STUDIES OF SCHWANN CELL PROLIFERATION

II. Characterization of the Stimulation and Specificity of the Response to a Neurite Membrane Fraction

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ABSTRACT

When prepared by methods utilized in our laboratory, pure populations of
Schwann cells in culture do not divide, but, after recombination with peripheral
sensory neurons or their processes, proliferate rapidly (Wood and Bunge, 1975,
Nature (Lond.) 256:661–664). In this paper, we demonstrate that a membrane
fraction prepared from sensory ganglion neurites is also mitogenic for Schwann
cells and increases the labeling index (assessed by autoradiography after incuba-
tion of cells with tritiated thymidine) from <0.2 to 10% for primary cells, and
from 0.4 to 18–19% for replated cells. The increased responsiveness of replated
cells may reflect their greater access to the neurite membranes which is a
consequence of the elimination of multiple cell layers after replating and the
removal of the basal lamina. This stimulation was specific; addition of membrane
preparations from other cell types (3T3, C1300, etc.) was not mitogenic. Ultrastruc-
tural analysis demonstrated apparent binding of neurite membranes to Schwann
cells as well as significant phagocytosis of the membranes by the cells. The uptake
of nonmitogenic membranes suggests that phagocytosis per se is not the stimulus
of proliferation.

KEY WORDS   Schwann cell proliferation   ·
mitogenic signal   ·   nerve tissue culture   ·
membranes   ·   autoradiography

In the first paper of this series we have reviewed
the evidence that proliferation of Schwann cells
during development is dependent on a mitogenic
signal provided by the growing axon (19). Evi-
dence is also presented that this stimulus for
Schwann cell proliferation is distinct from that
observed when Schwann cells proliferate during
Wallerian degeneration initiated by axotomy.
Modifying described culture techniques (27), we
have obtained pure preparations of either neurons
or Schwann cells. This has allowed preparation of
neurite fractions that can be applied directly to
Schwann cells to characterize the axonal mitogen.
The studies reported here document the stimula-
tion of Schwann cell proliferation by an axonal
membrane fraction, whereas membrane fractions
from several other cell and tissue sources were
ineffective. This stimulation of proliferation by
neurite membranes is compared to that engen-
dered by interaction with the intact neurite. The
present study also provides an ultrastructural anal-
ysis of the interaction of cultured Schwann cells

and added membrane fractions. A third paper of this series (20) presents evidence that the axonal mitogenic signal is located on the external surface of the axonal plasma membrane, and that direct contact is required for the delivery of the mitogenic signal from axon to Schwann cell.

MATERIALS AND METHODS

Culture Chambers

Details of the culture systems are provided in the preceding paper of this series (19). Two culture chambers were routinely used in these studies.

ACLAR MINIDISH: Circles 30 mm in diameter of 5-ml-thick Aclar (Allied Chemical Corp., Morristown, N. J.) were molded with a heated metal press into the shape of a small minidish (2.5 cm in diameter) (6). Cultures in this dish are fed with ~0.20 ml of medium.

SILASTIC MICROWELLS: We found it advantageous in many experiments to grow cells in a reduced volume of medium. This was accomplished by placing a Silastic double-ring ensemble into the Aclar minidish. Each ring (inside diameter of 0.65 cm) was manufactured by squeezing uncured Silastic (Corning Glass Works, Corning, N. Y.) adhesive through a number 19 needle onto an Aclar sheet, followed by curing overnight, and rinsing with several changes of double-distilled water for several days to remove the acetic acid generated during curing. After sterilization in 80% ethanol and drying, the flat surface of the rings adhered tenaciously to collagen substrates; media and cells introduced inside the Silastic wells could be maintained indefinitely without leakage.

Cultures in these wells were routinely fed with 0.025 ml of medium. The cured Silastic adhesive is hydrophobic, so that a smaller meniscus was formed by the medium compared with conventional dishes. As a result, when dissociated Schwann cells or neurite particulate were introduced into the chamber, they settled to the bottom homogeneously rather than concentrating at the periphery. After fixation, the Silastic rings could be peeled off the collagen before processing the cells for autoradiography or embedding for electron microscopy.

Culture System

Cells were obtained from fetal rat dorsal root ganglia (DRGs).[1] Methods of generating purified populations of Schwann cells and of transferring these cells are given in the first paper of this series. To generate unensheathed (bare) neurite ganglia (NDRGs), we have used ganglia from late 15- or early

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[1] Abbreviations used in this paper: BSS, balanced salt solution; BUDR, bromodeoxyuridine; CMF, balanced salt solution without Ca^{++} or Mg^{++}; DRG, dorsal root ganglion; FUDR, fluorodeoxyuridine; HPF, high-powered field; HPS, human placental serum; LI, labeling index; MEM, Eagle's minimal essential medium; NDRG, dorsal root ganglion composed of neurons only; NGF, nerve growth factor; PDE, alkaline phosphodiesterase; RER, rough endoplasmic reticulum; SNDRG, dorsal root ganglion composed of neurons and Schwann cells.

16-d embryos. At this stage in development virtually all neurons have undergone their last division (13), and the number of neurons is therefore at a maximum while the number of non-neuronal cells is at a minimum. At the embryonic stage employed, it is possible to remove the cord with all the sensory ganglia attached (Fig. 1). The ganglia can then be transferred directly to a collagen-coated dish. In this way, it was possible to routinely explant in a 3- to 4-h dissection several hundred ganglia (~15 DRGs/dish).

To generate cultures essentially free of non-neuronal cells, we initially used methods previously described (27). For reasons of convenience and reliability, we have substantially modified this procedure. Several different media are used in this modified protocol:

B Medium: 65% Eagle's minimal essential medium (MEM) with 2 mM glutamine, 25% human placental serum (HPS), 10% chick embryo extract, 6 mg/ml glucose, and 10–20 U of nerve growth factor (NGF).

BUDR medium I: B medium with 20 μg/ml bromodeoxyuridine (BUDR).

BUDR medium II: B medium with 40 μg/ml BUDR.

FUDR medium: 88% MEM, 10% HPS, 2% embryo extract, 6 mg/ml glucose, NGF, 10^{-5} M uridine, 10^{-5} M fluorodeoxyuridine (FUDR).

To prepare bare neurites, ~15 fetal rat DRGs are explanted onto a collagen-coated Aclar minidish. For the first 2 d in culture, the DRGs are maintained on BUDR medium I. On days 3 and 4, the cultures are switched to BUDR medium II. At this time there is an extensive neurite outgrowth from the ganglia and a large number of non-neuronal cells. The cultures are then fed with FUDR medium and UV irradiated through the covered dish at a distance of 7 inches from a General Electric germicidal lamp (C30T8) for 10 min. It is important to note that the dose of UV that penetrates to the cells is reduced by absorption by the plastic petri dishes, serum proteins, and nucleotides.

