antibodies

Ascites fluid containing mouse monoclonal antibody to GC was produced and characterized by Dr. B. Ranscht (22). It is of the IgG₃ subclass and was used at dilutions of 1:100 to 1:1,000. Clear visualization of GC on cultured cells was obtained at dilutions as low as 1:1,000. In teased nerves, a dilution of 1:200 was most often used, although GC could also be visualized using more dilute antibody solutions. Concentrated hybridoma supernatant containing mouse monoclonal antibody, 01, to GC (23) was used at a dilution of 1:100. Rabbit antiserum to GC (21, 22) was used at a dilution of 1:50. Rabbit antiserum to human glial fibrillary acidic protein (GFAP) was produced by Dr. R. Pruss. Its specificity has been described elsewhere (12), and it was used at a dilution of 1:1,000 or 1:2,000. Rabbit antiserum to cow S-100 protein (DAKO Immunoglobulins a/s, Copenhagen, Denmark) was used at a dilution of 1:200. Rabbit antiserum to P₀ was produced and characterized by Dr. J. P. Brockes (6). Rhodamine conjugated to goat anti-mouse Ig (Cappel Laboratories, Cochranville, PA), adsorbed with rabbit Ig to remove cross-reacting antibodies, was used at a dilution of 1:100. Fluorescein conjugated to goat anti-rabbit Ig (Nordic Laboratories, Ltd., Maidenhead, Berks, UK), adsorbed with mouse Ig to remove cross-reacting antibodies, was used at a dilution of 1:100. Control antibodies used were either normal mouse ascites (1:20 and 1:200) or myeloma AG8 ascites (1:200), in the case of GC antibodies, and normal rabbit serum at appropriate dilutions for the other antibodies used.

Immunofluorescence

TEASED NERVES: Immunostaining was done at room temperature. Antibodies were diluted in PBS containing 10% fetal calf serum and 0.1 M lysine. PBS was used for washes, and Citifluor (City Ig, Northampton Square, London) was used as an anti-fade mounting medium. The preparations were incubated with mouse monoclonal GC antibodies for 1 h, washed, and then

incubated with goat anti-mouse Ig rhodamine for 1 h, washed, and then fixed for 10 min in 95% ethanol/5% acetic acid at -12°C. Most often the nerves were then washed and treated for 20 min at room temperature in 0.3% Triton X-100 in PBS, since this maximized the GC fluorescence. This was not a necessary step for visualization of GC in teased nerves, and this treatment had no effect on GC fluorescence in cultured Schwann cells. Some preparations were mounted at this stage. In other experiments, after staining with antibodies to GC, the nerves were double labeled using rabbit antibodies to GFAP or P₀, essentially as described previously (13).

DISSOCIATED CELL CULTURES: Single- and double-label immunofluorescence experiments using antibodies to GC, P₀, and S-100 were done as described previously (5, 16).

GC Synthesis

Cervical sympathetic trunks (total 36 nerves) or sciatic nerves (total 2 nerves) were dissected from 8-10-d-old Sprague Dawley rats, desheathed, and placed in Eagle's minimum essential medium plus 0.02 M HEPES, pH 7.4. They were then chopped into small (1-5 mm) fragments and placed in 0.5 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 50 μ Ci of [³H]-galactose (10.4 Ci/mmol; Amersham International plc, Amersham, UK). The nerves were placed for 5 h at 37°C in 95% air/5% CO₂ in a humidified incubator. They were then separated from radioactive medium by centrifugation (1,000 g for 5 min), washed three times in PBS, resuspended in 0.25 ml of PBS, and homogenized by hand in a small glass homogenizer. A small volume of each sample was removed for protein estimation. Lipids were then extracted using the system described by Brammer and Carey (4). GCs and glucocerebrosides (Sigma Chemical Co., St. Louis, MO) were added to the chloroform phase containing the radioactive lipids at a concentration of 200 μ g/ml to act as carriers before the samples were dried down in a stream of N₂. The extracts were then analyzed using two different thin layer chromatography systems. A chloroform/methanol/water (17:7:1) system using Silica Gel 60 thin layer plates (E. Merck, Darmstadt, FRG) was used to separate cerebrosides from other lipids, and a chloroform/methanol/water (60:17:2) system and Silica Gel 60 plates impregnated with 1% sodium tetraborate were used to separate GCs from glucocerebrosides (3). After chromatography, carrier lipids were visualized with iodine vapor and the appropriate regions of the plate scraped off and counted in Aquasol (New England Nuclear, Dreieich, FRG) in a liquid scintillation counter.

Protein Estimation

Protein was estimated using the Lowry procedure (15).

RESULTS

GC in Teased Nerves

The cervical sympathetic trunk, in which >99% of the axons are enveloped by non-myelin-forming cells (2), and the sciatic nerve, which contains a mixture of myelin and non-myelin forming cells, were removed from 17-25- and 32-38-d-old rats, respectively, teased with fine syringe needles onto microscope slides, and air dried. This separated individual fibers and exposed Schwann cell surfaces. These preparations were then treated with monoclonal GC antibodies to locate GC, and polyclonal antibodies against the myelin-specific protein P₀ to visualize myelin. Surprisingly, we found that in both nerves the distribution of GC and P₀ was only partly overlapping. This was most obvious in the sympathetic trunk. Here, the GC antibodies revealed an unambiguous, speckled immunostaining covering most or all of the fibers. Collagen fibers and blood vessels were unstained (Fig. 1). In contrast, the P₀ antibody labeled only the occasional fiber, in agreement with morphological data. Thus, the overwhelming majority of the GC-positive fibers were P₀-negative unmyelinated Ramak fibers.² Similarly, in the sciatic nerve, GC immunoreac-

² The term unmyelinated fiber is used synonymously with the term Remak fiber and refers to a complex of several non-myelinated axons and the Schwann cell that they share. An unmyelinated (Remak) fiber thus usually contains more than one axon. The term myelinated fiber designates one axon and its myelin sheath.

