Developmental Changes in Myelin-induced Proliferation of Cultured Schwann Cells

Jun E. Yoshino, Patrick W. Mason, and George H. DeVries

Department of Biochemistry, Medical College of Virginia, Richmond, Virginia 23298

Abstract. Schwann cell proliferation induced by a myelin-enriched fraction was examined in vitro. Although nearly all the Schwann cells contained material that was recognized by antisera to myelin basic protein after 24 h, only 1% of the cells were synthesizing DNA. 72 h after the addition of the mitogen a maximum of 10% of the cells incorporated [^{3}H]thymidine. If the cultures were treated with the myelin-enriched fraction for 24 h and then washed, the number of proliferating Schwann cells decreased by 75% when compared with those cells that were incubated with the mitogen continuously. When Schwann cells were labeled with [^{14}C]thymidine followed by a pulse of [^{3}H]thymidine 24 h later, every Schwann cell labeled with [^{3}H]thymidine was also labeled with [^{14}C]thymidine. Although almost every Schwann cell can metabolize the myelin membranes within 24 h of exposure, a small population of cell initially utilizes the myelin as a mitogen, and this population continues to divide only if myelin is present in the extracellular media. The percentage of the Schwann cells that initially recognize the myelin-enriched fraction as a mitogen is dependent upon the age of the animal from which the cells were prepared.

CULTURED Schwann cells proliferate when treated with axolemma- or myelin-enriched fractions (15). The mitogenic activities of the membrane fractions could be differentiated by their dose-response curves and their sensitivity to the lysosomal inhibitor, ammonium chloride. Also, the rates of [^{3}H]thymidine incorporation by Schwann cells treated with the myelin-enriched fraction were not identical when compared with those cells incubated with the axolemmal membranes. Schwann cells that were exposed to the myelin-enriched fraction for 72 h accumulated three times as much [^{3}H]thymidine as those that were incubated with the mitogen for only 48 h. This sudden increase in proliferation coincided temporarily with similar periods of Schwann cell division during Wallerian degeneration in vitro (14) and in vivo (4).

In the present study, we have examined by autoradiography the kinetics of Schwann cell proliferation induced with a myelin-enriched fraction in vitro. The large increase in proliferation that occurs 72 h after the administration of the mitogen is the result of a small population of Schwann cells that initially divides in response to the mitogen and whose daughter cells subsequently continue to proliferate if the membrane fraction is present in the extracellular media. The number of Schwann cells that divide initially when treated with the myelin-enriched fraction is related to the age of the animal from which the cells were prepared.

Materials and Methods

HF[1] is DME (Gibco Laboratories, Grand Island, NY) with sodium bicarbonate and 10% FCS (HyClone Laboratories, Logan, UT). Saline G is a balanced salt solution containing 125 mM NaCl, 5 mM KCl, 0.7 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mM KH_2PO_4, and 1 mM Na_2HPO_4. Forskolin (Calbiochem-Behring Corp., La Jolla, CA) was dissolved in 95% ethanol to give a final concentration of 5 mM.

1. *Abbreviation used in this paper*: HF, DME with sodium bicarbonate and 10% FCS.

Preparation of Cultured Schwann Cells

Primary cultures of Schwann cells were prepared as described by Brockes et al. (5) and modified by Meador-Woodruff et al. (9). Briefly, the sciatic nerves of 2-d-old rat pups were excised, treated with collagenase and trypsin, and plated in 10-cm glass petri dishes in HF media. The cells were treated with the antimitotic drug, cytosine arabinoside, for 3 d to eliminate any rapidly dividing fibroblasts. After 72 h most of the surviving fibroblasts were removed from the cultures by using complement-mediated lysis with anti-Thy 1.1 (New England Nuclear, Boston, MA).