In the days immediately after irradiation there is a striking loss of non-neuronal cells, and the medium becomes laden with cell debris. In contrast, the neurons show no overt signs of radiation damage (14); nuclei remain centrally located and neurites elongate normally. These cultures may be maintained for periods of several months or more with normal viability; also, these unensheathed neurite preparations can stimulate Schwann cells to divide and subsequently myelinate, and therefore appear to function normally. Presumably, because the non-neuronal cells were actively dividing they incorporated BUDR, rendering them extremely sensitive to a sublethal dosage of UV radiation (8, 9). By contrast, the neurons are nondividing and are aggregated as ganglia, and may therefore be poorly penetrated by the UV radiation. In fact, because the ganglia are first cultured in a medium permissive for glial outgrowth, and because glia appear to be trophic for neurons (25), survival of neurons in the first few critical days after explantation seems enhanced.
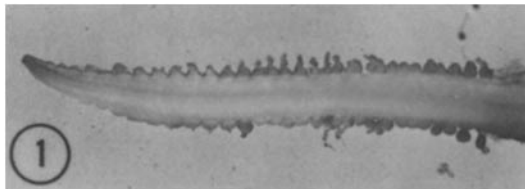

FIGURE 1   Spinal cord with attached DRGs. This cord was removed from a 15-d rat fetus. × 5.

Preparation of Cell Fractions

Ganglia free of supporting cells were maintained on FUDR medium for 3–5 wk. 3 or 4 d before harvesting the neurites, the ganglia were switched to B medium. At the time of harvesting the neuronal somas were still well confined in ganglia and could be mechanically excised using a sterile razor blade fragment. The appearance of the NDRG preparation before and after excision of the ganglia is shown in Fig. 2. The dishes were kept on ice until the neurites and collagen were pooled by scraping the tissue together with forceps. Material from four to eight dishes was homogenized with 35 strokes of a 7-ml Dounce homogenizer (Kontes Co., Vineland, N. J.; type B) in 4 ml of Tris buffer (50 mM, pH 7.4, with 0.2 M KCl, 0.5% bovine serum albumin, 0.2 mM $MgCl_2$, and 0.4 M sucrose). The homogenate was transferred with a siliclad-treated pasteur pipette to a 10-ml Pyrex tube and centrifuged at 300 g for 10 min. The pellet, which consisted primarily of collagen was discarded. The supernate was transferred to a Siliclad-treated Corex tube (Corning Glass Works) and centrifuged at 35,000 g for 1 h. The supernate was discarded and the pellet was resuspended in 1.1 ml of the same Tris buffer (without sucrose) by vortexing and homogenization in a Dounce homogenizer. A small aliquot was reserved for enzyme analyses and the remainder centrifuged at 35,000 g for 1 h. The supernate was again discarded. The pellet was rinsed briefly with B medium, resuspended at an appropriate concentration with a Vortex mixer (Scientific Industries, Inc., Bohemia, N. Y.) or Dounce homogenizer in B medium (containing 100 U/ml penicillin and streptomycin) and UV irradiated as described above for 15 min, to insure the sterility of the sample. Membranes were isolated on each day of the experiment as significant activity of the membrane preparation was lost on freezing at $-80°C$.

For experiments with cell lines, including C1300 (N 18) and Swiss 3T3 cells, the cells were grown to confluency in T-75 flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) in Dulbecco's modified Eagle's with 10% fetal calf serum, L-glutamine, and penicillin and streptomycin. Cells were rinsed three times with calcium- and magnesium-free Hank's solution (CMF), incubated with CMF containing 1 mM EDTA for 10 min at 34°C, and released by vigorous trituration. Cells were collected by centrifugation at 100 g for 10 min and resuspended in 4 ml of Tris buffer with sucrose. All further steps are as described bove.

Histological Techniques

Autoradiographic and histological procedures are given in the first paper of this series (19). Procedures for electron microscopy are described in detail elsewhere (4 and footnote 2). Briefly, cultures were rinsed in balanced salt solution (BSS), fixed in 2% glutaraldehyde in Sorensen's phosphate buffer (0.1 M, pH 7.2, with 86 mM sucrose) and subsequently fixed in 2% osmium in BSS. After dehydration in a graded series of alcohols and equilibrium with propylene oxide, cultures were embedded in Epon/Araldite. Thin sections were cut on an LKB-Huxley microtome (LKB Instruments, Inc., Rockville, Md.), stained with 4% uranyl acetate in methanol and then lead citrate, and viewed in a Phillips 300 electron microscope. To examine the neurite membrane preparation, a particulate fraction was pelleted at 35,000 g for 1

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[2] Wood, P., and A. K. Williams. Early morphogenesis and neurite growth in cultured embryonic rat retina. Manuscript in preparation.

h in a polypropylene centrifuge tube and fixed as described above.

RESULTS

Stimulation of Schwann Cells by a Neurite Membrane Fraction

To determine whether the neurite had to be in anatomic continuity with the neuron to provide the mitogenic signal, two preliminary experiments were done. Schwann cells were replated onto DRG neurites moments after excising the ganglion, and incubated for 48 h in the presence of tritiated thymidine. Under these conditions, there was substantial stimulation of Schwann cells by the axotomized neurites. Alternatively the neurites were transferred directly onto the Schwann cells with fine forceps on two successive days (in the presence of tritiated thymidine on the 2nd d) and processed for autoradiography. There was a significant increase in labeling, but only in areas to which neurites had been added. To obtain a more uniform interaction with the Schwann cells, we now routinely homogenize the isolated neurites. The amount of neurite membrane added was quantitated with a sensitive fluorescent assay for the membrane-associated enzyme, alkaline phosphodiesterase (PDE) (20).

In our initial experiments we found that multiple additions of neurite membranes gave the optimal stimulation of Schwann cells. A single 24-h exposure to the neurite particulate fraction was not stimulatory, whereas a 48-h incubation stimulated significant thymidine incorporation (~50–60% of maximal response), and two or three 24-h pulses gave the optimal response, approximately a 10% labeling index (LI) (Fig. 3).

