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Migration of Schwann cells and wrapping of neurites *in vitro*: A function of protease activity (plasmin) in the growth medium

(plasminogen activator/neuromuscular junction/peripheral nervous system/Schwann cell ensheathment)

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ABSTRACT *In vitro* conditions were defined under which Schwann cells, from a population of dissociated embryonic chicken spinal cord cells, migrate along the growing neuronal fibers and wrap bundles as well as individual axons, in a pattern similar to that found in a developing peripheral nervous system *in vivo*. The migration of Schwann cells and their wrapping of nerve fibers was found to be a function of plasmin activity in the growth medium. It was determined that at least one cell type among the spinal cord cells is producing plasminogen activator, the enzyme that activates the plasminogen that is a constituent of any serum. It is concluded that, to achieve wrapping of neurons by Schwann cells in culture, it is essential to have an active plasmin-generating system in the medium. It is hypothesized that the Schwann cell produces plasminogen activator. The possible role of both the Schwann cell and the plasminogen activator in the formation of the neuromuscular junction is discussed.

One of the striking, yet enigmatic, features of the developing peripheral nervous system is the ability of neuronal fibers to extend all the way to their target muscle cells. Moreover, these neuronal extensions are accompanied along their entire length by Schwann cells that multiply and migrate along with the axons between the other developing tissues (1, 2). The Schwann cells in turn send processes between the numerous axons, resulting in the ensheathment of either single axons or groups of separated axons (3). This developing nerve–Schwann cell system is a multivectorial one, and cell motility apparently is an important component in the entire process. In contrast, with the developing neuromuscular junction *in vivo* the mature active motor synapse represents a static cell system in which cell motility has apparently no function and in fact may be inhibited. At the adult neuromuscular junction the nerve and muscle cells are in a direct apposition separated by a basal lamina, whereas the Schwann cell envelops the neuronal bouton but does not enter the active synaptic cleft (4, 5).

Whereas *in vivo* the Schwann cells are capable of migration, this capacity is limited in cultures of the same developing cellular systems. This is so even though considerable effort has been exerted in an attempt to achieve the normal type of interaction between Schwann cells and nerve fibers in culture. The only successful attempts were those that have utilized micro conditions—cells grown in a drop of medium (6–11) rather than in standard culture dishes (12, 13). The significance of the volume of the medium will be discussed.

Recently the extracellular protease combined system—a plasminogen activator and its reaction product, the active plasmin—has been associated in various biological systems with tissue remodeling and the invasiveness or migratory potencies of certain cells, both normal and malignant. The plasminogen activator–plasmin system has been implicated as functioning, for example, in follicle rupture during mammalian ovulation (14), in blastocyst implantation and basement membrane metabolism (15), and in neoplasia in mammary involution (16).

In the present study I have tried to define and characterize the factors that control the migration of Schwann cells in culture. The preliminary efforts of this study were focused on proteolytic enzyme activities as possible participants in the process of migration/invasion during the development of both the peripheral nervous system and the neuromuscular junction.

MATERIALS AND METHODS

Materials. Fertilized hen's eggs were obtained from Shamrock Farms (North Brunswick, NJ). The following items were utilized for this study: minimal essential medium (Eagle) with Earle's salts and without L-glutamine, penicillin/streptomycin solution, L-glutamine solution, heat-inactivated horse serum (HS), and heat-inactivated fetal calf serum (FCS), GIBCO; 1–300 trypsin (hog pancreas), ICN; soybean trypsin inhibitor, Worthington; sterile aqueous solution of [^{3}H]thymidine (6 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), Schwarz/Mann. For the plasminogen activator assay, multiwell dishes coated with ^{125}I-labeled fibrinogen, plasminogen-depleted fetal calf serum (FCS − P), and acid-treated plasminogen-depleted fetal calf serum (ATFCS − P) were kindly donated by W. H. Beers, The Rockefeller University. Falcon culture dishes (BioQuest, BBL, and Falcon Products, Cockeysville, MD) were utilized. Chicken embryo extract (CEE) was prepared as described (17).