Indirect Immunoperoxidase Labeling of Myelin Basic Protein in Cultured Schwann Cells Treated with a Myelin-enriched Fraction

Schwann cells were incubated with the myelin-enriched fraction for 24 h. The cells were washed twice with Saline G and fixed to the coverslips with a solution of 90% ethanol and 1% paraformaldehyde for 10 min, placed in absolute ethanol for 1 min, and air dried. The cells were stained for myelin basic protein by the indirect peroxidase method of Nakane and Pierce (11) using rabbit anti-bovine myelin basic protein antiserum (a generous gift of Dr. M. Kies, National Institute of Mental Health) at 1:100 and swine anti-rabbit IgG–horseradish peroxidase conjugate at 1:150. The cells were incubated for 30 min in each antiserum followed by 15-min washes in PBS. Immunoreactive material was visualized with diaminobenzidine (25 mg/100 ml) in Tris-HCl buffer, pH 7.6, containing 0.003% hydrogen peroxide.

Autoradiography of Mitogen-treated Schwann Cells

Schwann cells were plated onto glass coverslips in 24-well plastic culture dishes at a density of 40,000 cells per well after treatment with anti-Thy 1.1. Mitogens or HF were added to give a final volume of 500 μl/coverslip. 3 h before the end of the experiment, 1.5 μCi of [^{3}H]thymidine (15 Ci/mmol; New England Nuclear) in 125 μl of HF was added to each coverslip. The

experiment was stopped by removing the media and washing twice with Saline G. The cells were then processed for autoradiography as previously described (7). A minimum of eight randomly selected fields with at least a total of 1,000 cells were counted for each coverslip.

Double Labeling of Proliferating Schwann Cells with [^{3}H]Thymidine and [^{14}C]Thymidine

Schwann cells were incubated with the myelin-enriched fraction 24 h before the addition of 0.3 μCi of [^{14}C]thymidine (56 mCi/mmol; Moravek Biochemicals, Brea, CA) in 125 μl of HF. After 24 h, the labeled thymidine was washed out and replaced with HF and the myelin-enriched fraction. The cells were incubated with 1.5 μCi of [^{3}H]thymidine in 125 μl of HF 21 h later. After a 3-h incubation with the label, the cells were washed and processed for autoradiography using a modification of the procedures of Baserga and Nemeroff (1). Briefly, the coverslips were mounted on microscope slides and coated with a 1:2 dilution of Nuclear Track Emulsion (Eastman Kodak Co., Rochester, NY) and left at 4°C for 48 h. The coverslips were developed and stained as described previously (7). The slides were dipped in a solution of collodion (J. T. Baker Chemical Co., Phillipsburg, NJ) diluted with an equal volume of ethyl ether to provide an inert but transparent layer of material to separate the two layers of emulsion. The slides were air dried and then coated with an undiluted solution of Nuclear Track Emulsion. The coverslips were left at 4°C for 96 h and then developed.

Determination of Free [^{14}C]Thymidine in Schwann Cells

Schwann cells (100,000 cells/ml) were plated in 2 ml of HF in 35-mm wells. The cultures were incubated with the myelin-enriched fraction and labeled with 1.2 μCi of [^{14}C]thymidine in 0.5 ml of HF as described in the previous section. After the cells were washed once with HF and treated with 2 ml of 1 N NaOH for 15 min, the total [^{14}C]thymidine within the Schwann cells was determined by neutralizing an aliquot with glacial acetic acid and counting in a liquid scintillation counter (model LS 5801; Beckman Instruments, Fullerton, CA). The results were corrected for quench with an external standard. Aliquots of the cell suspension were also spotted on filters (Whatman Inc., Clifton, NJ) and dried overnight. The filters were treated with 5% trichloroacetic acid for 1 h at 4°C and 70% ethanol for 1 h at 4°C to precipitate the DNA onto the filters. The filters were dried and counted as described previously. The amount of free [^{14}C]thymidine was calculated as the difference between the total label within the Schwann cells less the amount recovered on the filters after precipitation with trichloroacetic acid and ethanol.

Preparation of Myelin-enriched Fractions

Myelin-enriched fractions were obtained from rat brainstem as previously described (15). Protein was determined using the Bradford dye binding assay (3) in the presence of 0.1 N NaOH using gamma globulin as a protein standard.

