Proc. Natl. Acad. Sci. USA
Vol. 77, No. 11, pp. 6902–6906, November 1980
Neurobiology

Schwann cells proliferate but fail to differentiate in defined medium

(peripheral nerve development/extracellular matrix/myelination/nerve tissue culture)

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Communicated by Sanford L. Palay, August 11, 1980

ABSTRACT **Primary cultures of dorsal root ganglia cells from 18- to 21-day rodent embryos were studied for their ability to express Schwann cell function in a defined medium lacking serum and embryo extract. It was confirmed that Schwann cells, but not fibroblasts, are able to proliferate in response to contact with axons when cultured in this defined medium. We here report that in this medium, however, differentiation of Schwann cells was arrested before completion of ensheathment and before initiation of myelin formation. Electron microscopic analysis confirmed this ensheathment failure and showed that the extracellular matrix components (basal lamina and thin collagenous fibrils) normally produced by axon-related Schwann cells had not been formed. This absence of extracellular matrix, as well as the presence in the Schwann cell of an increased cytoplasmic granularity (observed in the light microscope) and numerous distended cisterns of rough endoplasmic reticulum, suggest a failure in Schwann cell secretion. However, within one week after addition of serum and embryo extract to the culture medium, the ensheathment failure was corrected and myelination occurred; electron microscopic observations showed the presence of basal lamina and collagen fibrils in association with Schwann cells. These results suggest the presence in serum or embryo extract (or both) of factors necessary for the full expression of Schwann cell function (although a similar requirement is not present for the expression of oligodendrocyte function in culture). We propose that these observations indicate a linkage between Schwann cell secretion and axonal ensheathment, including myelin formation.**

Nerve tissue culture systems designed for the study of cellular interaction have traditionally used complex media that contain serum and, in some cases, embryo extract. For example, Peterson and Murray (1, 2) have described media requirements for myelin formation by Schwann cells in relationship to chicken sensory neurons in culture. In recent years, we have worked with peripheral nervous system tissues in culture in which media containing serum and embryo extract were used for studies of axon-Schwann cell interactions. Our investigations have included the expression of histiotypic organization in culture (3), Schwann cell proliferation (4–7), extracellular matrix formation (8), and myelin injury (e.g., ref. 9; for review, see ref. 10). Recently, Varon and collaborators (11, 12) have cultured dissociated dorsal root ganglia (DRG) cells in the N2 serum-free defined medium designed by Bottenstein and Sato (13) to support proliferation of neuroblastoma cells; they observed neuronal growth and Schwann cell (but not fibroblast) proliferation in their dissociated cell cultures. In this paper, we report that, when this defined medium is used in our standard sensory ganglion preparations, Schwann cell proliferation occurs and differentiation is initiated but fails to progress to typical axonal ensheathment and myelination, even after many weeks in culture. Electron microscopic study of these cultures shows that secretory products of the neuron-related Schwann cells [basal lamina and collagen fibrils; (8)] are absent from the extracellular space. Ensheathment, appearance of basal lamina and fibrils, and myelination occur within several days, however, after serum and embryo extract are added to the defined medium. We will discuss the implication of these observations on the role of secretion in the expression of Schwann cell function.