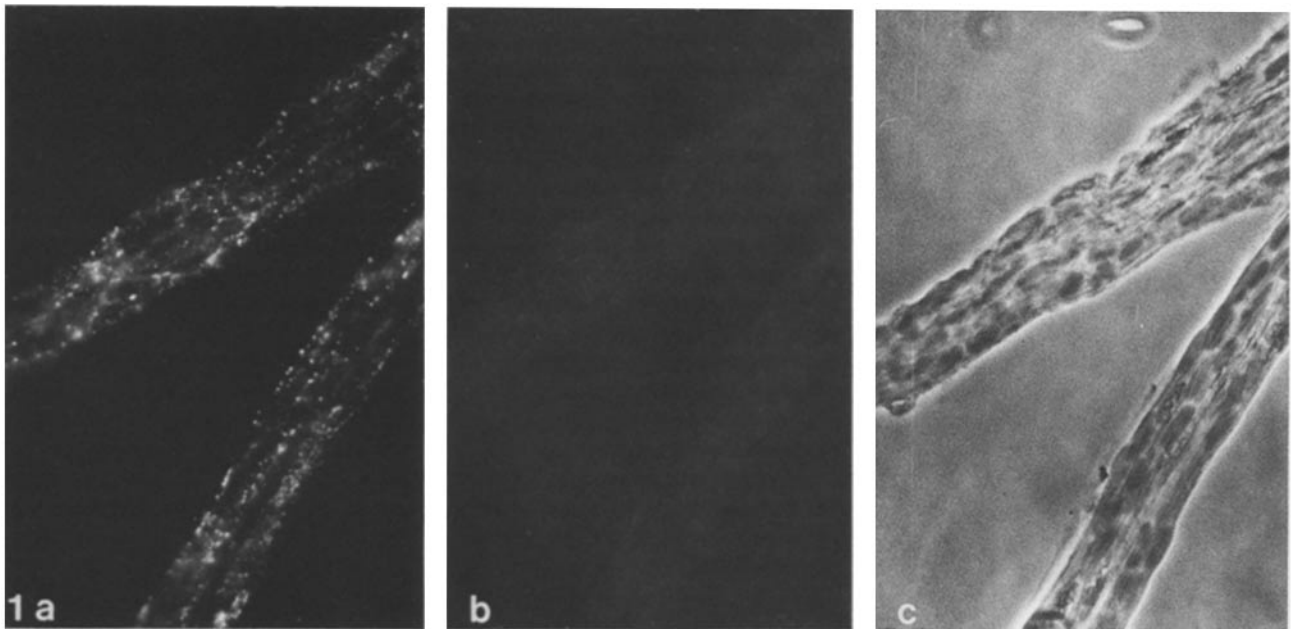


FIGURE 1 Double-label immunofluorescence using antibodies to GC and P_0 in a teased preparation of cervical sympathetic trunk from 10-d-old rats. (a) Rhodamine optics to visualize GC. (b) Fluorescein optics to visualize P_0 . (c) Phase contrast. Note speckled GC-positive fiber bundles, which are P_0 -negative, indicating that they are unmyelinated fibers. $\times 600$.