Preparation of Glial Growth Factor

Glial growth factor was prepared from bovine pituitaries as described by Raff et al. (13). Briefly, six pituitaries (Pel-Freez Biologicals, Rogers, AR) were homogenized in 20 ml of 0.15 M $NH_4(SO_4)$ using a motor driven Teflon-glass homogenizer. The pH of the homogenate was adjusted to 4.5 using HCl. The homogenate was centrifuged at 13,000 *g* for 30 min after a 90-min incubation at 4°C. The resulting supernatant was dialyzed overnight against distilled water. The dialyzed supernatant was centrifuged at 13,000 *g* for 30 min to generate a pellet and supernatant. This supernatant was lyophilized and reconstituted with 3 ml of distilled water. Protein concentration was 17 mg/ml as determined by the Bradford dye binding assay (3).

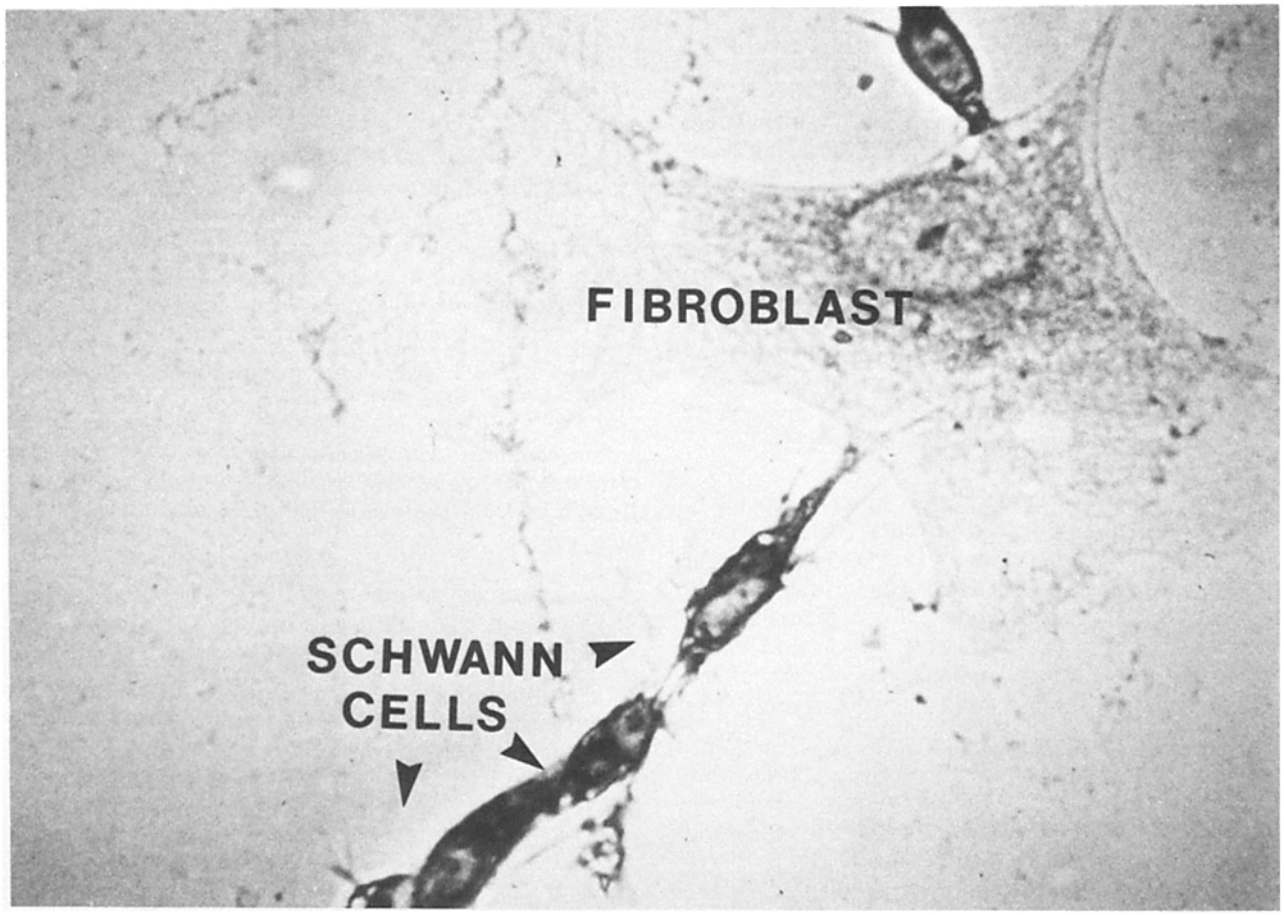

Figure 1. Localization of myelin basic protein in Schwann cells treated with a myelin-enriched fraction. Schwann cells were incubated with the myelin fraction (20 μg/ml) for 24 h. The cultures were reacted with an antisera to myelin basic protein and visualized by indirect immunoperoxidase.