MATERIALS AND METHODS

DRG were dissected from either rat or mouse 18- to 21-day embryos, and their connective tissue capsules were removed by using forceps (Dumont, modified) having exceptionally fine tips. Ganglia were used as explants or were dissociated by incubation at 37°C for 30 min in 0.25% trypsin, followed by trituration with a pipette and filtration through a nylon membrane having 15-μm pores. The ganglia or dissociated cells were plated on Aclar minidishes coated with reconstituted rat tail collagen and incubated as described (14). Explant cultures were established and maintained in either N2 medium alone or in N2 medium supplemented with serum, serum and embryo extract, or specific additions (Table 1). Dissociated cell cultures were initially plated in a starter (i.e., antimitotic) medium (AMM; see below) containing serum, embryo extract, and an antimitotic agent and designed to (*i*) promote vigorous initial axonal growth, (*ii*) permit optimal neuronal differentiation, and (*iii*) suppress the development of nonneuronal cells. Cell cultures were shifted to N2 medium 10–14 days later. Cultures were fed every 2–3 days with an appropriate medium. Compositions were as follows: the standard medium (SM) contained 65% Eagle's minimal essential medium (15), 25% human placental serum, 10% 9-day chicken embryo extract, glucose at 6 g/liter, and crude nerve growth factor (16) at ≈50 biological units per ml; the AMM had the same basic composition as the SM, but also included 10 μM uridine and fluorodeoxyuridine and had only 10% serum and 2% embryo extract; the N2 medium was prepared as described (13) with the addition of nerve growth factor at the same concentration as in the SM. For microscopic analysis, cultures were fixed in buffered glutaraldehyde, followed by treatment with OsO_4, and either stained with Sudan black (17) for observation under the light microscope or further processed for electron microscopic analysis as described (8, 18). In brief, the cultures were stained en bloc with uranyl acetate, dehydrated and embedded in Epon/Araldite, all within the Aclar minidish. After curing, the cultures were studied by phase microscopy to select desired areas that were cut out and mounted to allow cross-sectioning of outgrowth fascicles in a plane perpendicular to the collagen substratum. Thin sections were stained with uranyl acetate and lead citrate. In both the light and the electron microscope, Schwann cells in defined medium can be recognized by their general morphology (smaller nuclear and cytoplasmic profiles than the fi-

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Abbreviations: DRG, dorsal root ganglion; AMM, antimitotic medium; SM, standard medium.

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Table 1. Effect of medium on myelin formation by Schwann cells

Medium	Myelin formation
Explant cultures*	
N2	0
N2 plus glucose (6 mg/ml)	0
N2 plus ascorbic acid (50 μg/ml)	0
N2 plus 10% human placental serum	+
N2 plus 20% human placental serum	++
N2 plus 25% human placental serum and 10% EE†	+++
Dissociated cell cultures‡	
SM	++
N2 plus 20% human placental serum	+/0
N2 plus 20% EE†	++
N2 plus 20% serum and 20% EE†	+++
N2 plus fibronectin (40 μg/ml)§	0
N2 plus fibroblast growth factor (10–100 ng/ml)§	0

* In every case, these were maintained from the onset in the indicated medium and evaluated after 6 or more weeks in culture.

† EE: Extract from 9-day chicken embryos diluted 1:1 with Earle's salts.

‡ In every case, these were initiated in AMM, shifted for 2 weeks to N2 medium, and then shifted for 2 weeks to the medium indicated.

§ Obtained from Collaborative Research, Waltham, MA.

broblast), association with axons, and failure to occupy regions devoid of axons. By the addition of serum and embryo extract to the N2 medium, Schwann cells were easily identified as usual by their ensheathment of axons, generation of basal lamina and, in some cases, formation of myelin.

RESULTS

Our observations on both explants and dissociated cell cultures from rodent sensory ganglia confirm recent reports (11, 12) of sustained proliferation of Schwann cells, but not fibroblasts, in the presence of nerve cells grown in a serum-free medium. The ability of N2 medium to support Schwann cell proliferation was best shown in the culture illustrated in Fig. 1 *Upper*. Dissociated DRG cells were plated and maintained in AMM to suppress initial cell proliferation. During this antimitotic treatment, networks of neurons interconnected by bundles of axons were formed; these lacked obvious supporting cells and, after two weeks in culture, contained primarily neurons. Some Schwann cells survived the antimitotic treatment, however; after withdrawal of the AMM and replacement by N2 medium, substantial Schwann cell proliferation occurred and, after 2 additional weeks, the network of nerve fibers was populated with Schwann cells. A similar proliferation of fibroblasts in N2 medium after withdrawal of the antimitotic agent was not observed. Explant cultures both established and maintained in N2 medium behaved similarly; large numbers of Schwann cells were generated in relation to nerve fibers forming the outgrowth, but few fibroblasts appeared.

These observations raise the question of whether contact with nerve fibers is the only mitogenic stimulus for Schwann cells under these culture conditions or whether components of N2 medium are also mitogenic for Schwann cells. Other work (12) as well as our own (L. Wartels and R. Bunge, unpublished observations) indicate that the main proliferative stimulus for Schwann cells in N2 medium is contact with axons. We have observed that cultures established as explants and allowed to generate a substantial outgrowth of axons and Schwann cells in N2 medium show little Schwann cell proliferation after explant removal and maintenance in N2 medium.