This requirement for extended exposure to homogenized neurites could result from the accumulation of a degeneration product with time or represent the lability of the neurite membranes under our culture conditions. To distinguish these possibilities, we added some neurite membranes directly to Schwann cells, others were incubated at 34°C for 24 h before addition to Schwann cell cultures. The results, run in duplicate, are given in Table I. After addition of freshly isolated neurite membranes, there was approximately a 20-fold stimulation over control; membranes incubated at 34°C for 24 h gave a markedly lower stimulation of thymidine incorporation (three- to fourfold of
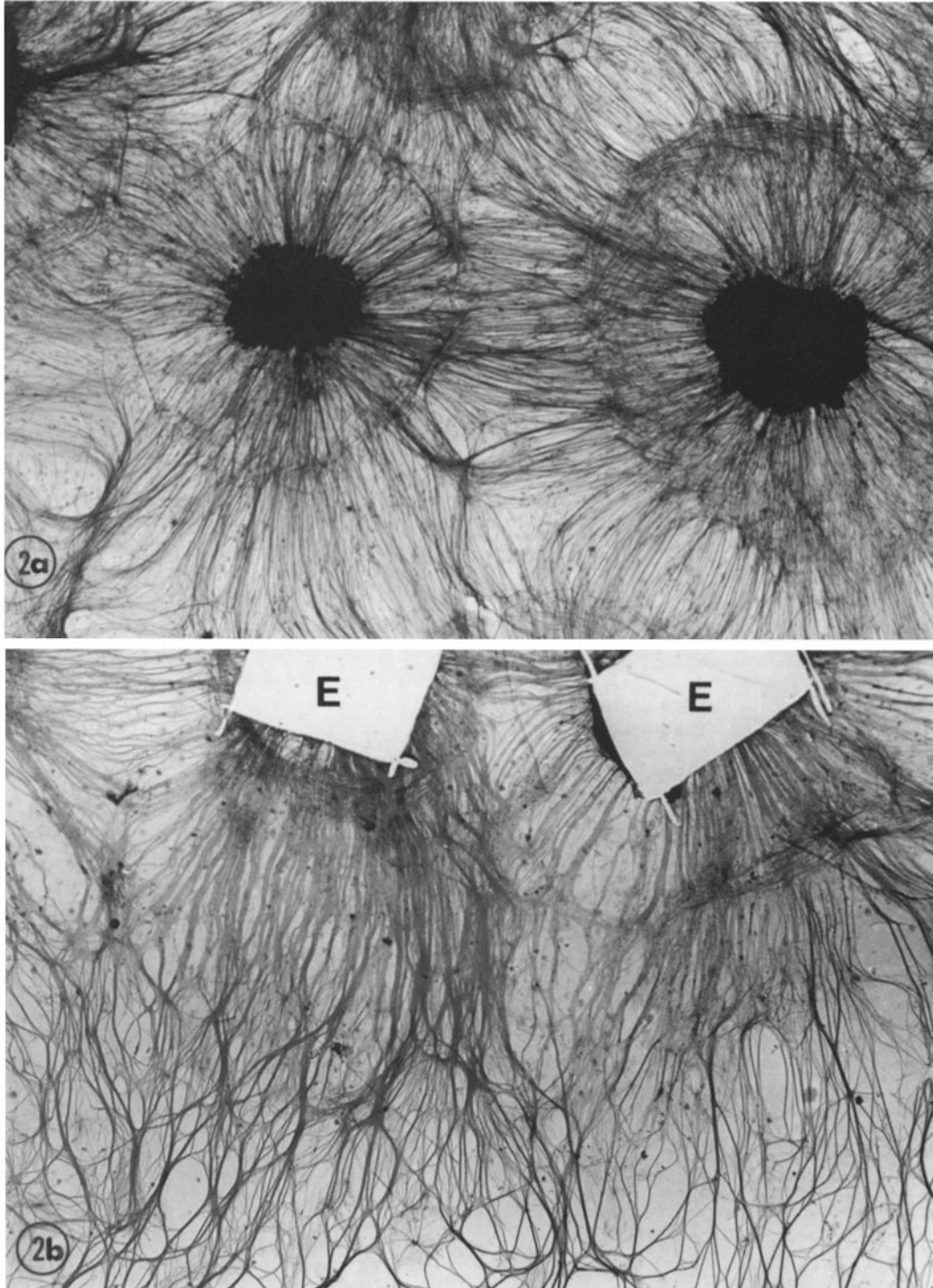
FIGURE 2 Bare neurite preparations before and after excision of the ganglia (NDRGs). This pair of micrographs shows the appearance of two cultures shortly (a) before or (b) after removing the ganglia. NDRGs were from 16-d rat fetuses treated as described in the text and maintained for 1 mo on FUDR medium. The halo of neurites around the ganglia (a) is caused by retraction of neurites on the collagen. In Fig. 2b, the razor blade cuts around the former site of the explant (E) can be seen. × 20.
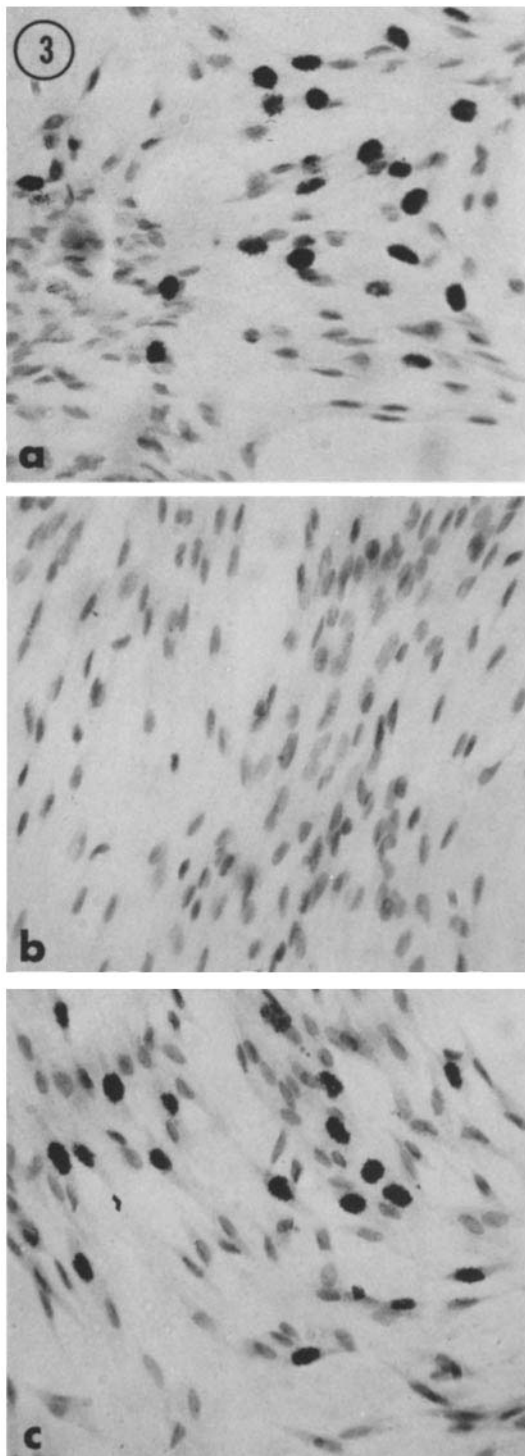
controls). These data suggest that the requirement for prolonged exposure does not represent accumulation of a breakdown product of the neurite membranes.

Schwann cells responding to neurite membranes have an altered morphology and increased propensity to migrate. After excision of the ganglion, Schwann cells retain their spindle shape and alignment in fascicles, showing very little tendency to migrate, especially if the DRGs had been maintained in vitro for periods of 30 d or more. This presumably reflects the elaboration of the "limiting" basal lamina, and its persistence after excision of the ganglion (5). When neurite membranes are added to Schwann cells at high doses ($\geq$1.2 PDE units/microwell), the cells lose their spindle shape, assume a more irregular, flattened appearance, and migrate into small islands of cells several cell layers thick.

Titration and Variation of Response

In a large number of experiments, we observed substantial variation in the LI of Schwann cells after addition of neurite membranes. This is illustrated by the results of a titration experiment in which increasing amounts of neurite membranes were added to primary Schwann cells in microwells (Fig. 4). At low concentrations there is an intermediate response; saturation occurs at 0.6–1.2 PDE units of membrane—addition of greater

TABLE I

Effect of Incubation at 34° C on the Neurite Mitogen

Sample	LI
No addition	0.23, 0.25
Neurite membranes	5.36, 4.13
Neurite membranes incubated at 34°C for 24 h	0.64, 1.01

In this experiment neurite membranes (0.6 PDE units/well) were either added directly to Schwann cells or incubated at 34°C for 24 h before addition to cells. The following day the procedure was repeated with the addition of [^{3}H]thymidine. After an additional 24-h incubation, the cultures were fixed and processed for autoradiography as previously described. Results of duplicate dishes are shown.

neurite membrane homogenates on two successive days. In this experiment, cells were incubated with tritiated thymidine for 24 h, processed for autoradiography, and poststained with toluidine blue to stain cell nuclei. Labeled nuclei appear to be black because of the high grain density. × 440.