Results

Immunohistochemical Localization of Myelin Basic Protein in Cultured Schwann Cells Treated with a Myelin-enriched Fraction

Mixed cultures of Schwann cells and fibroblasts were treated with a myelin-enriched fraction to determine whether both types of cells were capable of phagocytosis of myelin membranes. Myelin basic protein was localized in the cultures by indirect immunoperoxidase. After incubation with the myelin fraction for 24 h, almost all of the Schwann cells were labeled with antisera to myelin basic protein (Fig. 1). In contrast, the fibroblasts did not possess any immunoreactive material.

Kinetics of Schwann Cell Proliferation Induced by Myelin Membranes

The percentage of Schwann cells that were dividing 24 h after the addition of the myelin fraction was only 1% (Fig. 2). Schwann cells were incubated with the myelin-enriched fraction for 48, 72, and 96 h. The percentage of cells that were synthesizing DNA was determined by adding [^{3}H]thymidine 3 h before the end of the experiment. A large increase in the number of labeled cells was observed between 48 and 72 h after the addition of myelin. This large increase in labeled Schwann cells coincided temporarily with a rise in the rate of [^{3}H]thymidine incorporation by Schwann cells treated with a myelin-enriched fraction (15). The highest dose of myelin produced a maximal labeling of only 10% of the cells.

Is the Continuous Presence of Myelin Required to Elicit Schwann Cell Proliferation?

To determine whether the continuing presence of myelin membranes in the media was absolutely required for maximal Schwann cell proliferation, the cells were treated with the myelin-enriched fraction for 24 h and then washed. If the mitogen was left in the media for 72 h, the percentage of labeled Schwann cells was fourfold greater than those cells that were pulsed with the myelin membranes for only 24 h (Fig. 3).

Labeling of Schwann Cells with [^{14}C]- and [^{3}H]Thymidine

To promote maximal proliferation of the Schwann cells, the myelin-enriched fraction must be present for the duration of the experiment. One interpretation of this result is that only a certain population of cells is proliferating and their daughter cells are utilizing the mitogen to divide again. Alternatively, new cells could be stimulated to divide from the initial population of quiescent cells. To distinguish between these possibilities, the following study was undertaken. Schwann cells were incubated with the meylin-enriched fraction and [^{14}C]thymidine for 24 h to label all of the cells synthesizing DNA. The cells were washed and media containing the myelin-enriched fraction was added. [^{3}H]Thymidine was added 21 h later for 3 h to label any cells synthesizing DNA. An artifact of the staining process that always occurred caused the inert layer of collodion to expand and contract differentially. The upper layer of emulsion was shifted and its image was displaced slightly when compared with the lower layer (Fig. 4). Almost all of the cells that had incorporated [^{3}H]thymidine in the last 3 h of the experiment also were labeled with ^{14}C (Table I). The few cells treated with the myelin-enriched fraction which were labeled only with ^{3}H can be accounted for by the number of cells which were labeled by ^{3}H but in absence of the mitogen (control).

The presence of free [^{14}C]thymidine with the Schwann cells, after removal of the media containing the label, could cause difficulty in the interpretation of the results from the double labeling experiment. The percentage of [^{14}C]thymidine recovered after precipitation with 5% trichloroacetic acid was 88, 99, and 112% for three separate experiments. Thus, nearly all the [^{14}C]thymidine taken up by the Schwann cells was incorporated into DNA and was not available for subsequent DNA synthesis when [^{3}H]thymidine was added 21 h later.