Although N2 medium supports proliferation of Schwann cells, which are seen to be associated with axons by light microscopic observation, these cultures (either explant or dissociated cells) did not form myelin even when maintained in N2 medium for 7 weeks. Dissociated cell or explant cultures maintained in SM generally exhibited myelin formation beginning in the third or fourth week. After prolonged exposure to N2 medium, a substantial Schwann cell population is retained, but these cells show little proclivity to gather axons into definite fascicles. A consistent and striking observation is the accumulation in Schwann cell cytoplasm of granules of variable density after Sudan black staining.

Electron microscopic analysis of preparations maintained in N2 medium (Fig. 2 *Upper*; see also ref. 19) showed that Schwann cell processes make contact with and partially surround axons but do not develop the degree of ensheathment typical for this type of culture (8). A striking feature was the frequent occurrence of thin "meandering" Schwann cell processes that had not begun to enclose nerve fibers (arrows, Fig. 2 *Upper*). Coincident with this ensheathment failure was a complete absence of basal lamina and extracellular fibril formation; cultures in serum-containing medium exhibit these morphological features after a few weeks of axon-Schwann cell contact (8). Dilated cisterns of rough endoplasmic reticulum were present in the Schwann cell cytoplasm (as illustrated in ref. 19). These cisterns, filled with flocculent material, may correspond to some of the many cytoplasmic granules seen in the light microscope, an observation that suggests that secretory products are synthesized but not released by Schwann cells. No myelin was observed.

This differentiation failure in the absence of serum and embryo extract is reversed by adding these components to the N2 medium; Fig. 1 *Lower* shows a culture of dissociated cells after 6 weeks *in vitro*. In this preparation, the cells were initially established in AMM for 2 weeks and, by the end of this period, many of the supporting cells had disappeared. During the next two weeks, the culture received N2 medium, which allowed repopulation by Schwann cells; the culture was then fed SM. Five days after addition of SM, myelin began to appear at the light microscopic level. Electron microscopic examination of a sibling culture of the one shown in Fig. 1 *Lower* showed that basal lamina and extracellular fibrils had been formed during this period and that the myelin present was normal in appearance (see Fig. 2 *Lower*).

We have used both explant and dissociated cell cultures to test the efficacy of adding various substances to N2 medium to promote Schwann cell differentiation and myelination (see Table 1). In all cases, only the undefined components—serum and embryo extract—were effective; addition of extra glucose, ascorbic acid, fibronectin, or fibroblast growth factor was not. In the dissociated cultures, embryo extract was more effective than serum (at the levels used) in correcting the failure of Schwann cell differentiation in N2 medium alone.

DISCUSSION

Recent work (8) has shown that, when cocultured with neurons in medium containing human placental serum and embryo extract, Schwann cells form basal lamina, thin collagenous fibrils, and myelin. Because contact with axons is required for the formation of basal lamina on Schwann cells (20, 21), it is possible that, in the experiments reported here, the absence of serum and embryo extract results in a failure of neuronal differentiation and thus in an inability of the axon to deliver this necessary signal to the Schwann cell. In this case, the failure of secretion of extracellular matrix materials would result from an axonal rather than a Schwann cell defect. Alternatively, this signal may be delivered normally, but there may be a failure