FIGURE 3 Autoradiographs of (*a*) Schwann cells proliferating on neurites, (*b*) quiescent Schwann cells, and (*c*) Schwann cells induced to divide by the addition of

amounts does not increase the LI. We have generally used 1.2 PDE units to obtain maximal stimulation; this corresponds to 2 or 3 μg of membrane protein/microwell of cells. Also the variation observed in Fig. 4 existed not only between duplicates within the same experiment, but also between experiments.

Two important interrelated variables in determining the level of stimulation were: (a) the length of time the explants had been maintained in vitro before excision of the ganglia, and (b) the Schwann cell density. In general, we have observed a diminished responsiveness of Schwann cells, derived from ganglia which had been maintained in vitro for periods in excess of 1 mo, which parallels the gradual decline in the Schwann cell LI observed as intact dorsal root ganglia composed of neurons and Schwann cells (SNDRG's) are maintained in vitro (19). For example, in one experiment in which the explant had been maintained for 35 d in vitro, after addition of 1.2 PDE units of membranes, the maximal response was only 5%, however, another culture which had been maintained for only 20 d before excision exhibited an LI of 18.3%.

FIGURE 5 Effect of cell density on the labeling index. Neurite membranes (1.2 PDE units/well) were added to four primary Schwann cell cultures. The LI decreased in regions of high cell density (as defined by the number of cells in the microscope field). The LI at each range of cell density was normalized to the mean LI per culture; the results for all cultures were then averaged.

The effect of cell density on the LI of four Schwann cell cultures to which 1.2 PDE units of neurite membranes was added is summarized in Fig. 5. When these slides were counted at × 400, the LIs from each separate high-powered field (HPF) and the total number of cells in that field were recorded. Schwann cells are confluent at ~100 cells/HPF which corresponds to 2 × 10^5 cells cm^{-2}. When ~5,500 cells (~70 HPFs) had been counted in each culture, the LIs for HPFs whose total cell counts were between or equal to 0–50, 51–75, 76–100, 101–125, 125–151, ≥151 cells/HPF were averaged. These averaged LIs were normalized to (divided by) the mean LI for that culture. The results of the four cultures, averaged at each density, demonstrate a consistent decline in the LI with increasing cell density (Fig. 5). The mitogenic effect of membranes declines throughout the range of Schwann cell densities examined, rather than sharply dropping at confluent cell densities. This suggests that the effect of density may represent competition for membranes or a problem in membrane access to the cells rather than a contact-mediated inhibition of proliferation. Also, cells at the lowest densities generally were located at the periphery of the outgrowth, and were therefore cells which had been actively dividing at the time of excision (19).
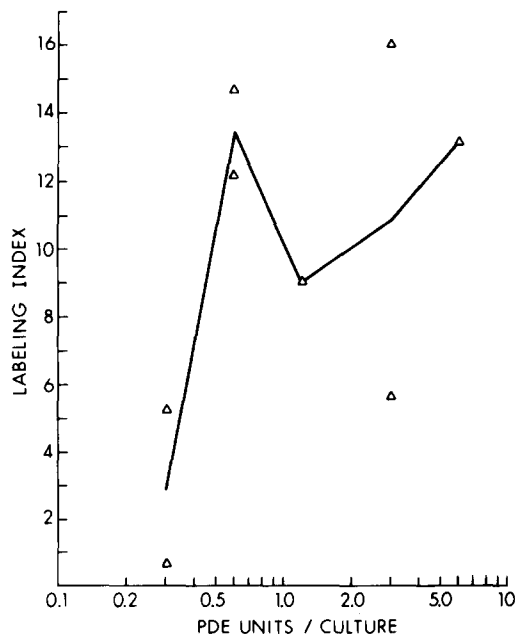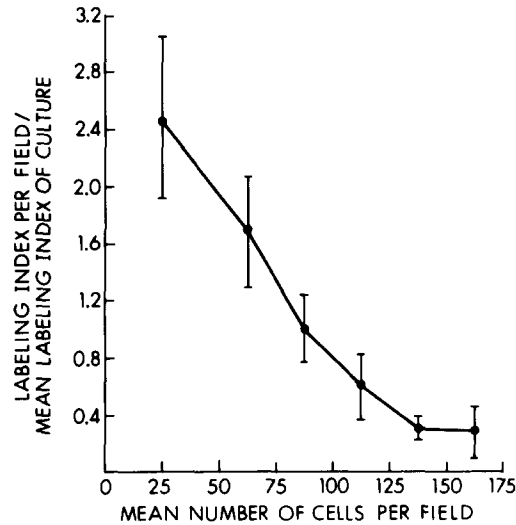

FIGURE 4 Titration of the mitogenic response of primary Schwann cells to neurite membranes. This graph illustrates an experiment in which neurite membranes were prepared as usual and added at various concentrations on two successive days to Schwann cells in the presence of tritiated thymidine as described in the text.

Replating Schwann cells at sparse densities increased their responsiveness to neurite membranes. Thus, addition of neurite membranes (1.2 PDE units/microwell) for 2 d resulted in an average LI of 18% in replated Schwann cell cultures vs. 9.9% in primary cultures. A titration experiment where increasing quantities of neurite membranes were added to replated Schwann cells is shown in Fig. 6. At every concentration of neurite membranes replated, Schwann cells demonstrated an elevated LI compared to primary Schwann cells (data not shown). Control cultures in this experiment had an LI of 0.76 and 0.83%. This increased response may reflect the greater accessibility of replated cells caused by elimination of multiple cell layers and the basal lamina with the enzymatic treatment (5); although selection for a more responsive population during replating cannot be ruled out at present.

Specificity of the Stimulation of Schwann Cell Proliferation

As was reported earlier (27) Schwann cells do not proliferate in isolation or if they are in contact with fibroblasts. We examined fibroblast (3T3) particulate fractions (prepared as described in Ma-
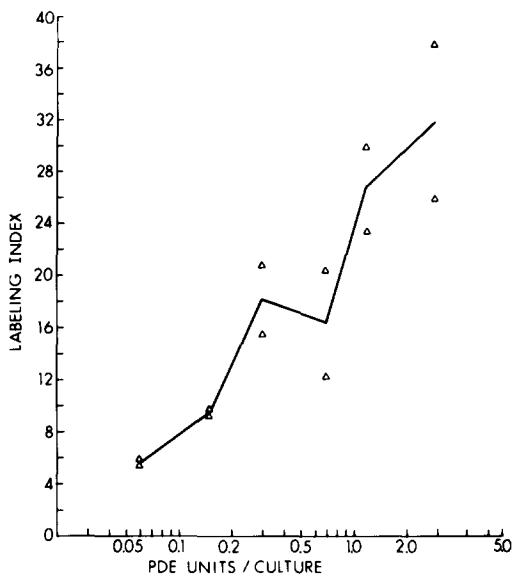

FIGURE 6 Titration of neurite membranes on replated cells. Neurite membranes were prepared and incubated with replated Schwann cells as described in the text. Cells were processed for autoradiography, and the labeling index was determined. Duplicate points from a single experiment are shown at each concentration.

terials and Methods) for their ability to stimulate Schwann cells. In four separate experiments in which 3T3 membranes were added to Schwann cells (both primary cells and replated cells) at concentrations between 0.3 and 6.0 PDE units/microwell, we observed no stimulation over background (compare to Figs. 4 and 6).