Comparison of Schwann Cells Treated with a Myelin-enriched Fraction or Glial Growth Factor and Forskolin

An alternative interpretation of these results would suggest that only one population of Schwann cells exists that is capable of dividing. The combination of glial growth factor and forskolin has been utilized as a mitogen to expand Schwann cell populations (12). Schwann cells were treated with increasing concentrations of the myelin-enriched fraction or with forskolin and glial growth factor (Fig. 5). The myelin-enriched fraction produced a maximal labeling of 5% of the Schwann cells, whereas the combination of forskolin and glial growth factor stimulated the incorporation of [^{3}H]thymidine by 14% of the cells.

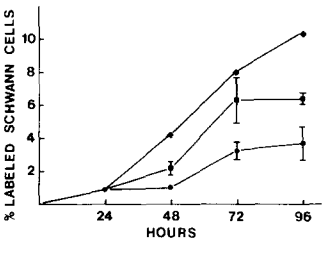

Figure 2. Dose-dependent incorporation of [^{3}H]thymidine by Schwann cells stimulated with myelin membranes. Values are expressed as the means ± SEM for n = 3. The absence of mitogen produced between 0.1 and 0.4% labeled Schwann cells. Schwann cells were treated with 5 μg/ml (*solid circles*), 10 μg/ml (*solid squares*), or 20 μg/ml (*solid diamonds*) for 24, 48, 72, and 96 h. [^{3}H]Thymidine was added to the cultures 3 h before the end of the experiment.

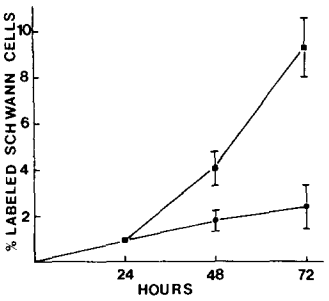

Figure 3. Schwann cell proliferation after treatment with myelin membranes for 24 h. Values are the means ± SEM for n = 3. The absence of mitogen produced between 0.1 and 0.4% labeled Schwann cells. Schwann cells were stimulated with 20 μg/ml of the myelin-enriched fraction for 24 h. The cells were washed and media was added back without (*solid circles*) mitogen for another 24 and 48 h. For comparison, sister cultures were incubated with the myelin-enriched fraction at a final concentration of 20 μg/ml for 24, 48, or 72 h (*solid squares*). [^{3}H]Thymidine was added to the cultures during the last 3 h.