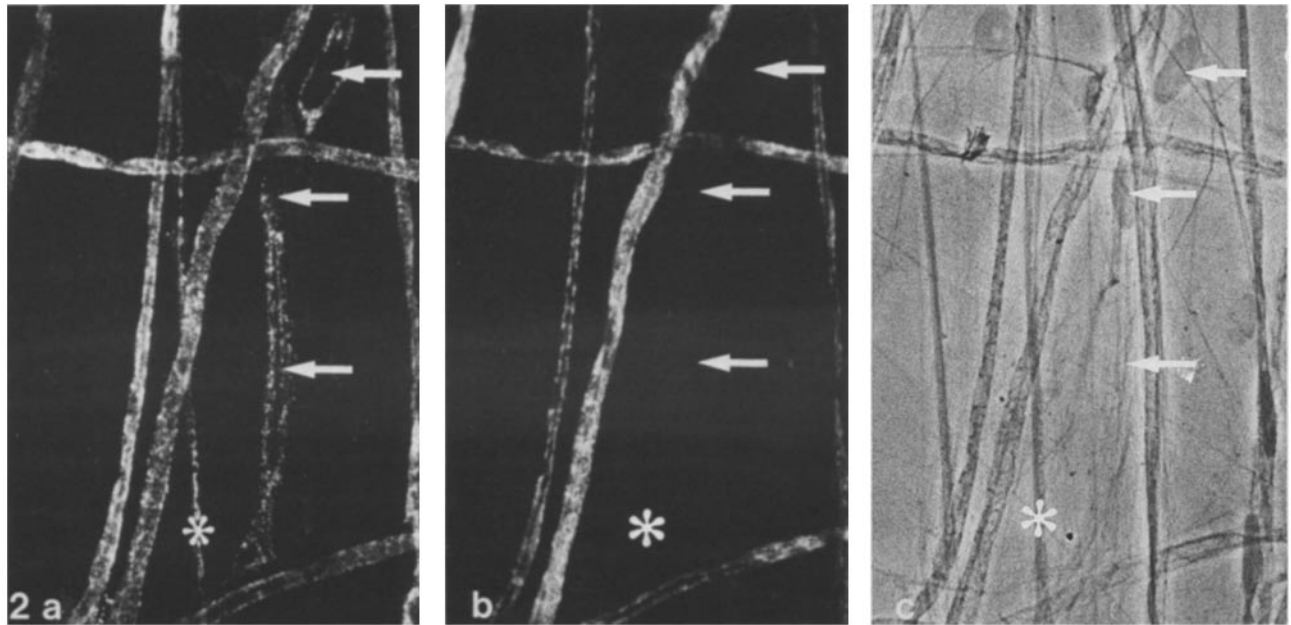


FIGURE 2 Double-label immunofluorescence using antibodies to GC and P_0 in a teased preparation of sciatic nerve from 35-d-old rats. (a) Rhodamine optics to visualize GC. (b) Fluorescein optics to visualize P_0 . (c) Phase contrast. Note that many fibers are both GC-positive and P_0 -positive, indicating that they are myelinated, but several unmyelinated fibers are GC-positive and P_0 -negative. Three arrows indicate the course of one such unmyelinated GC-positive, P_0 -negative fiber, and the asterisk indicates another. $\times 600$.

tivity was not restricted to the many P_0 -positive myelinated fibers, but also covered P_0 -negative fibers, which by phase contrast conformed morphologically to non-myelin-forming cells (Fig. 2).

The identity of the GC-positive, P_0 -negative fibers as Remak fibers was confirmed in double-label experiments using the monoclonal GC antibodies, this time together with rabbit antiserum raised against GFAP. This procedure took advantage of the observation that, in rat nerves, GFAP filaments

are present in non-myelin-forming Schwann cells only (14, 30). In these experiments, the myelin-forming Schwann cells, generally identifiable by phase contrast, were GFAP-negative. Most of the GFAP-positive non-myelinated fibers, however, were GC-positive (Figs. 3 and 4).

GC on Schwann Cells after 3 h in Culture

To confirm the results obtained from teased nerves, we studied GC expression by Schwann cells in dissociated cell

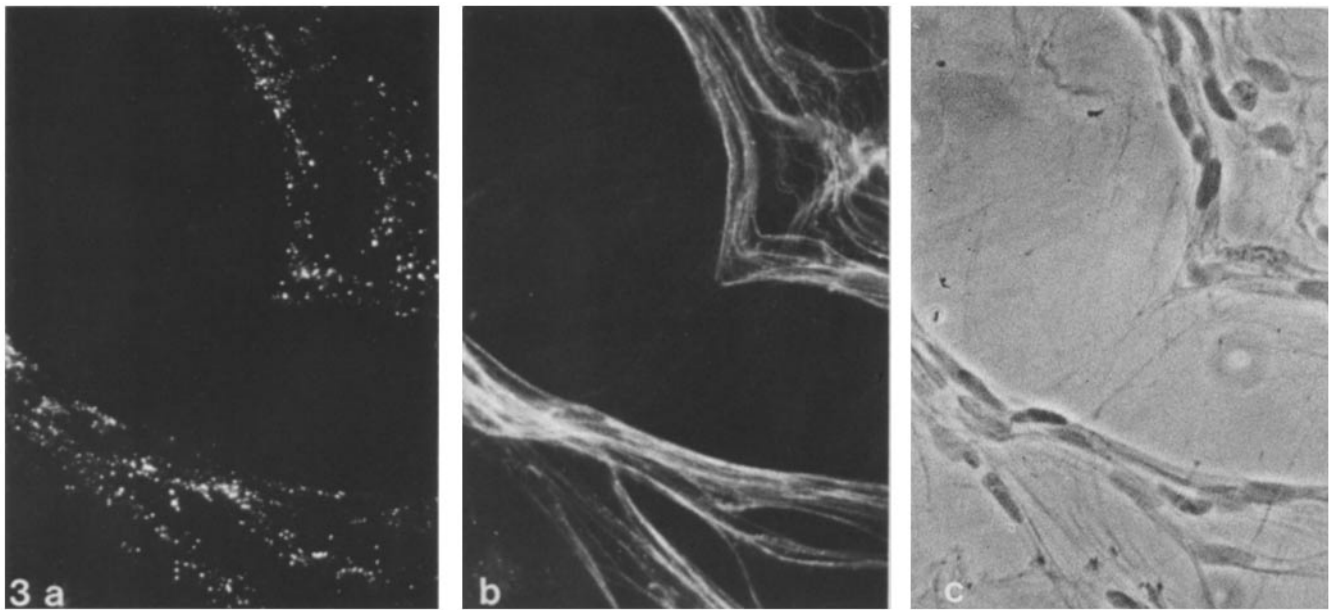


FIGURE 3 Double-label immunofluorescence using antibodies to GC and GFAP in a teased preparation of cervical sympathetic trunk from 10-d-old rats. (a) Rhodamine optics to visualize GC. (b) Fluorescein optics to visualize GFAP. (c) Phase contrast. Note the overlap between GC-positive and GFAP-positive fluorescence in the two unmyelinated fiber bundles in which the Schwann cell nuclei can be seen prominently in the phase-contrast micrograph (c). $\times 600$.