Cultures. Spinal cord cells from 7- to 9-day-old chicken embryos were dissociated by a minor modification of the procedure of Fischbach and Dichter (13). The small pieces of the spinal cord were trypsinized with 0.1% trypsin in Ca^{2+}, and Mg^{2+}-free Puck's saline G (17) for 30 min at 37°C. The cells were rinsed once with a 0.1% soybean trypsin inhibitor in minimal essential medium and then suspended in the appropriate medium. All the media that were used contained 2 mM glutamine, penicillin (50 units/ml) and streptomycin (50 μg/ml). The cells were plated either on multiwell dishes that were coated with ^{125}I-labeled fibrinogen and pretreated with minimal essential medium containing 10% FCS − P (14) or on 35-mm plastic petri dishes that were coated with either collagen (17) or fibrinogen and pretreated with minimal essential medium containing 10% FCS − P. This treatment results in the conversion of fibrinogen to fibrin (14). The cultures were maintained in a 5% CO_2 incubator at 37°C.

Plasminogen Activator Assay. Extracellular plasminogen activator was assayed by monitoring the fibrinolytic release of ^{125}I-labeled peptides into the medium as described by Strickland and Beers (14). The cells were plated in minimal essential medium supplemented with 19% FCS-P and 0.01% soybean trypsin inhibitor into multiwell dishes that had been coated with ^{125}I-labeled fibrin (14). After 2 hr the medium was removed

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Abbreviations: FCS, fetal calf serum; HS, horse serum; CEE, chicken embryo extract; AT, acid-treated; − P, plasminogen-depleted.

and the appropriate media were added. Maximal, or 100%, activity was defined as the amount of ^{125}I-labeled peptides solubilized by an excess of trypsin. For the fibrinolytic activity assays the data are expressed as a percent of this maximal available substrate. Acid treatment of FCS and CEE, to decrease protease inhibitor activity, was conducted as described (14). Attempts to acid treat HS failed, because the serum components gelled when neutralized with NaOH.

Ultrastructural Analysis. All the materials and methods utilized for this analysis were identical to those described (17), with one exception. The samples were treated for only 30 min with 1% OsO_4 in barbital/acetate buffer (pH 7.4).

Light Microscopic Autoradiography. All the materials and methods utilized to monitor the radiolabeling of nuclei with [^{3}H]thymidine were identical to those described (17). The cells were plated on collagen-coated 35-mm culture dishes, and [^{3}H]thymidine (10 μCi/ml) was added to the medium right after the initial plating medium was replaced by the appropriate growth medium.

RESULTS

Dissociated spinal cord cells from 7- to 9-day-old chicken embryos produce some proteolytic enzyme in culture as determined by their extracellular fibrinolytic activity (Fig. 1). The spinal cord cells were cultured on a multiwell ^{125}I-labeled fibrin-coated dish and the fibrinolytic assay was started by replacing the plating medium with minimal essential medium that contained one of the following: (*i*) regular medium (10% HS + 5% CEE), (*ii*) acid-treated medium (10% ATFCS), (*iii*) acid-treated medium (10% ATFCS + 5% ATCEE) [AT(FCS + CEE)], (*iv*) plasminogen-depleted medium (10% FCS − P), or (*v*) plasminogen-depleted acid-treated medium (10% ATFCS − P). Fibrinolytic activity was detected only in the wells in which the media both contained plasminogen and had the protease inhibitors eliminated by the acid treatment, namely,

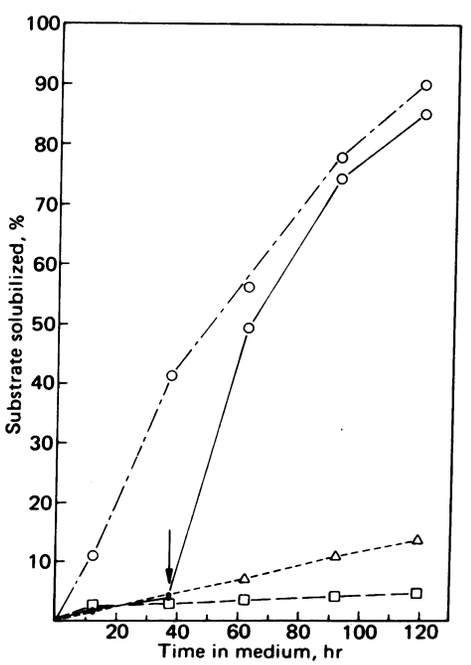

FIG. 1. Fibrinolytic activity of dissociated embryonic spinal cord cells as a function of presence or absence of plasminogen and protease inhibitors in the growth media. The dissociated spinal cord cells were plated at 1.5 × 10^5 cells per well, and the fibrinolytic assay was started by removing the plating medium and adding different media at 2 ml per well: ○, AT(FCS + CEE); •, regular medium, HS + CEE; □, FCS − P. After 36 hr (arrow) the regular medium was replaced with the following media: △, ATFCS − P; ○, AT(FCS + CEE).