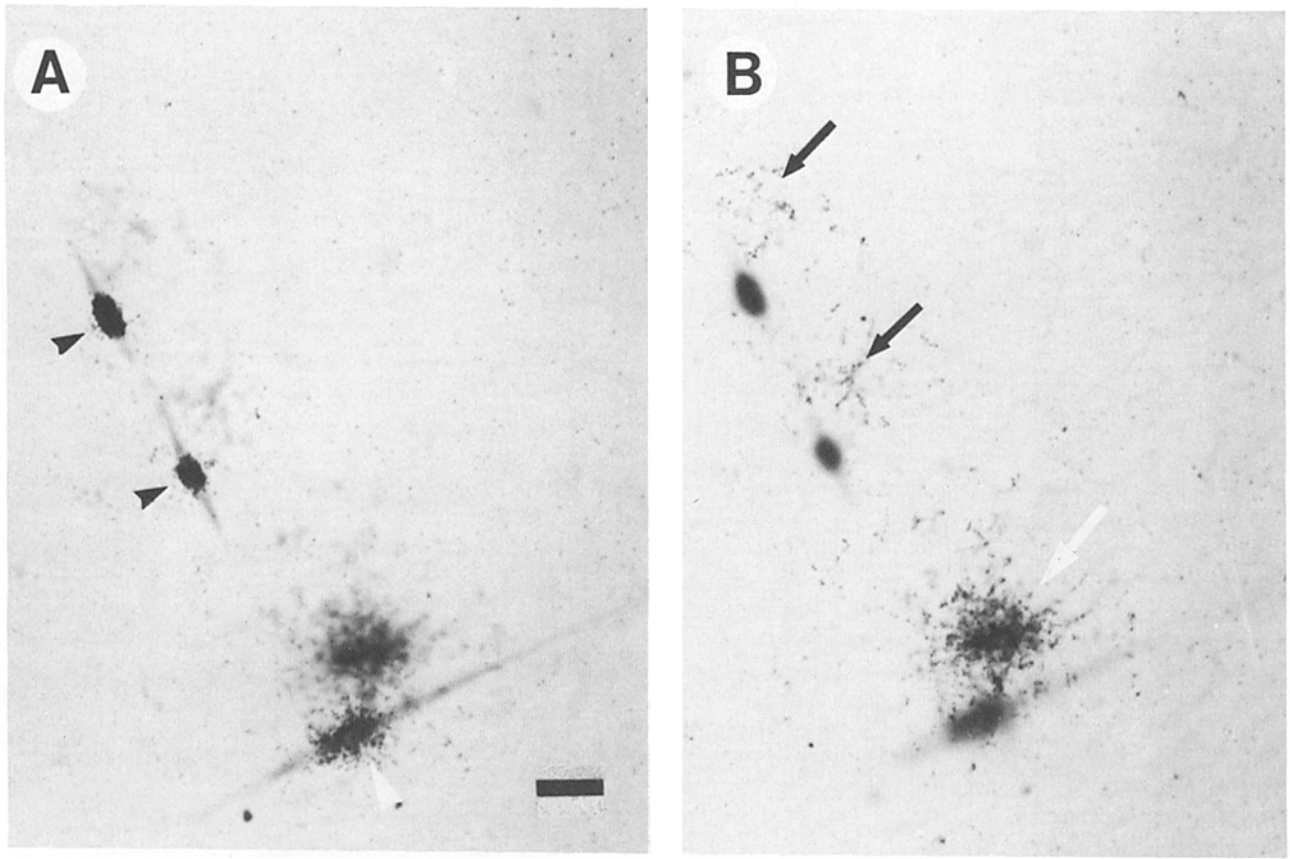

Figure 4. Double labeling of the Schwann cells. Schwann cells were treated with myelin membranes for 24 h followed by a 24-h incubation with [^{14}C]thymidine. The cultures were washed, the mitogen added back, and [^{3}H]thymidine was added 21 h later for a 3-h period to label specifically those cells that were synthesizing DNA at that time. (*A*) Lower emulsion. Two Schwann cell nuclei (*black arrowheads*) labeled with [^{3}H]thymidine. A cell containing [^{14}C]thymidine will be labeled in both layers of emulsion. The pattern of labeling with [^{14}C]thymidine is not confined to the area directly above the nucleus (*white arrowhead*). Some scattered grains from [^{14}C]thymidine can be seen above the [^{3}H]thymidine labeled nuclei. (*B*) Upper emulsion. An artifact of the processing that always occurred caused the movement of the inert layer between the emulsions, slightly offsetting the upper emulsion from the lower (*black and white arrows*).

The Age of the Animals from which Cultured Schwann Cells Are Prepared Affects the Kinetics of Cellular Proliferation

The results obtained from the double label experiment support the interpretation that a small population of Schwann cells initially recognize the myelin-enriched fraction as a mitogen and their daughter cells continue to proliferate when myelin is present. Schwann cells were prepared from 6-d-old rat pups (Schwann cells–6 d) and treated with the myelin fraction to determine whether the age of the animal from which the cells were obtained affects the rate of proliferation.

Table I. Double Labeling of Schwann Cells Treated with a Myelin-enriched Fraction

	Schwann cell nuclei		
Treatment	^{3}H-Labeled	^{3}H- and ^{14}C-labeled	Total nuclei counted
Myelin	208 (11.0)‡	204 (10.8)	1,889
Myelin*	262 (8.6)	–	3,056
None*	13 (0.4)	–	3,217

* For comparison, these cells received only [^{3}H]thymidine.
‡ Values in parentheses are the percentage of labeled Schwann cell nuclei.