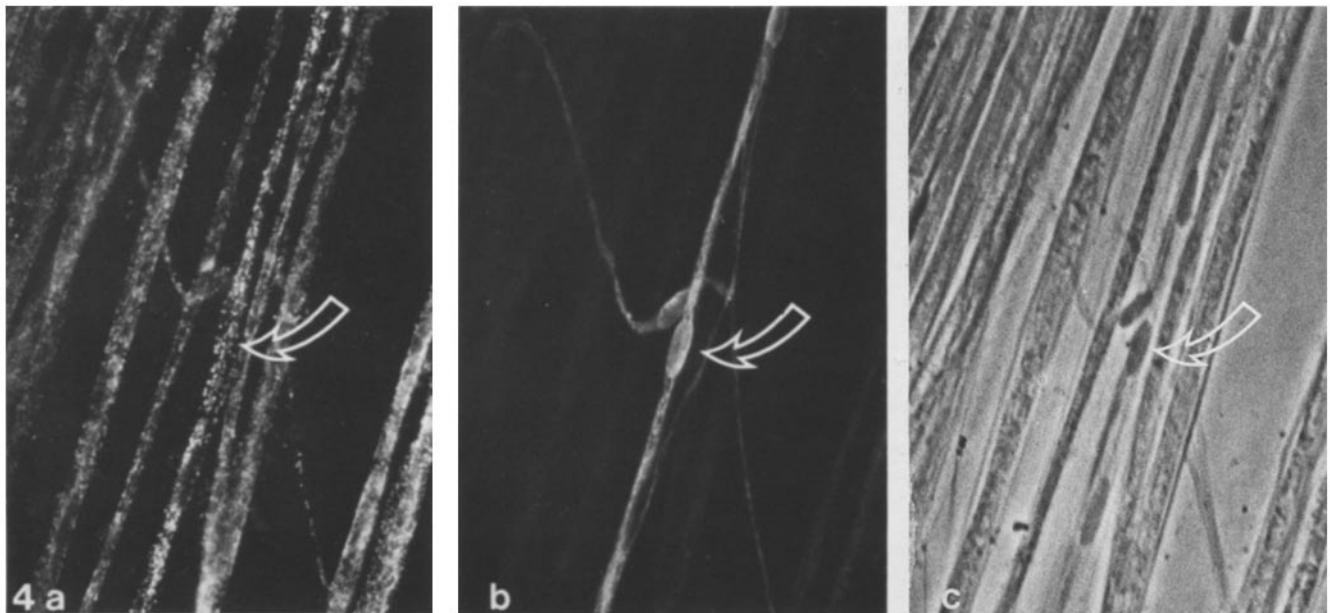


FIGURE 4 Double-label immunofluorescence using antibodies to GC and GFAP in a teased preparation of sciatic nerve from 35-d-old rats. (a) Rhodamine optics to visualize GC. (b) Fluorescein optics to visualize GFAP. (c) Phase contrast. Note the large number of GC-positive fibers, representing both myelinated and unmyelinated fibers. The open arrow points to a GC-positive, GFAP-positive unmyelinated fiber, with the Schwann cell nucleus clearly visible in the phase-contrast micrograph (c). $\times 600$.

cultures. Significant loss of GC expression due to removal from axonal contact (see below and reference 16) was avoided by staining the cells after only 3 h in culture. In double-labeled immunohistochemical experiments, using the monoclonal GC antibody and S-100 as a specific Schwann cell marker (see Materials and Methods), $95 \pm 0.85\%$ (mean \pm SEM) of the Schwann cells in cultures from the sympathetic trunk removed from 10-d-old animals expressed GC (total of 1,066 cells counted, $n = 4$). Less than 0.5% of the Schwann cells in these cultures expressed P₀, and therefore represented cells

that in situ had formed myelin. In the same type of experiment on sciatic nerve cultures obtained from 35-d-old animals, $94 \pm 0.92\%$ (mean \pm SEM) of the Schwann cells expressed GC (total of 1,010 cells counted, $n = 4$) (Figs. 5, 6, and 7).

The monoclonal GC antibody also recognizes psychosine in an ELISA assay (22). We therefore checked our results using a GC antiserum that does not recognize psychosine and that has been used by us in previous studies on GC (21, 22). In double-label immunofluorescence experiments using the monoclonal and polyclonal GC antibodies, >90% of the