in a medium that was supplemented with either ATFCS or AT(FCS + CEE) (Fig. 1). The fact that this fibrinolytic activity is dependent on the presence of plasminogen in the medium demonstrates that some of the cultured spinal cord cells produce plasminogen activator (plasminogen is a normal constituent of any serum). Some fibrinolytic activity was detected in control wells that contained acid-treated medium without any cells. This activity amounted to approximately 5% of the fibrinolytic activity monitored in the presence of cells at any given time point. No fibrinolytic activity could be detected in the cultures that were grown in the regular medium (13), which is supplemented with HS + CEE (Fig. 1). However, when the regular medium was changed (36 hr after the assay was started) to an acid-treated medium [AT(FCS + CEE)], there was a sharp rise in the fibrinolytic activity (Fig. 1). Again, this fibrinolytic activity is a result of plasminogen activator, because no significant activity above background could be detected when the regular medium was changed to either the plasminogen-depleted medium or the plasminogen-depleted and acid-treated medium (Fig. 1). Growing cells in the regular medium apparently does not eliminate their capacity to produce plasminogen activator.

Light microscopic examination of the dissociated spinal cord cells grown in the different media revealed some striking differences in the relative spatial organization of these cells (Fig. 2). The spatial organization of the cells can be correlated with the expression of plasminogen activator activity in the growth media. In cases in which fibrinolytic activity was found in the medium (acid-treated medium *ii* or *iii*), the neuronal cell bodies aggregated in tight clusters often resembling ganglia (Fig. 2*D*). From these aggregates there was an extensive outgrowth of bundles of axons (Fig. 2 *C–E*), and these bundles sometimes seemed to be accompanied by other cells (Fig. 2*E*). The large aggregates of cell bodies from which bundles of axons are extending in Fig. 2 *C* and *E* are not shown. The most prominent feature of these cultures was that the neuron cell bodies were almost never found to exist individually, and they could be traced and identified only by the complex fiber network in the culture. The fibers themselves were either bare axons or bundles of axons accompanied by some cells (Fig. 2 *C–E*). In the other cases in which the spinal cord cells were grown either in regular medium supplemented with HS + CEE (Fig. 2*A*) or in medium in which either plasminogen or both plasminogen and protease inhibitors were eliminated (Fig. 2*B*), the cells for the most part displayed a dispersed organization pattern. In these cultures (Fig. 2 *A* and *B*) the survival rate of the neurons was low and most of the neurons were individually spread on a carpet of nonneuronal cells as described (13). The neurons in these cultures can be identified by either their extended processes in unipolar and bipolar forms or the network of fine neurites associated with their cell bodies. Rarely, aggregates of neuronal cell bodies could be found also in media in which no fibrinolytic activity could be expressed (Fig. 2*B*). Still, the growth from these aggregates was primarily as single processes without any accompanying cells.

The accompanying/wrapping cells in the bundles were identified as nonneuronal cells, and part of them possibly as Schwann cells, by two independent criteria. First, these cells are dividing cells as determined by their incorporation of [^{3}H]thymidine into the nuclei. Neurons at this stage of differentiation for the most part do not divide (13), whereas the Schwann cells and other glial cells do. When [^{3}H]thymidine was added to the cultures in acid-treated medium, the nuclei of the wrapping cells were radiolabeled, whereas the associated neurons did not incorporate any label (Fig. 3). Second, groups of axons as well as individual axons were found to be enveloped or enclosed within nonneuronal cells, as determined by ultra-