The cells were stimulated with myelin membranes for 24, 48, and 72 h (Fig. 6). After 24 h all three doses of the mitogen produced a greater labeling of Schwann cells than observed for cells prepared from 2-d-old animals. Two other preparations of Schwann cells (6 d) were treated with the myelin-enriched fraction at a concentration of 20 μg/ml. After a

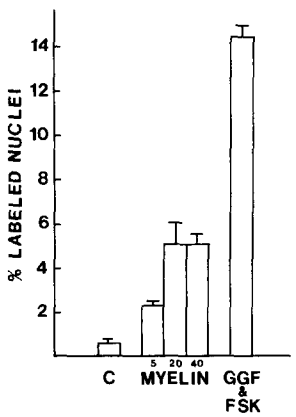

Figure 5. Cultured Schwann cells stimulated with a myelin-enriched fraction or glial growth factor (GGF) and forskolin (FSK). Values are the means ± SD for n = 3. The myelin-enriched fraction was tested at three concentrations: 5, 20, and 40 μg/ml. Glial growth factor and forskolin were added to give final concentrations of 500 μg protein/ml and 10 μM, respectively. The cells were incubated for 72 h with the mitogens. [^{3}H]Thymidine was added 3 h before termination of the study. The control condition (*C*) is the absence of mitogens.

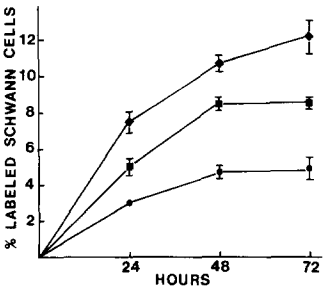

Figure 6. Proliferation of Schwann cells prepared from 6-d-old sciatic nerve stimulated with myelin membranes. Values are the means less controls ± SEM for $n = 3$. Schwann cells were treated with 5 μg/ml (*solid circles*), 10 μg/ml (*solid squares*), and 20 μg/ml (*solid diamonds*) of the myelin-enriched fraction. [^{3}H]Thymidine was added to the cultures during the last 3 h.

24-h incubation with the membranes, 5.8 ± 0.2% and 5.3 ± 0.7% of the Schwann cells (6 d) were labeled with [^{3}H]thymidine after correcting for controls. Thus, a greater percentage of the Schwann cells prepared from 6-d-old animals would appear initially to recognize myelin as a mitogen when compared with cells obtained from 2-d-old animals.

Discussion

Immunohistochemical localization of myelin basic protein revealed that nearly all of the cultured Schwann cells that were treated with a myelin-enriched fraction for 24 h possessed immunoreactive material; however, under the same conditions fibroblasts did not stain. These results are in agreement with previous observations that used a fluorescein-conjugated myelin fraction (9).

Despite the presence of immunoreactive material in almost every Schwann cell, only 1% of the cells treated with the myelin-enriched fraction for 24 h were labeled [^{3}H]thymidine. Furthermore, if the mitogen was removed from the media after 24 h, a dramatic decrease in Schwann cell proliferation was observed 48 h later. One interpretation of this phenomenon is that a small population of Schwann cells initially recognize myelin as a mitogen, and the resulting daughter cells continue to divide if the myelin membranes are present in the media. To examine this possibility, Schwann cells were incubated for 24 h with the myelin-enriched fraction, and then [^{14}C]thymidine was added for 24 h to label the DNA of any cell that divided. The cells were washed and incubated with the myelin-enriched fraction for another 24 h. 3 h before the end of the experiment, [^{3}H]thymidine was added to label any cells that were dividing at that time. All the cells that were labeled with [^{3}H]thymidine were also labeled with [^{14}C]thymidine. Thus, a population of Schwann cells had divided 24 h earlier, and the resultant daughter cells were also proliferating in response to the mitogen.

Two earlier studies (6, 8) reported that myelin-enriched fractions were slightly mitogenic for cultured Schwann cells; however, the authors were not able to determine whether the activity was intrinsic to myelin membranes or due to contamination by axolemmal membranes. If the Schwann cells were treated with the myelin membranes for 72 h instead of 48 h (15), the cells were able to accumulate three times as much [^{3}H]thymidine. From our present investigation, this large increase in the incorporation of [^{3}H]thymidine can be traced back to a small population of Schwann cells that initially recognize the myelin-enriched fraction as a mitogen. This population expands by successive cell divisions until the number of myelin-sensitive Schwann cells has grown to 8% of the population after treatment with the mitogen for 72 h. Furthermore, the dose dependent relationship between the incorporation of [^{3}H]thymidine and the myelin-enriched fraction is consistent with a small population of cells that divides and continues to divide (15). The dose-response curve for the myelin fraction rises and plateaus quickly, indicative of a limited number of myelin-responsive cells that can be saturated readily.