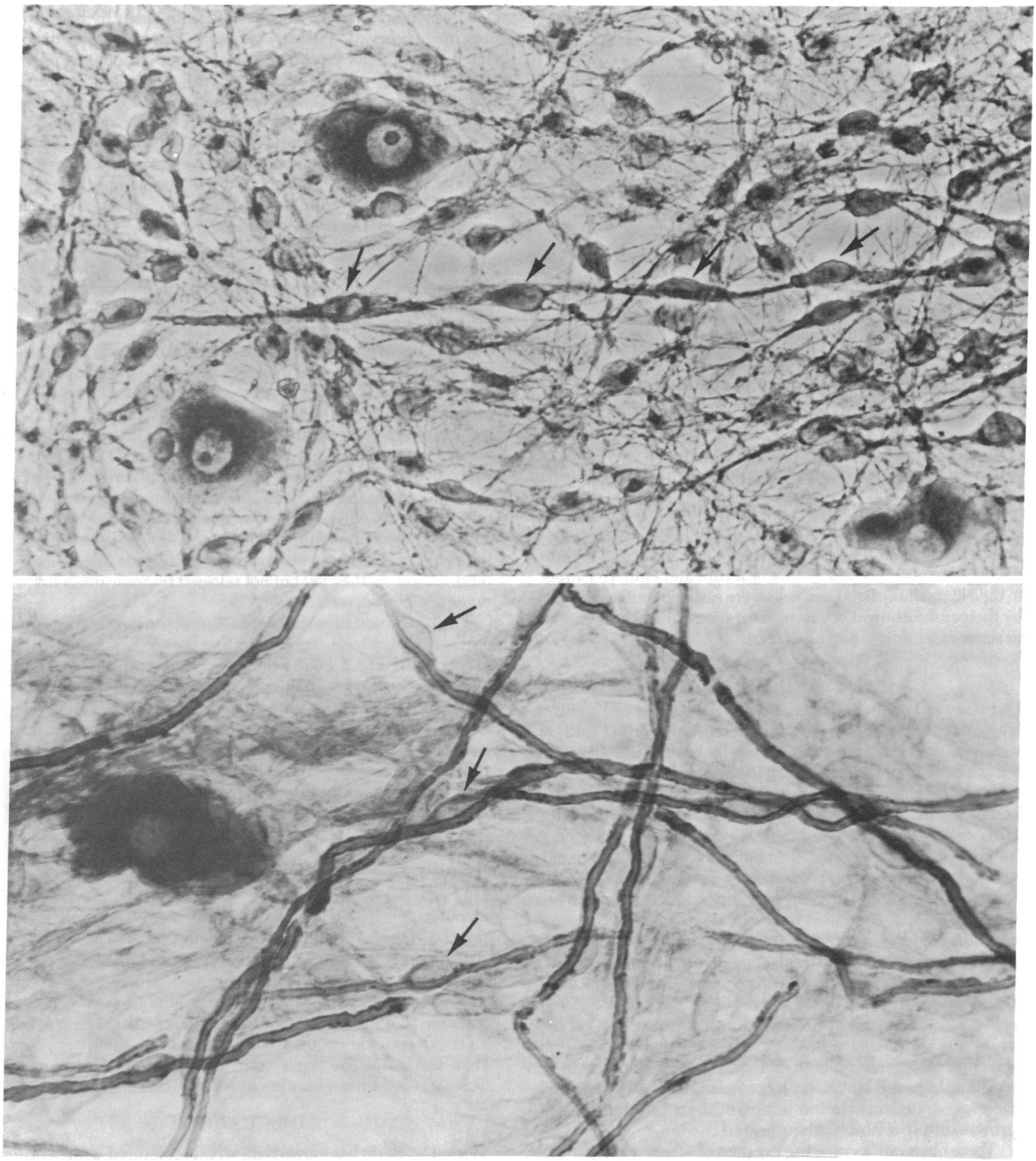

FIG. 1. Comparison of dissociated DRG cells grown in serum-free medium (*Upper*) and in a medium containing serum and embryo extract (*Lower*). In the first 2 weeks *in vitro*, both cultures were fed a starter medium (AMM) that contained serum and embryo extract; over the next 2-week period, they were grown in N2 medium. During the final 2 weeks, one culture continued to receive N2 medium, whereas the other was fed SM containing serum and embryo extract. In the culture grown in N2 medium, large numbers of Schwann cell nuclei are visible and, in favorable areas (arrows), are seen to be associated with axons. In the culture grown in SM, after a relatively short period, myelin sheaths are formed and Schwann cell nuclei (arrows) are situated in a normal position and are less frequent along axons than in the absence of serum. A few nerve cell bodies are seen. Cells from a 20-day-old rat embryo DRG. (Sudan black; ×450.)

of the Schwann cells to synthesize or secrete (or both) the necessary components for basal lamina assembly and for development of a stable association with axons. We believe the latter to be a more likely explanation for the following reasons: (*i*) When serum and embryo extract are added to cultures established and maintained in N2 medium, full Schwann cell func-