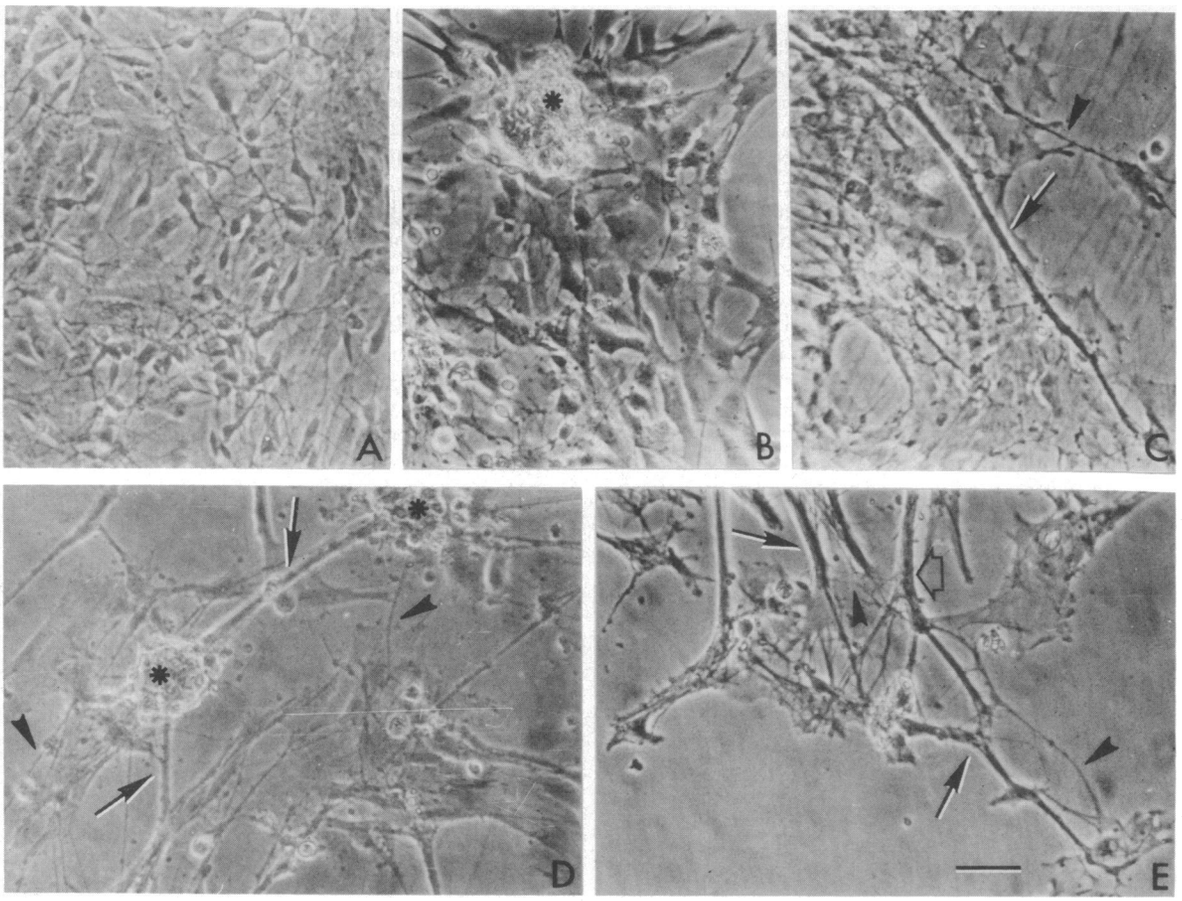

FIG. 2. Photomicrographs of the spinal cord cells in culture, in different growth media. (Bar = 50 μm; ×170.) The cultures were photographed at 4 days (*A*) and at 7 days (*B–E*) after plating. The composite contains a culture in a regular medium, HS + CEE (*A*), a culture in ATFCS −P medium (*B*), and cultures in AT(FCS + CEE) medium (*C–E*). (*A* and *B*) The neurons are randomly spread on a carpet of nonneuronal cells, and aggregates are rarely seen (*) (*B*). (*C–E*) Bundles of axons (arrows) as well as individual bare neurites (arrowheads) are observed in the cultures. In certain cases (*E*) the nuclei of the accompanying cells can be discerned (clear arrow). Aggregates of cell bodies (*) of various sizes are found in *D*, but in *C* and *E* the aggregates are not shown. The neuronal fibers extend in bundles from clumps of cells and in certain cases (*D*) form connections between the aggregates. Note the absence of individual neurons in *C–D* in contrast to their abundance in *A* and *B*.