Another possible interpretation of these results is that in any given culture there exists a finite number of Schwann cells capable of proliferation. If glial growth factor and forskolin were utilized as mitogens, almost three times as many Schwann cells were labeled with [^{3}H]thymidine when compared with those cells treated with the myelin-enriched fraction. Thus, the myelin-enriched fraction appears to stimulate a select population of cells and not every cell that possessed the capacity to undergo proliferation.

The significance of this small population of cultured Schwann cells that can recognize myelin membranes as a mitogen may be related to the developmental state of the sciatic nerve from which the cells were prepared. Salzer and Bunge (14) reported that if Wallerian degeneration were initiated in myelinated cultures of dorsal root ganglion, the Schwann cells that proliferated were only those that had formed myelin sheaths. Schwann cells were prepared from sciatic nerves of 6-d-old pups to determine whether a more differentiated nerve would produce a larger population of Schwann cells that would initially recognize the myelin-enriched fraction as a mitogenic signal. 24 h after the addition of the myelin fraction, a dose-dependent proliferation of the Schwann cells (6 d) was observed. The percentage of labeled Schwann cells (6 d) range from 2 to 8%. Therefore, the number of Schwann cells that can divide 24 h after the addition of the myelin-enriched fraction appears to be a function of the maturation of the sciatic nerve from which the cells were prepared.

The absolute size of this population of cells that initially respond to the mitogen may not be directly comparable to the number of myelin sheaths present in the sciatic nerve because the procedures required to prepare the cultures may select against the survival of a cell that has differentiated. For example, the cytosine arabinoside which is added to the cultures 24 h after dissection of the nerve may eliminate a population of Schwann cells stimulated to divide by myelin membranes present in the initial preparation as a result of the developmental state of the sciatic nerve. Mirsky et al. (10) reported that after 16–20 h in culture, 5% of the Schwann cells prepared from newborn and 38% of the Schwann cells obtained from 5–7-d rat sciatic nerves were positive for myelin basic protein. When the maximal number of Schwann cells that divided 24 h after the addition of myelin membranes are compared, an eightfold difference exists between cells prepared from 6-d and 2-d-old animals. This difference is equivalent to that observed by Mirsky et al. (10) when examining the presence of myelin basic protein in Schwann cells prepared from newborn and 5–7-d-old animals. Therefore, the relative magnitude of the percentage of Schwann cells that initially divided 24 h after the addition of the mitogen appears to be related to the percentage of Schwann cells that contained immunologically detectable myelin basic protein.

The kinetics of cellular proliferation for the cells prepared from the 6-d animals was not identical to that observed for cells obtained from 2-d pups. The apparent doubling of the proliferating population of Schwann cells prepared from the 2-d rat pups is conspicuously absent from the cells of the 6-d preparation (Fig. 6). One possible explanation is that another factor in the culture other than the mitogen is limiting the rate of proliferation. Beuche and Friede (2) have reported that the proliferation of Schwann cells that accompanies Wallerian degeneration does not occur if macrophages are excluded from the site of the lesion. Macrophages can be identified in our cultures (unpublished observations) and may be a contributing factor in the expression of the mitogenic activity of the myelin-enriched fraction. It is possible that a limited number of macrophages exist in the Schwann cell cultures, and these macrophages can assist in stimulating the division of a certain number of cells. As the percentage of proliferating Schwann cells increases to ~8% (see Figs. 2 and 6), the number of macrophages becomes limiting and consequently the percentage of cells dividing 24 h later does not double.

Material that reacts with antisera to myelin basic protein can be identified within almost every Schwann cell in culture after exposure to a myelin-enriched fraction for 24 h; however, only 1% of these cells are dividing. The percentage of cells that divide after treatment with the myelin membranes for 24 h is dependent upon the developmental state of the sciatic nerve from which the cells were prepared. These observations are consistent with the hypothesis that only Schwann cells that have differentiated to produce myelin sheaths are capable of recognizing myelin membranes or a by-product of myelin sheaths as a mitogen.

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