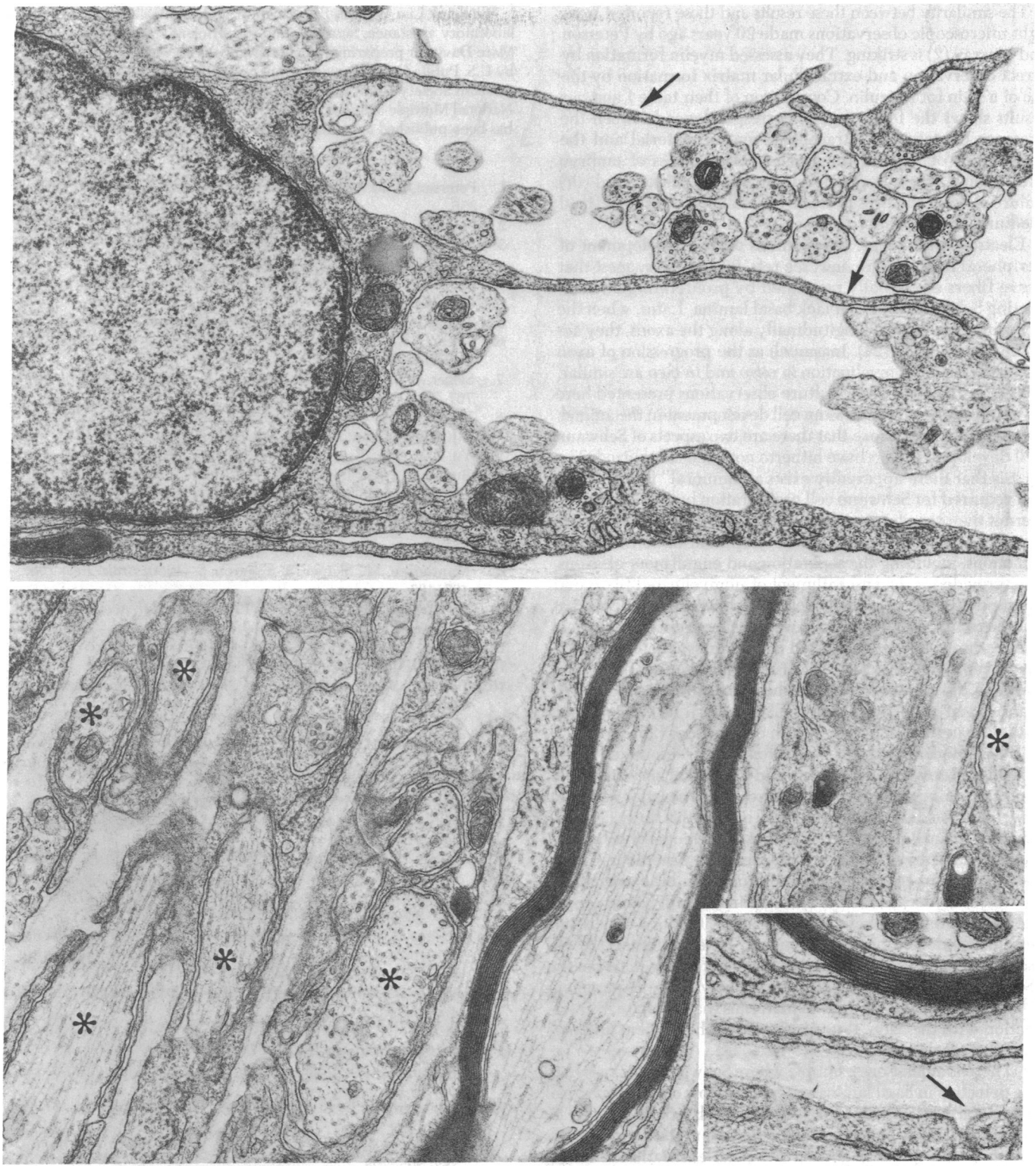

FIG. 2. Sibling cultures of those shown in Fig. 1, grown finally in a serum-free medium (*Upper*) or a medium containing serum and embryo extract (*Lower*). Schwann cells in N2 medium do not ensheathe axons in the typical way; some axons are nestled against the cell or are resting on thin processes (arrows) of the cell. Only after culture in SM are individual axons (such as those marked by asterisks) enclosed by Schwann cell cytoplasm; also, basal lamina and extracellular fibrils are initially absent from the Schwann cell exterior but are generated in 2 weeks in SM (see *Inset*, particularly at arrow). Formation of normal myelin also occurs in 2 weeks in SM. (*Upper*, ×31,000; *Lower*, ×30,000; *Inset*, ×42,000.)