structural analysis (Fig. 4). After 8 days in culture the majority of the axons appear in a still immature form, unseparated, in groups enveloped by one or two nonneuronal cells (Fig. 4*A*). This type of nerve bundle organization is similar to the one described in the developing peripheral nervous system in 16-day-old rat fetus (3), or in developing fast and slow muscles in chicken embryo (18), or in 14-day-old mouse fetus intercostal muscle (unpublished observations). It is important to note here that, within its ventral roots, embryonic spinal cord includes Schwann cells (19) that accompany the developing motoneurons. These Schwann cells originate from the neuronal tube (20, 21), whereas those in the dorsal root ganglia originate from the neuronal crest (20). The cultures utilized in the present study probably contain both oligodendrocytes and Schwann cells as glial cells that envelop neurons. The mode of wrapping of neurons by either oligodendrocytes or Schwann cells can serve as a criterion to distinguish between the two: "In the periphery, Schwann cells associate themselves with large numbers of axons, and by cell division which gradually increases the number of cells, they isolate a single one of the larger axons for their attention. In the central nervous system a single oligodendrocyte merely encompasses a single axon with one of its processes and straight away starts the irregular process of spiral wrapping" (22). I have used this criterion with some of the ultrastructural features of Schwann cells *in situ* (22) to identify the Schwann cells in my cultures. Examples are the abundance of free ribosomes, microtubules, and filaments in the cytoplasm, and numerous processes found in between the cross-sectioned axons. These features suggest that part of the enveloping cells are indeed Schwann cells (Fig. 4). Ultrastructural analysis of spinal cord cells maintained in the regular medium revealed, as reported (13), that the nerve processes were neither enveloped nor covered by Schwann or other glial cells.

DISCUSSION

It is possible to conclude from the data presented above that Schwann cells migrate along and wrap the growing neurites as a result of the expression (presence) of the proteolytic activity of plasmin in the growth medium. The plasminogen probably is activated by plasminogen activator produced by one of the cell types in the culture. Thus, to achieve wrapping of neurons by Schwann cells in a culture of dissociated chicken embryo spinal cord cells, it is essential to have in the medium a plasmin-generating system containing plasminogen activator and plasminogen. Plasminogen is supplied with the supplemented serum (23), whereas plasminogen activator is produced by the cells.

Whereas the wrapping of neurons by Schwann cells is a function of proteolytic activity, it is not clear yet which factors are involved in the aggregation of the cells. It seems from preliminary experiments that the cell aggregate size is probably a function of cell density (cell number per plating surface) rather than the difference in substrate—i.e., fibrin or collagen. Nevertheless, whatever the factors are that promote aggrega-