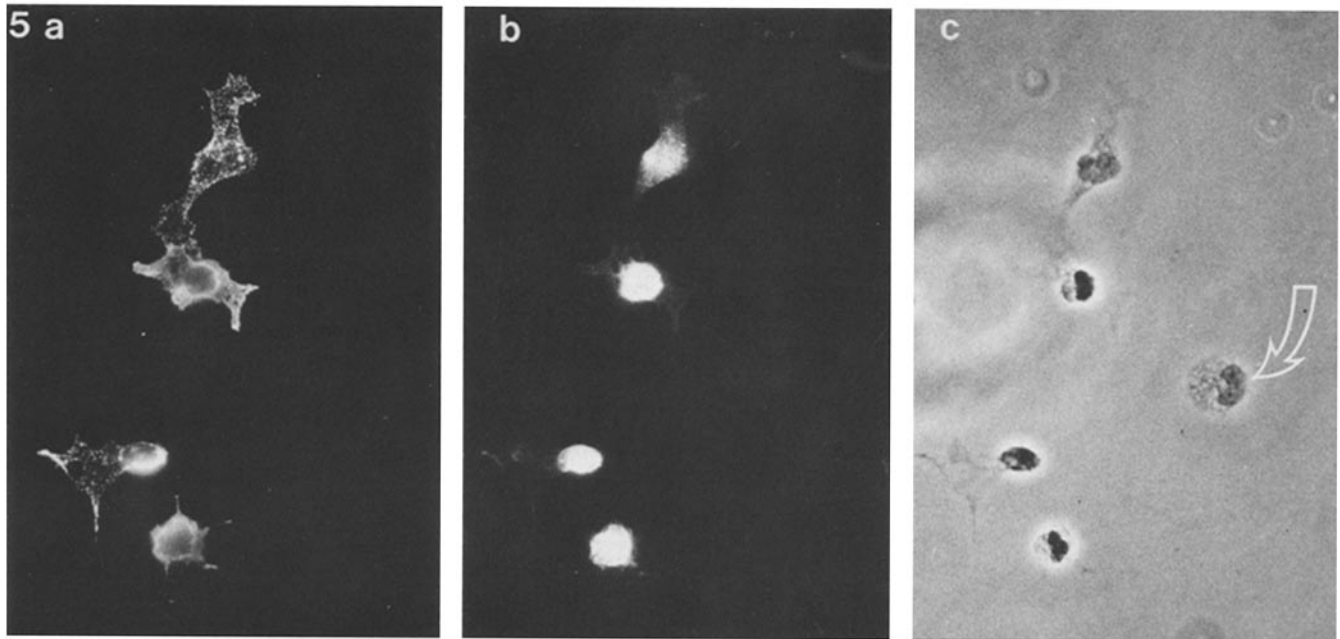


FIGURE 5 Double-label immunofluorescence using antibodies to GC and S-100 in a dissociated cell culture from cervical sympathetic trunk from 10-d-old rats, 3 h after plating. (a) Rhodamine optics to visualize GC (b) Fluorescein optics to visualize S-100. (c) Phase contrast. Note the four GC-positive, S-100-positive Schwann cells, and one GC-negative, S-100-negative cell, presumably a fibroblast (arrowed). $\times 600$.

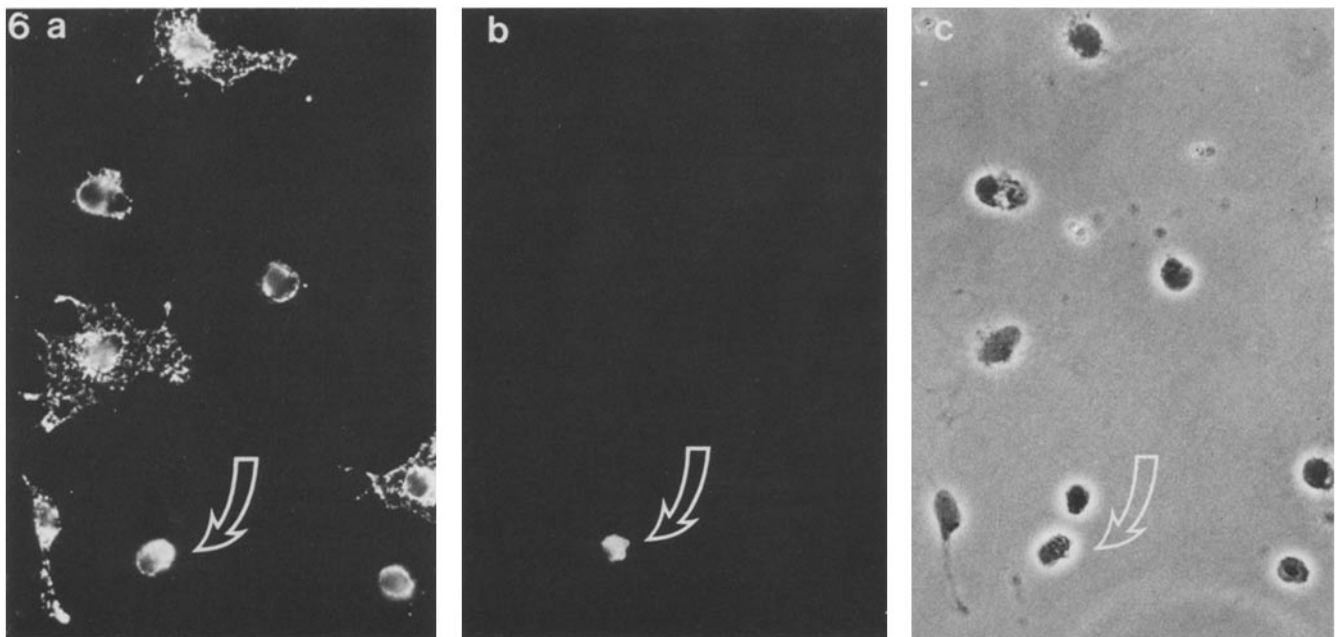


FIGURE 6 Double-label immunofluorescence using antibodies to GC and P₀ in a dissociated cell culture from cervical sympathetic trunk from 10-d-old rats, 3 h after plating. (a) Rhodamine optics to visualize GC. (b) Fluorescein optics to visualize P₀. (c) Phase contrast. Note the single GC-positive, P₀-positive Schwann cell (arrowed), surrounded by several GC-positive, P₀-negative Schwann cells. One cell, probably a fibroblast, is unlabeled with either antibody. $\times 600$.

Schwann cells were double labeled in each of three separate experiments, indicating that these Schwann cells do indeed express GC. We also used another monoclonal antibody, 01, against GC (23) to confirm the results obtained on the Schwann cell cultures from the sympathetic trunk.

GC Synthesis from [³H]Galactose

To demonstrate that GC synthesis could occur in the nerves of the cervical sympathetic trunk, we incubated freshly dis-

sected trunks with [³H]galactose to see whether they could incorporate it into GC; parallel experiments were done on sciatic nerves.