Next we investigated whether a more readily available alternate source of the mitogen might exist. We also therefore examined several neuroblastomas, including C1300 N-18, a mouse sympathetic cell line, and two rat CNS cell lines, B-103 and B-65, isolated by D. Schubert and his colleagues (23) and described as having neuronal properties. In each case the cells were grown for the 2 d before harvesting in the presence of dibutyryl cAMP which induces process formation in these clonal lines (22). Cells were harvested and a particulate fraction isolated. In the two experiments in which particulate fractions were added to primary cultures of Schwann cells at concentrations of 1.5, 3, and 6 PDE units/microwell, we again observed no elevation of the LI.

We also tested embryonic CNS (telencephalon or spinal cord, stripped of the meninges, from 16- to 17-d fetal rats) as an alternate mitogenic source. We observed a variable but weak stimulation of primary cells with whole brain particulate (average LI of 2.1 ± 2.1%) and a nonsignificant effect of spinal cord. More recently, we have found an axolemma enriched membrane fraction from adult rat brain to be significantly mitogenic for replated cells.[3]

Because the neurons were grown on a collagen substrate, there is a small collagen contaminant found in our neurite preparation,[4] but not in these heterologous preparations. In an experiment in which medium-conditioned collagen was harvested, homogenized, and added to replated Schwann cells, no stimulation of the cells was observed. We also treated intact neurites with

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[3] Salzer, J. L., R. P. Bunge, and G. H. deVries. Isolation of an axolemma enriched fraction from rat brain that is mitogenic for Schwann cells. Manuscript in preparation.
[4] We estimate that greater than three-fourths or more of the collagen is lost on the low-speed centrifugation step, whereas only about one-fourth of the neurite membranes are. These estimates are based on hydroxyproline measurements of the supernate and pellet (assay courtesy of Dr. J. Jeffries, Washington University School of Medicine). Neurite membrane losses are based on PDE and Na^+-K^+ ATPase (20) measurements.

0.05% collagenase (Sigma Chemical Co., St. Louis, Mo., type VI, protease-free) in CMF at 34°C for 1 h using 0.30 ml/minidish before isolating membranes. Results from this experiment are summarized in Table II. Membranes prepared from collagenase-treated neurites retain full stimulatory

TABLE II

Effect of Collagenase on the Mitogenic Activity of Neurite Membranes

Units of PDE	Treatment of membranes	
	Buffer	Collagenase
	LI	
No addition	0.00, 0.25	
0.60	8.19	9.98
	9.97	13.31
1.20	10.21	19.05
	11.70	12.29

Neurite membranes were incubated with 0.05% collagenase (or 0.05% bovine serum albumin) in MEM for 1 h at 34°C. Membranes were centrifuged at 35,000 g. Pellets were resuspended in medium, and added to Schwann cells following our standard protocol.

activity for Schwann cells—in fact are somewhat more active than preparations with a small collagen contaminant. Thus collagen does not appear to be sufficient by itself, or to be required in this system for Schwann cell proliferation (however, for the role of collagen in Schwann cell differentiation see reference 7).

Ultrastructural Analysis

An extensive description of Schwann cells and NDRGs in tissue culture has been reported (5); we describe here only those features unique to the culture system employed in the present study. We show in Fig. 7 the appearance of our neurite membrane preparation (after centrifugation). The pellet consists primarily of membranes, in the form of small empty vesicles, ranging in size up to 0.10 μm in diameter. There is a significant amount of nonmembranous floculent material both outside and inside the vesicles and occasional cytoplasmic organelles such as mitochondria and lysosomes. During homogenization, neurites apparently vesiculate, trapping some of their cytoplasmic contents (20) and these are pelleted with the plasma membranes.
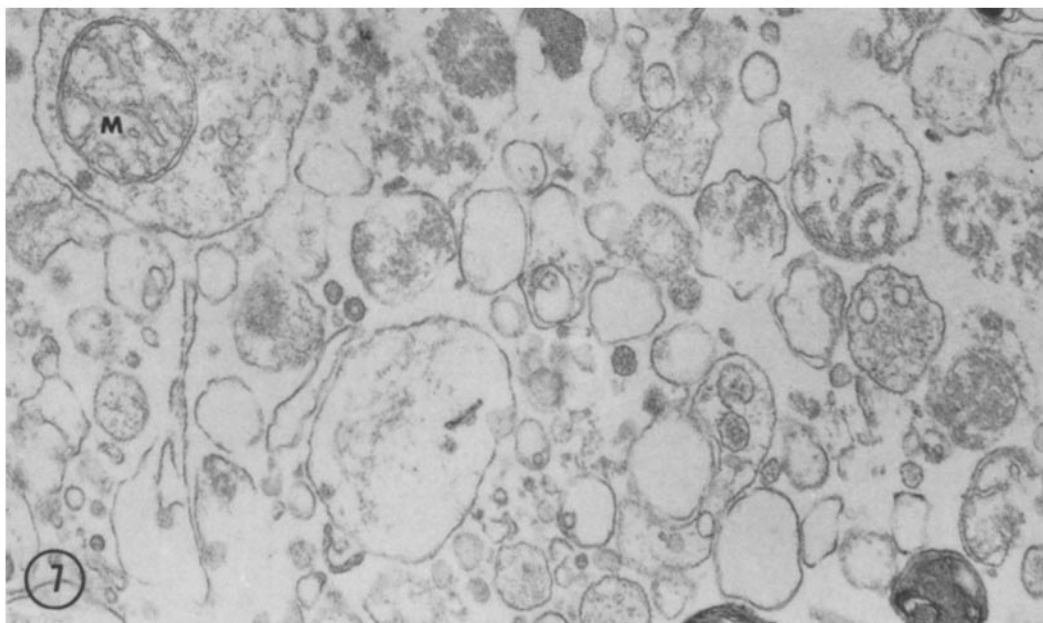

FIGURE 7 Electron micrograph of neurite membranes. A neurite membrane pellet was fixed for electron microscopy as described in Materials and Methods, and processed as usual. The majority of the preparation consists of small membrane vesicles, although there is a fair amount of flocculent material which may represent partially disrupted cell organelles. One large vesicle contains an intact mitochondrion (M) and has the general appearance of an intact neurite. × 44,000.

The appearance of Schwann cells in culture is shown in Figs. 8 and 9. Sections were cut perpendicular to the orientation of the fascicle and the collagen substrate. In contrast to Schwann cells associated with neurites, quiescent Schwann cells have a relatively less dense cytoplasm, a deficit of polyribosomes and rough endoplasmic reticulum (RER), and an abundance of microfilaments. There is an extensive basal lamina present, which seems to provide a scaffolding for the cells (Fig. 8). Replated Schwann cells are similar in appearance (Fig. 9), although they lack a basal lamina

and appear to be somewhat more active (more RER).