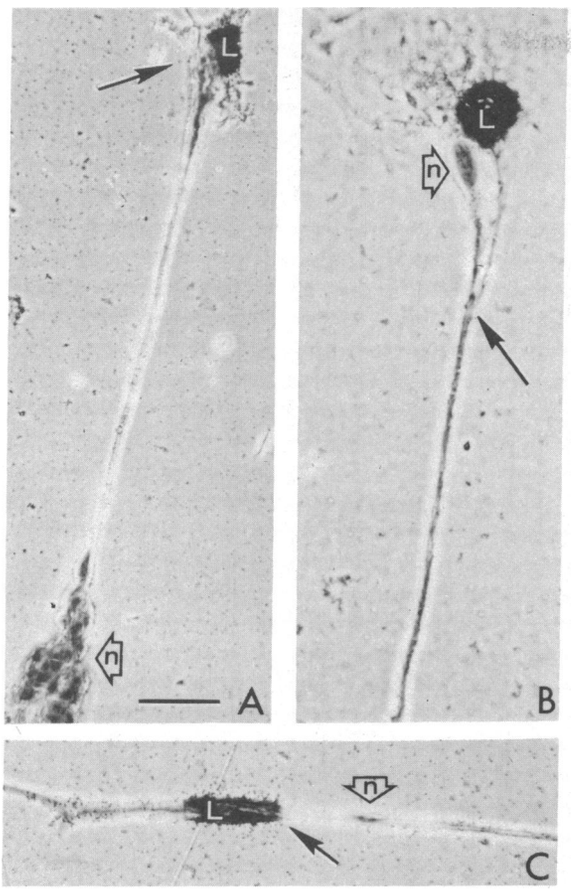

FIG. 3. Association of neurons with dividing nonneuronal cells. Phase-contrast autoradiographs of spinal cord cells grown in acid-treated medium and [^{3}H]thymidine. After 3 days in this medium the cultures were fixed and processed for autoradiography. The nonneuronal cells with the radiolabeled nuclei (L) are the cells that underwent mitosis. The neurons extend long axons and their nuclei (n in a clear arrow) did not incorporate any [^{3}H]thymidine. A nonneuronal cell is associated in a specific manner with the neurons (arrows); it either accompanies a bundle of axons coming from an aggregate of neuronal cell bodies (*A*) or sends a process that accompanies the axon (*B*). Sometimes the nonneuronal cell covers and is aligned along a neuron, as in *C*, where the unlabeled axon can be discerned (arrow) in the midst of the labeled nucleus (L). (*A*, ×430, bar = 25 μm; *B*, ×580; *C*, ×690.)

tion, we have to remember that the aggregates were found mainly in media in which the activity of plasmin was expressed (Fig. 2).

As mentioned in the introduction, attempts have been made previously to achieve ensheathing of neurons by Schwann cells as well as myelination in culture; indeed, some of these attempts have been successful (6–9, 11). In all these cases the dissociated cells or the explants from spinal cords or dorsal root ganglia were cultured in a drop of medium in a Maximow depression slide, a minidish (24), or the Rose multipurpose chamber (9). In these cases the volume was probably minimal (≤0.25 ml), and the ratio (number cells per volume of medium) was at least 20 times larger than the ratio, for example, in cultures described in Fischbach and Dichter (13) or in those described in this report (Fig. 2 *A* and *B*) where no wrapping was found. In media in which the cell density is high the protease inhibitors are probably neutralized by the comparatively high amounts of the enzymatic chain reaction product, plasmin (25). The amount of plasmin is probably determined by the amount of the plasminogen activator available, which is produced by the cells. This pattern of development may reflect the actual process *in vivo*, where protease inhibitors do exist in the serum but plasminogen activator is produced in high concentrations in a localized manner. As a result of an excessive plasmin activity the way is cleared, in this microenvironment, for the growth of neurons and migration of Schwann cells. For example, the rupture of the follicular wall during mammalian ovulation by plasmin is explained by the localized release of plasminogen activator from the hormonally stimulated granulosa cells (14, 25). In fact, the only neuromuscular junction that included the Schwann cell as well was obtained in an organ culture (10) grown in a drop in the Maximow slide assembly. In all the other cases of regular nerve–muscle cocultures, only naked nerve–muscle contacts were observed (refs. 26–29; unpublished results). The difference in the pattern of development in these two systems can be accounted for by the difference in the ratio of cell number to volume.

The identity of the cell type in the spinal cord cell population that produces the plasminogen activator is still unknown. However, it is possible at the present to speculate on the type of cell that might be the producer of plasminogen activator, mainly by deduction from some published data: (*i*) Several studies have demonstrated that glial cells can produce nerve growth factor-like proteins (for review see ref. 30). It was also hypothesized (30) that glia may act as a source of nerve growth factor *in vivo* for ganglionic and possibly other neural tissues. Additionally, it was found that nerve growth factor is a serine protease that can activate plasminogen; it thus behaves as a plasminogen activator (31). (*ii*) It was suggested (32) that displacement of degenerated synaptic terminals is carried out by microglial cells. In another study it was suggested that glial processes are responsible for the spontaneous elimination of synapses on cat spinal motoneurons after birth (33). O'Brien *et al.* (34) suggested recently that the elimination of multiple innervation of the skeletal muscle is carried out by proteolytic activity. And, finally, Glazer *et al.* (35) have studied the neuropathology at the cat soleus neuromuscular junction after one injection of diisopropyl fluorophosphate. The induced damage of the soleus nerve–muscle in this system was biphasic, composed of subacute and delayed effects. The subacute injury was confined to the junctional region and mainly involved postjunctional structures, presumably as a result of the irreversible inhibition of acetylcholinesterase at the endplate. On the other hand, the delayed effects were in response to the delayed degeneration of motor nerve terminals, and the postjunctional changes that were observed were similar to those seen in denervation studies. The delayed effect of diisopropyl fluorophosphate in this study remains unresolved.