[³H]Galactose was clearly incorporated into GC in the cervical sympathetic trunk and sciatic nerve under the conditions used. To establish the identity of the radiolabeled lipids, two different thin layer chromatography systems were used (see Materials and Methods). The first of these systems, using Silica Gel 60 plates and chloroform/methanol/water

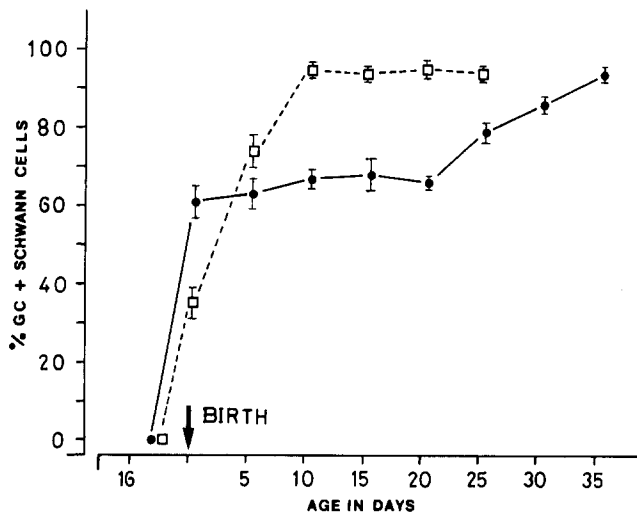


FIGURE 7 Development of GC-positive Schwann cells in sciatic nerve and cervical sympathetic trunk. Sciatic nerves (●) and cervical sympathetic trunks (□) from rats of different ages were dissociated and double labeled with antibodies to GC and S-100 after 3 h in culture as described in Materials and Methods. The percentage of GC-positive Schwann cells at each time point is shown on the graph. Results at each time point were obtained from a minimum of three experiments, with a total of at least 1,000 Schwann cells counted.

TABLE I. Percentage Distribution of Radioactivity between Lipid Classes

Lipid classes	Sciatic nerves	Sympathetic trunks
A. Distribution of label between phospholipids and cerebroside		
Cerebrosides	85.5 ± 3.2%	74.8 ± 4.5%
Ethanolamine phosphoglycerides	3.6 ± 0.9%	11.1 ± 2.5%
Sulfatide	8.6 ± 1.8%	7.5 ± 4.3%
Choline, serine, and inositol phosphoglycerides	2.3 ± 1.9%	6.7 ± 10.3%
B. Distribution of label within cerebroside		
Galactocerebrosides	82.0 ± 2.1%	59.3 ± 9.4%
Glucocerebrosides	18.9 ± 2.4%	40.7 ± 9.4%

Results are the means ± SEM of four experiments done on separate preparations of sciatic nerves or sympathetic trunks.

(17:7:1) as the developing solvent, separates cerebroside from other lipid classes, but results in the co-migration of glucosyl and galactocerebrosides. A second system, using borate-impregnated plates, is required to effect separation of the two classes of cerebroside. Chloroform-soluble radioactive lipids were examined using both of these systems, and the zones co-migrating with authentic cerebroside and other lipids were analyzed for radioactivity. The percentage distribution of radioactivity between lipid classes is shown in Table I. Table I, A shows that, using the first of the two systems described above, the bulk of the radioactivity co-migrates with authentic cerebroside after incubation of either sympathetic trunks or sciatic nerves with labeled galactose. Table I, B indicates that most of the radioactivity co-migrating with cerebroside on nonimpregnated silica gel plates is recovered in the same zone as authentic GC standard when cerebroside is subfraction-

ated using borate-impregnated plates. The absolute levels of incorporation of radioactive galactose into GC in sciatic nerves and cervical sympathetic trunks were 229.6 ± 48.5 dpm/ μ g protein and 75.1 ± 14.6 dpm/ μ g protein, respectively (mean ± SEM, $n = 3$), giving a ratio of incorporation in sciatic nerve to cervical sympathetic trunk of 3.04. In addition to GC, radioactive sulfatide was also formed in sciatic nerves at levels of 29 ± 8.5 dpm/ μ g protein and in cervical sympathetic trunks at levels of 5 ± 1.8 dpm/ μ g protein (mean ± SEM, $n = 3$). It is known that *in vivo* sulfatide is formed from GC.

Although both sympathetic trunks and sciatic nerve are clearly synthesizing GC, the relative proportion of glucocerebroside is higher in the sympathetic trunks than in the sciatic nerve (see Table I). Glucocerebroside is a precursor of gangliosides and appears to be formed mainly in neuronal cell bodies (3). Scattered neuronal cell bodies are often found along the sympathetic trunk but are absent from the sciatic nerve.

Development of GC Expression in Schwann Cells

The developmental appearance of GC expression was studied using dissociated cultures maintained for 3 h before immunostaining as described above. The results are shown in Fig. 7. Briefly, in the sympathetic trunk, GC expression was first detected at embryonic day 19, whereas in the sciatic nerve GC first appeared at embryonic day 18. The maximum number of GC-positive cells in the sympathetic trunk was reached at 10-d postnatal, whereas in the sciatic nerve the number of GC-positive Schwann cells continued to rise until postnatal day 35.

Regulation of GC Expression in Non-Myelin-forming Schwann Cells

To study the regulation of GC expression in non-myelin-forming cells, two types of experiments were done on Schwann cells from the sympathetic trunk.

First, we investigated whether GC would appear developmentally on schedule in Schwann cells removed from axonal contact at embryonic day 18, i.e., ~1 d before the first Schwann cells normally start expressing the molecule *in situ*. Dissociated cell cultures prepared from trunks removed from 18-d-old embryos were maintained for 13 d (reaching an age equivalent to postnatal day 10). No GC was detected in immunostaining of these cultures.

Second, we determined whether non-myelin-forming Schwann cells that had acquired GC during normal development maintained this expression when they were removed from axonal contact and grown in culture. Cultures were prepared from 10-d-old sympathetic trunks, at which stage ~95% of the Schwann cells are GC-positive, and maintained for 4 d. GC expression was monitored during this period using S-100 as a Schwann cell marker in double-label immunofluorescence experiments. The results are expressed in Fig. 8. They show that the number of Schwann cells that express GC steadily declines under these conditions, reaching zero after ~3 d. Similar experiments were done on the Schwann cells from sciatic nerves, also removed from animals at a stage when GC expression was fully developed, in this case at 35 d. GC disappeared from these cultures with a time course similar to that seen in the sympathetic trunk (Fig. 8).