We examined the interaction of neurite membranes with both primary and replated Schwann cells. Neurites were added in two pulses (1.2 PDE units/pulse) and fixed 20 h after the second pulse. One set of cultures were fixed for electron microscopy and the second set which received [^{3}H]thymidine for the second 24-h period was fixed and processed for light microscope autoradiography (to validate the mitogenicity of the neurite preparation used).
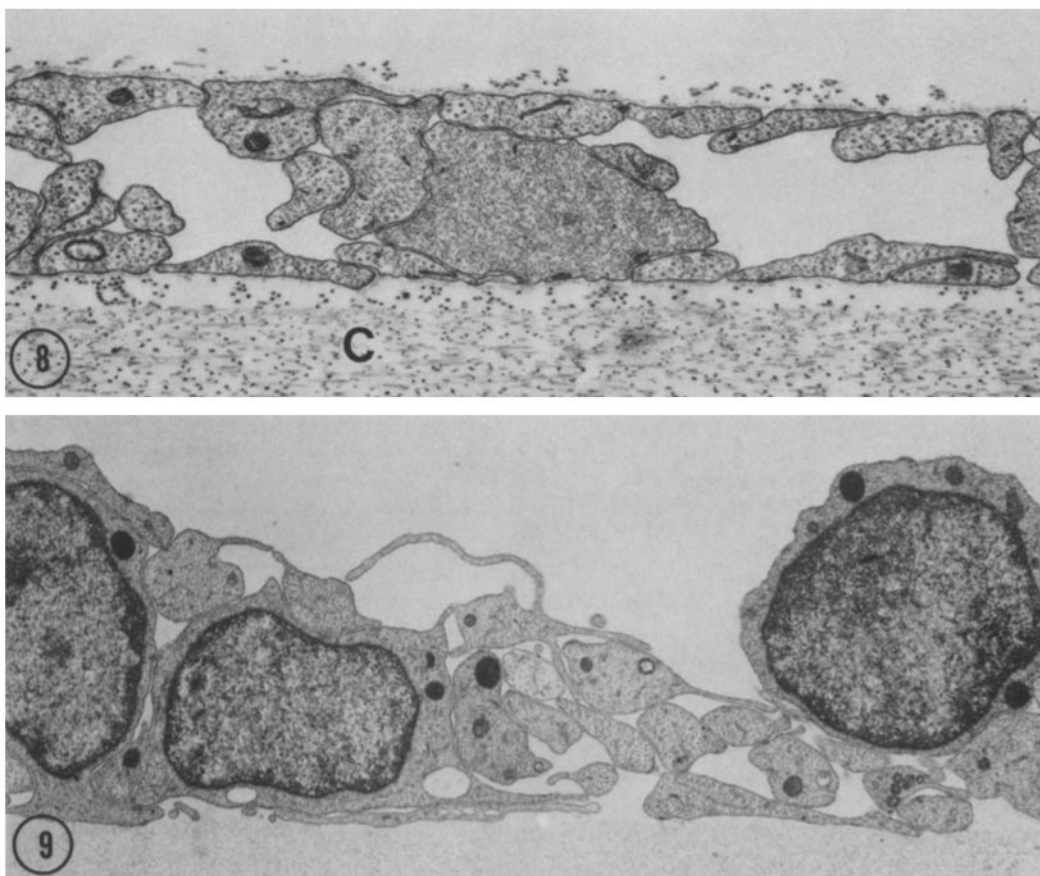

FIGURE 8 Electron micrograph of primary Schwann cells in culture. This micrograph shows the appearance of a fascicle of Schwann cells 1 wk after excision of the ganglion. The collagen substrate (C) is shown below. The basal lamina is well visualized in this preparation; it covers the external surface of the cells and appears to provide a scaffolding. Regions formerly occupied by neurites appear as empty spaces; the surfaces of the Schwann cell facing these spaces display no basal lamina. Schwann cell processes resemble neurites in cross section and have numerous filaments and microtubules. Photo courtesy of Dr. M. Bunge. Tannic acid stain. × 8,000.

FIGURE 9 Electron micrograph of replated Schwann cells. Cells resemble primary Schwann cells but lack a basal lamina. Several nuclear profiles may be seen in this section. Schwann cell membranes are closely opposed in both Figs. 8 and 9. × 12,000.
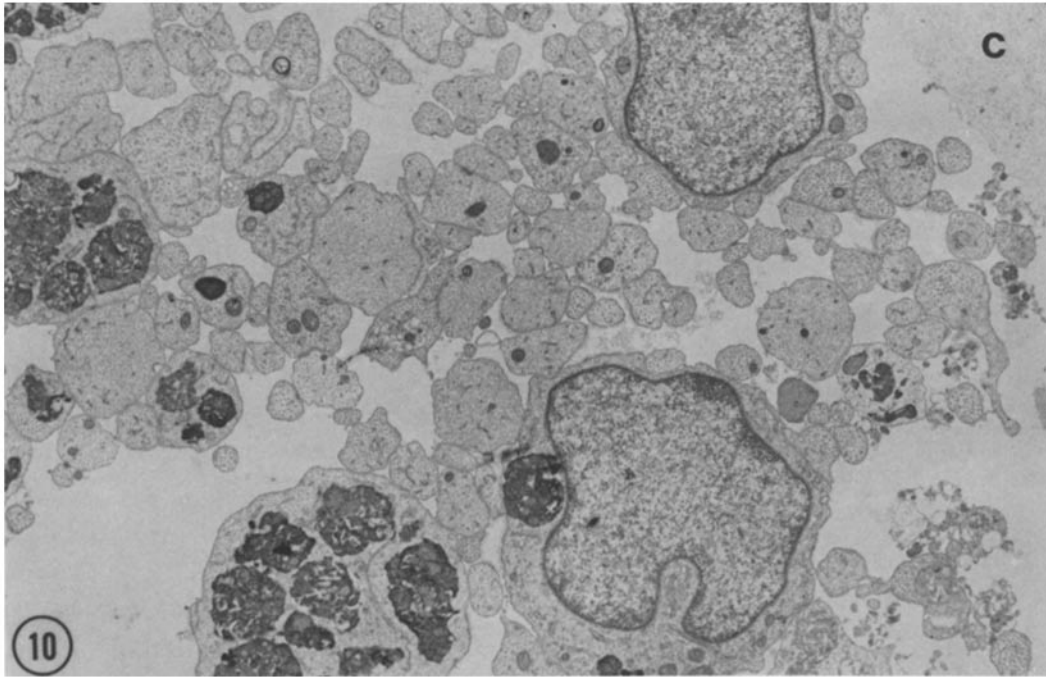
FIGURE 10 Phagocytosis of neurite membranes by Schwann cells at culture surface. In this electron micrograph the collagen substrate is at C, with the culture surface at the left. After 2 d of exposure to neurite membranes, those Schwann cells near the culture surface have ingested substantial amounts of membranes, apparently shielding those near the substrate surface. Rounded profiles are extensions of adjacent Schwann cells. × 8,000.

There are several changes that may be observed after neurite membrane addition, the most striking of which is the dramatic phagocytosis of the membranes (Fig. 10). Cells are laden with membrane debris that has been sequestered in phagolysosomes. This is consistent with observation of living cultures; after addition of neurite membranes the substrate is covered with the fine particulate except in areas where there are Schwann cells. Also associated with the uptake of debris is the presence of occasional vacuoles. In several cases, we have observed apparent binding of neurite membranes to Schwann cells and cells that appear to be actively phagocytosing membranes (Fig. 11).