Let us assume that glial cells (including Schwann cells) produce plasminogen activator and that it is crucial for the normal function of the cell; then all four of the separate observations described above (in *ii*) have a common denominator: namely, the Schwann cell is involved both in the elimination of multiple innervation and in the displacement of degenerated synaptic terminals. This process would be accomplished at least in part by utilizing some proteolytic activity; such activity was reported in the case of the elimination of multiple innervation (34). Moreover, because plasminogen activator is a serine protease (36) that is irreversibly blocked by diisopropyl fluorophosphate, as is acetylcholinesterase, this might be the reason for the delayed degeneration of the motoneurons observed by Glazer *et al.* (35). In this context it is tempting to hypothesize that in the mature active endplate the Schwann cell is stopped from penetrating between the nerve and the muscle at their site of apposition by some protease inhibitors that are present in the synaptic cleft, perhaps a component of the basal lamina. This inhibitor might actually be a product of a functional nerve–muscle contact. It might be that the data presented in this report

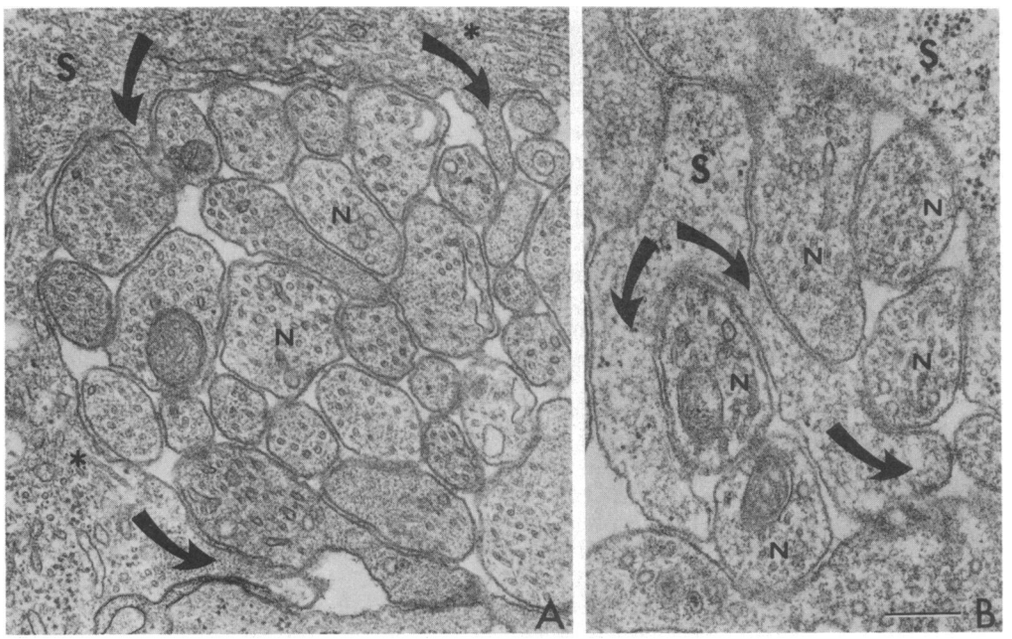

FIG. 4. Thin section electron micrographs of neurons enveloped and in the process of being wrapped by Schwann cells. The cells that were grown in an AT(CEE + FCS) were fixed 8 days after plating and were sectioned in a plane vertical to the dish. (*A*) A group of neurites (N) is enveloped by a Schwann cell (S). Note the small processes (arrows) that extend from the Schwann cell to penetrate between the neurons. Groups of microtubules in the Schwann cell are indicated (*). (*B*) Two or three neurites (N) are in the process of being wrapped and separated from each other by processes (arrows) of the Schwann cells (S). (*A*, ×27,500; *B*, ×33,000, bar = 0.3 μm.)

together with these bits of information will eventually fit into one common mechanism.

The fact that the behavior of Schwann cells appears to be a function of plasmin in the growth medium does not necessarily imply that this is either the only or a sufficient requirement for their proper development or development of other cell types. The proteolytic activity of plasmin is probably one of the necessary culture conditions required to generate proper model systems for the study of the factors that are involved, for example, in the formation of the neuromuscular junction. One can expect that each of the three synaptic components—the nerve, muscle, and Schwann cell—will mutually modulate and control the activity and the behavior of the two other partners. Even if, for example, plasminogen activator is produced by the Schwann cell, the amount of production might actually be under a direct control of the nerve cell, dependent on the neuronal activity.

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