tion is rapidly expressed, which suggests that axonal signals are in place and active; (*ii*) the presence of distended cisterns of granular endoplasmic reticulum in Schwann cells related to axons in N2 medium suggests failure to release secretory products, rather than failure to synthesize these products; and (*iii*) preliminary electrophysiological observations on neurons allowed to mature in N2 medium suggest normal resting and action potentials (D. Higgins, personal communication).

The similarity between these results and those reported from light microscopic observations made 20 years ago by Peterson and Murray (2) is striking. They assessed myelin formation by direct observation and extracellular matrix formation by the use of a stain for reticulin. Comparison of their table 1 and our results shows the following: (*i*) correspondence between the presence of stainable extracellular matrix material and the appearance of myelin, (*ii*) greater effectiveness of embryo extract than serum in promoting myelin formation, and (*iii*) failure of Schwann cells to relate normally to axons in defined medium lacking embryo extract.

Electron microscopic observations on the development of peripheral nerve (for review, see refs. 22 and 23) suggest that nerve fibers are initially populated by proliferating and migrating Schwann cells that lack basal lamina. Later, when the Schwann cells extend longitudinally along the axons, they acquire basal lamina (24). Inasmuch as the progression of axon ensheathment and myelination *in vitro* and *in vivo* are similar, we believe that the tissue culture observations presented here have implications for Schwann cell development in the animal. On this basis, we believe that there are two aspects of Schwann cell development that have hitherto not been recognized. The first is that there apparently exists a "humoral" factor that is not required for Schwann cell proliferation but is necessary to permit their secretory activity. The second is that this secretory activity appears linked to the development of Schwann cell functions, including the separation and engulfment of axons, that provide for the ensheathment of unmyelinated fibers and must precede the myelination of larger fibers. The concept that there is a linkage between Schwann cell secretion and axonal ensheathment is also supported by the observation that *cis*-4-hydroxyproline, an agent that disrupts collagen synthesis (25), causes a diminution in both ensheathment (including myelination) and basal lamina and collagen fibril formation when added to nerve cell plus Schwann cell cultures (26).

These findings suggest a difference between the developmental patterns of Schwann cells and the central nervous system myelin-forming cells, the oligodendrocytes. The latter will form myelin in the defined medium, N2, used in this study (27) and in other defined media (28). Electron microscopic observations suggest that myelination within the central nervous system occurs without concomitant deposition of visible extracellular matrix components, such as basal lamina; except where central nervous system parenchyma interfaces with blood vessels, it is essentially devoid of these elements. Taken together, the observations suggest that a fundamental difference between the Schwann cell and the oligodendrocyte may be the requirement that the Schwann cell secrete materials that mediate its interaction with the axon, thus promoting ensheathment and permitting myelination.

There is at least one example where Schwann cells known to be defective in basal lamina production show certain functional characteristics of oligodendrocytes. Cells considered to be Schwann cells in peripheral nerves of the mutant mouse, dystrophic, may provide abnormal axonal ensheathment along with defective basal lamina coverage; the myelin formed under these circumstances shows certain resemblances to central nervous system myelin [e.g., ensheathment of two axons by one Schwann cell (29)].

We thank Lisa Wartels, Robert Smith, and Ann Williams for expert laboratory assistance, Susan Mantia for efficient secretarial aid, and Marc Davis for preparing the photographs. This work was supported by U.S. Public Health Service Grant NS 09923 and National Multiple Sclerosis Society Grant RG 1118. F.M. is a Postdoctoral Fellow of the National Multiple Sclerosis Society. A preliminary report of this work has been published as an abstract (30).

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