Primary and replated cells have a similar response to neurite membranes. However, the pattern of interaction with primary cells is complicated by problems of access to the neurite membranes. A fascicle of Schwann cells to which membranes were added is shown in Fig. 10. It can be seen that cells which are located at the top and edge of the fascicle have taken up material, whereas those cells located in the interior of the
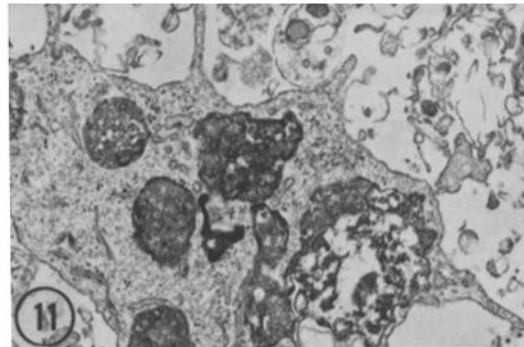

FIGURE 11 Interaction of neurite membranes with Schwann cell surface. After a 2-d incubation with neurite membranes, cells were fixed as usual. This electron micrograph shows the formation of filopodial-like extensions of the Schwann cell surface. Between these filopodia are several examples of apparent neurite membrane binding to the Schwann cell surface. This cell has already internalized a substantial amount of membrane. In the lower right portion of the micrograph are membranes which appear to be in the process of being internalized. × 19,000.

fascicle apparently have been shielded and display no uptake. Also, primary Schwann cells retain at least part of the basal lamina produced during their prior axonal contact; this may impede access to the added neurite membranes.

Other responses to membranes are largely morphologic. Schwann cells, especially replated cells, display filipodial-like extensions of the plasma membrane (Fig. 12) which may play a role in uptake of membranes or represent redundant membrane as the cells round-up and compact. Also, cells are more rounded and generally foreshortened longitudinally, as indicated by the large number of nuclear profiles observed in our sections (Fig. 12).

To determine whether phagocytosis and cell stimulation were necessarily linked, we examined Schwann cells to which several nonmitogenic membrane fractions were added. The membranes which were examined included fibroblast particulate and heat-treated neurites. Some uptake of each type of membrane was observed (for example, Fig. 13) and, although it is difficult to quantitate whether the extent of phagocytosis is altered by heat treatment, phagocytosis and stimulation are dissociable under these conditions.

DISCUSSION

We have presented evidence that isolated neurites or neurite membranes are mitogenic for Schwann cells and that this stimulation is specific. The interpretation of this finding is complicated by the ability of Schwann cells to proliferate in vivo and in vitro in response to both unensheathed intact neurites and, at least indirectly, to degenerating neurites (19). We will consider the mitogenic and
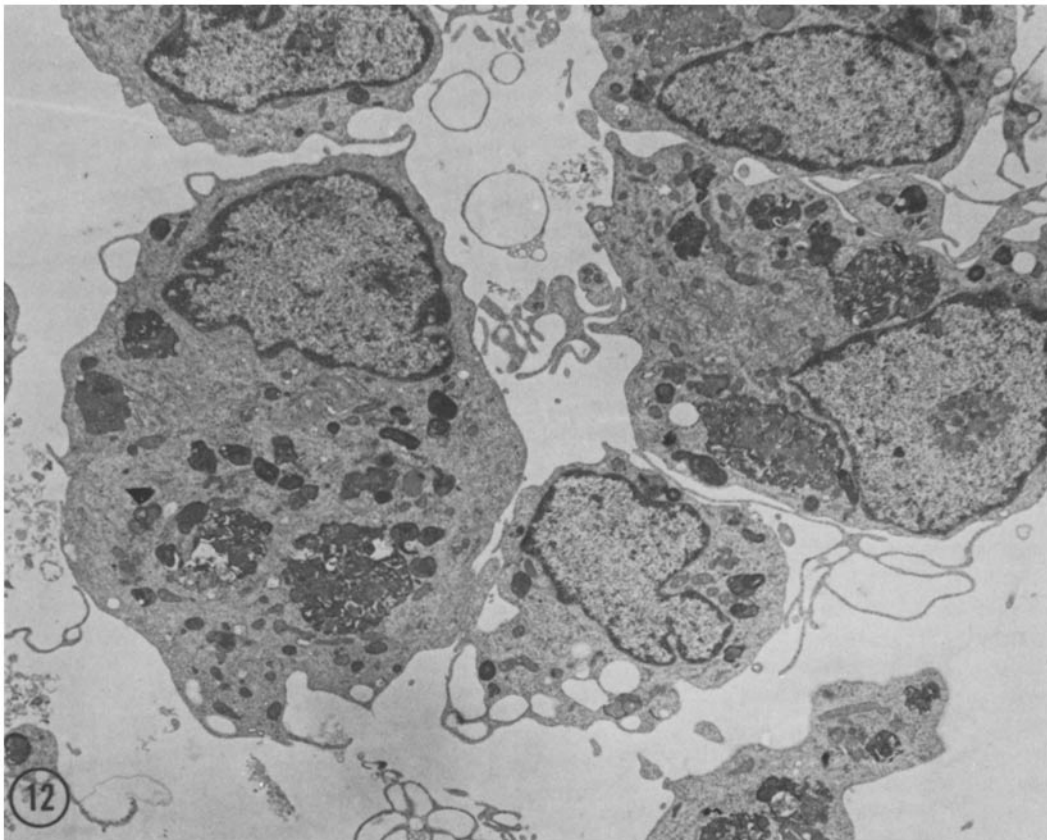