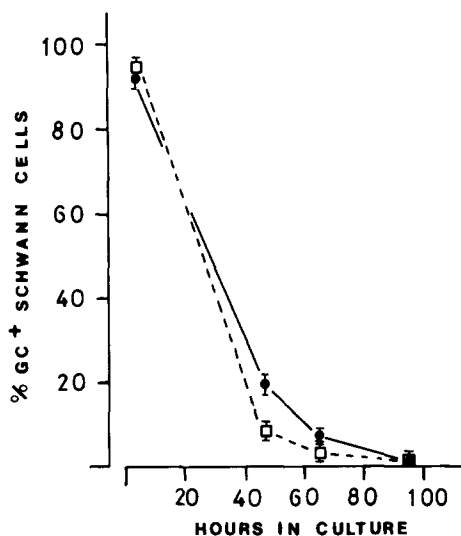


FIGURE 8 Disappearance of GC from Schwann cells in dissociated cell cultures of sciatic nerve (●) and cervical sympathetic trunk (□). Cells were labeled with antibodies to GC and S-100 at different times in vitro as described in Materials and Methods. Results at each time point were obtained from a minimum of three experiments with a total of at least 1,000 Schwann cells counted.

DISCUSSION

In this paper, we show that the glycolipid GC is expressed in the surface membrane of mature non-myelin-forming Schwann cells in situ in the rat peripheral nervous system. The evidence rests on immunohistochemical experiments, using both monoclonal and polyclonal GC antibodies, and is supported by biochemical data on GC synthesis.

This observation contradicts the previous consensus of opinion regarding the role and distribution of GC, which holds that this molecule is specifically involved in some event connected with myelin formation. This view was based on several lines of evidence: GC is found in high quantities in the nervous system and in particular in isolated preparations of oligodendrocytes, while only trace amounts are present elsewhere, and constitutes ~15% of the total lipid found in myelin (e.g., references 8, 18, and 20). In tissue sections from brain and peripheral nerves, GC immunoreactivity is associated with myelin sheaths, whereas in these experiments no immunoreactivity is described elsewhere (25). Furthermore, GC largely co-distributes with the myelin-associated protein P₀ among Schwann cells in early cultures of dissociated sciatic nerve, taken from 4–6-d-old rats (unpublished observations). In similar cultures from the superior cervical ganglion, where myelin-forming cells are infrequent, very few Schwann cells expressed GC (16). Similarly, GC is restricted to oligodendrocytes in dissociated cultures from 4–6-d-old rat brain (16, 21, 22). Antibodies to GC can reversibly inhibit myelination in culture and induce demyelination in situ (7). On the basis of these data, it has become accepted that synthesis and expression of GC is a major and characteristic feature of those cells that elaborate myelin, i.e., oligodendrocytes and myelin-forming Schwann cells.

A number of factors may have contributed to the failure of these studies to detect GC on non-myelin-forming Schwann cells. These experiments have not included nerves, such as the sympathetic trunk, in which non-myelin-forming cells are obvious, since they make up almost the whole tissue. With

light microscopy, such fibers are much harder to detect and study in those nerves in which they are found among many myelinated axons, as for instance in the sciatic nerve. In this situation it is helpful to use immunohistochemical tools that will selectively mark non-myelin-forming Schwann cells and distinguish them from the myelin-forming ones. For this purpose, we have used GFAP antibodies to visualize the GFAP-containing intermediate filaments selectively present in non-myelin-forming Schwann cells in situ (14). Alternative markers for non-myelin-forming Schwann cells in situ include the cell surface molecules Ran-2 and A5E3 antigen (13). In tissue sections, the immunohistochemical visualization of GC in non-myelin-forming cells is also more difficult than in myelin-forming cells because of the layered structure of myelin. This ensures that the quantity of GC surrounding a myelinated axon is many times that associated with non-myelinated fibers, even if the quantity of GC per unit area of membrane were similar in myelin and in the plasma membrane of the non-myelin-forming cells.

Our developmental studies show that GC expression is a relatively late maturational event. In the cervical sympathetic trunk, GC is first detectable on day 19 in utero. The number of Schwann cells expressing the molecule then rises fairly evenly to level off at postnatal day 10, when ~95% of the S-100-positive cells are also GC positive (Fig. 7). This time course is similar to that of several other maturational events in the same nerve. Thus, axonal diameter, total number of Schwann cells, and the ratio of axons to Schwann cells all show a postnatal phase of rapid development toward mature levels that ends at ~day 14. The segregation and enclosure of axons by Schwann cell processes into individual troughs does not coincide with the appearance of GC, since it occurs significantly earlier and is to a large extent completed by day 7 (2). Previously, we reported that very few glial cells in the superior cervical ganglion expressed GC at day 5 (16). In repeat experiments, we have found that the glial cells in the ganglion develop GC even later than those in the sympathetic trunk. Under the present experimental conditions, using monoclonal GC antibodies and shorter times between cell plating and immunostaining, ~10% of the S-100-positive cells expressed GC in dissociated cultures obtained from 5-d-old ganglia maintained for 3 h before staining, while full GC expression, or ~95%, was reached at day 35 (unpublished observations).