FIGURE 12 Filopodia and phagocytosis. After addition of neurite membranes, a substantial amount of phagocytosis has occurred. Also, numerous filopodia and membrane extensions characterize the response to neurite membranes. The large number of nuclear profiles apparent in this and other micrographs suggests that Schwann cells tend to foreshorten after interaction with the neurite membranes. × 6,000.
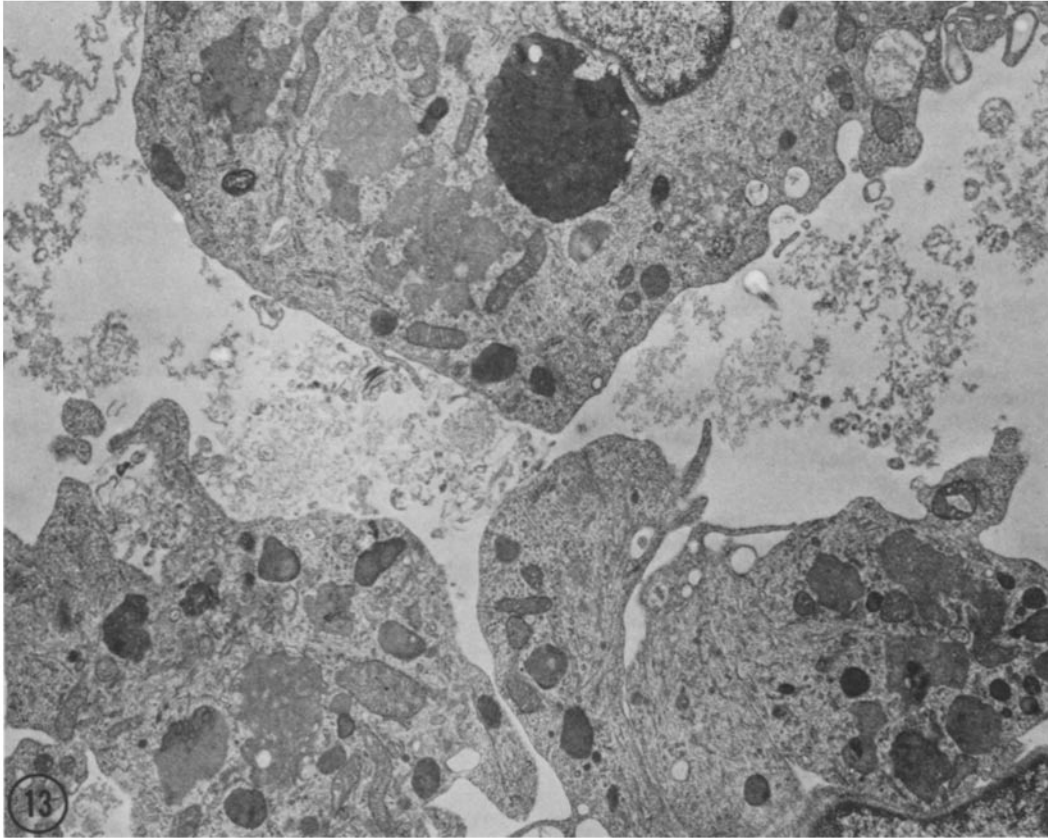
FIGURE 13   Phagocytosis of heat-treated membranes. Briefly heating (60°C, 10 min) neurite membranes abolishes their mitogenic effect (20) but, as this electron micrograph illustrates, there is still considerable internalization of the membranes. × 11,000.

phagocytic response of Schwann cells to neurite membranes in this context, and discuss evidence that the mitogenic stimulation is a model for the stimulation engendered by the intact neurite. It is worthwhile to note that the stimulation of proliferation by neurite membranes has direct implications for the localization of the neurite mitogen to the plasma membrane. We have considered this possibility in some detail in the final paper of this series, and defer further discussion on this point.

The stimulation of proliferation induced by neurite membranes simulates that induced by the intact axon in several ways. In response to neurite membranes, Schwann cells flatten (appearing polygonal in shape) and become more migratory; similar responses are observed when growing neurites interact with quiescent Schwann cell cultures. The stimulation provided by membranes is specific, 3T3 cells are not mitogenic nor are membranes isolated from 3T3 cells. Thus, Schwann

cells are not simply activated by particulate debris as are fibroblasts (2, 18). There is a rapid decline with time in the ability of membrane fractions to initiate division (Table I); this parallels the sharp decline in labeling of Schwann cells after excision of recently explanted ganglia (cultures in which there had been substantial ongoing proliferation). Finally neurite membranes stimulate those cells in primary cultures which were most actively dividing just before excision (i.e., cells at the outgrowth periphery); the resultant level of stimulation in the presence of neurite membranes approaches (as an upper limit) the level of proliferation expressed in that population of cells in the presence of the intact neurite.

By contrast there are a number of differences between the proliferative response after addition of membranes and the proliferative response to axotomy. The time-course of response to membranes is comparable to that observed when

Schwann cells are transferred onto intact neurites (20), but several days earlier than the peak response observed for myelin-related cells during Wallerian degeneration in vitro (19). After axotomy, Schwann cells do not normally divide in response to the presence of degenerating neurites alone, the cells also need to have been mechanically injured or to have previously formed myelin (19); this was not true for the Schwann cells to which neurite membranes were added. Observations on neurites harvested from the outgrowth of autonomic neurons are pertinent to this point. This neuronal species does not myelinate in vivo or in vitro. It is also known that there is little Schwann cell response in Wallerian degeneration of autonomic nerves (17). Thus this type of neurite is not stimulatory for Schwann cells during degeneration whereas it is mitogenic during development in vivo (24) and in vitro (27). When harvested neurites of this type were applied to quiescent Schwann cells they were stimulatory (data not shown). Taken together these data suggest that the stimulation by neurite membranes provides a model for the developmental rather than the degenerative mitogenic signal.

With respect to the phagocytic response to neurite membranes, Schwann cells do resemble reactive cells in Wallerian degeneration. For example, myelin and axonal debris have been reported to be sequestered in vacuoles inside of Schwann cells ("phagosomes") 3 or 4 d after peripheral (myelinated) nerve transection or crush (10–12, 15, 21). Also, in addition to increased lysosomal activity, the debris was generally membrane bound and there was an increased number of vacuoles in the reactive Schwann cells (12, 21). Schwann cells displayed cytoplasmic invaginations and filopodia, and were generally shortened in length (10, 12). In addition, most authors reported persistence of the basal lamina and increased numbers of ribosomes and endoplasmic reticulum. These observations parallel our own ultrastructural findings.

Our results strongly suggest that some of the cells engaged in phagocytosis observed in the distal degenerating peripheral nerve stump are derived from endogenous Schwann cells. The role of Schwann cells as phagocytes is a controversial subject (1). In this study, as well as others using this system (5, 27), we have not observed conversion of Schwann cells into macrophages as originally reported by Weiss and Wang (26). However, after the excision of the ganglion in SNDRGs cultures, the clearing of myelin proceeds rapidly despite the absence of macrophages, and myelin debris is clearly visible within the Schwann cell cytoplasm (19). These observations are consistent with several in vivo studies of Wallerian degeneration (3, 16, among others) which demonstrated that endogenous cells within the nerve, i.e., Schwann cells, resorbed the degenerating tissue. Also, it is evident from our studies that Schwann cells can avidly phagocytize a variety of cell membranes and are not merely autophagocytic (1). During this process of phagocytosis, the cells undergo a substantial change of morphology, which supports the notion of Fisher and Turano that "the unusual appearances assumed by these cells undoubtedly have led to their confusion with macrophages of systemic or local origin" (10).

Phagocytosis of particulate matter is not sufficient for stimulation. Other membranes which were not mitogenic (fibroblast membranes, heat-treated neurite membranes) were also taken up. It is worthwhile to note that cells were examined in these studies at a relatively late time (48 h of incubation), at which time neurite membranes had begun to degenerate and this may have stimulated their clearing by the cells. Alternatively, it is possible that neurite membranes (vesicles) bind to Schwann cells rapidly and that phagocytosis represents an exaggeration of a process that normally leads to ensheathment. Thus, when Schwann cell cultures were fixed and examined after only a 3.5-h incubation with neurite membranes, instances of membranes binding to the cell surface were more common and significant uptake already was in progress (data not shown).

Is uptake of neurite membranes necessary for stimulation? We cannot rule out this possibility at present. It may be that introduction of the neurite membrane (and entrapped cytoplasm) inside the Schwann cell is the stimulus; although the apparent sequestration of membranes within the phagolysosomal system does not make this an attractive possibility. Further work will be required to settle this point, but because phagocytosis is not a component of the stimulation provided by the intact neurite, we believe that stimulation and uptake are likely to be separable events.

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