In the sciatic nerve, the time course of GC development is quite different from that in the cervical sympathetic trunk (Fig. 7). It shows two separate phases of rapid increase—the first between embryonic day 19 and postnatal day 1 and the second approximately between days 20 and 35, at which stage ~94% of the S-100-positive Schwann cells also express GC. This time course is not similar to that of the appearance of myelin sheaths in this nerve. Myelin sheaths are first seen at postnatal day 1 and their number increases rapidly during the first week of life and more slowly thereafter (11, 26, 27). As far as can be judged from the literature, however, the first very rapid phase of increase in cells expressing GC coincides with another important development, namely the period during which most of the axons that will later become myelinated segregate to achieve a 1:1 relationship with the Schwann cell. The attainment of the 1:1 ratio between axons and Schwann cells is presumed to be accompanied by cessation of division of this Schwann cell population (27). From the qualitative ultrastructural studies of Diner (9), Peters and Muir (19),

Friede and Samorajski (11), and Aguayo and his collaborators (1, 2), it can be inferred that Remak fibers develop significantly later in the sciatic nerve than in the sympathetic trunk. As discussed above, their development in the sympathetic trunk appears to have reached a mature stage at day 15, while at that date only the very first mature Remak fibers are appearing in the sciatic nerve (9, 11). Similarly, Schwann cell proliferation, which in the trunk is largely over at day 15 (2), is still significant at that date in the sciatic nerve, reaching very low levels by day 28 (11). Comparing our results to those data, we suggest that the second rise in the number of GC-positive cells in the sciatic nerve is related to the late emergence of mature Remak fibers in this nerve.

A clue to how GC expression may be related to other aspects of cellular differentiation comes from studies on oligodendrocytes. In these cells, there is good evidence that GC first appears at about the same time as cell division stops in the precursor cell (10, 17, 29). A similar relationship may exist in Schwann cells, irrespective of their differentiation into myelin- or non-myelin-forming cells. If this is correct, and GC appears at about the time at which a mitotic immature cell gives rise to a more mature postmitotic cell, then the developmental curves for GC appearance in Fig. 7 would parallel the rate at which Schwann cells come out of division, i.e., their rate of maturation. In the sympathetic trunk, this would occur fairly evenly with time. This is compatible with data on the development of the number of Schwann cell units in this nerve, although detailed studies on mitotic frequency are not yet available (2). In the sciatic nerve, our data would predict the existence of two periods of relatively rapid Schwann cell maturation with the accompanying cessation of division and GC acquisition, i.e., between embryonic day 18 and postnatal day 1, and between postnatal days 20 and 35. These phases would be separated by a period of slower Schwann cell maturation, i.e., between days 1 and 20. Presumably the increase in the number of GC-positive cells during this period is slow enough to be balanced by ongoing division of GC-negative cells, thus causing the relative number of GC-positive Schwann cells to stay approximately constant. As in the sympathetic trunk, this hypothesis is compatible with previous studies on myelination and development of Remak fibers in the sciatic nerve (9, 11, 19, 26, 27), but cannot be vigorously tested by comparison with existing data. Those studies on Schwann cell proliferation in the rat sciatic nerve that we are aware of deal mostly with the period between days 1 and 15 and do not provide data of the type needed to test our suggestion. We are at present using a combination of [³H]thymidine injections and GC immunostaining to test more directly whether cessation of division is accompanied by GC expression in the three Schwann cell populations studied here.

Our results suggest that an axonal signal is needed to trigger and maintain GC expression in non-myelin-forming Schwann cells, as in myelin-forming cells. It is therefore likely that, contrary to current thought, the signal that induces GC expression is different from the myelination signal. There is some evidence from experiments done on sciatic nerve cultures that the signal triggering GC expression operates via an increase in intracellular cyclic AMP levels (23). In these experiments, GC could be recalled in a proportion of Schwann cells in dissociated cell cultures that had previously lost GC expression *in vitro*, by exposing the cells to high concentrations of cyclic AMP derivatives. We have found that similar results

can be obtained using Schwann cell cultures obtained from the sympathetic trunk of 10-d-old rats, although >99.5% of the Schwann cells in these cultures derive from non-myelin-forming cells (unpublished observations).

More GC was synthesized from [³H]galactose per microgram of protein in excised sciatic nerves than in cervical sympathetic trunks. In the sciatic nerve, 80% of the fibers are myelin-forming, whereas 20% are Remak fibers (11). On the basis of counts in the cervical sympathetic trunk that 99% of the axons are unmyelinated (2) and that there are on average 5.7 axons per Schwann cell in mature Remak fibers and 1 axon per Schwann cell in the myelinated fibers (1), it can be estimated that only 5–6% of the fibers in this nerve are myelin-forming. Furthermore, it seems reasonable to assume that the rate of GC synthesis in 8–10-d-old rats might be considerably higher in myelin than in non-myelin-forming Schwann cells, given the multilamellar nature of the myelin sheath and the need to make large quantities of myelin membrane. It is therefore not surprising that GC synthesis is higher per microgram of protein in the sciatic nerve than in the trunk. The difference between the two is only threefold, however, making it very unlikely that the Schwann cells surrounding the myelinated axons in the trunk are responsible for all the GC synthesis observed, and supporting the idea that a significant proportion of the synthesis is contributed by the Schwann cells of the unmyelinated fibers.

Apart from being one of the building blocks of the myelin sheath, the functional role of GC is still unclear. Developmentally, it appears on the surface of non-myelin-forming Schwann cells as they reach maturity and is expressed by oligodendrocytes and myelin-forming Schwann cells as cell division stops but before the onset of myelination. This points to an involvement in those membrane–membrane interactions that establish the mature relationship between axons and their glial cells, quite apart from any specific role this lipid may have in the membrane-wrapping events involved in myelination.

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