Quantitation of Sprouting of Dorsal Root Axons

Abstract. *The axonal sprouting that occurs after denervation resulting from a spinal hemisection can be quantified. Rats were subjected to hemisection of the spinal cord at birth, and the myelinated and unmyelinated axons in dorsal roots three segments cranial and three segments caudal to the lesion were counted 1 month after surgery. The number of unmyelinated axons in the dorsal root on the side of the hemisection increased 22 percent for the roots one segment from the lesion and 13 percent for the roots two and three segments from the lesion.*

When neural tissue is damaged, intact axons in the damaged area often develop collateral branches. This phenomenon, known as sprouting, may represent a mechanism of neural regeneration. It has been generally accepted, however, that the mammalian spinal cord does not regenerate (*1*), and as might be expected, axonal sprouting after injury has been reported to be sparse and ephemeral (*1*). In 1958, however, Liu and Chambers reported the appearance of significant sprouting of dorsal root axons in the spinal cord after neighboring dorsal roots were cut (*2*). Some subsequent studies (*3*) confirmed and extended these observations by showing that any of several lesions that resulted in denervation of an area of cord seemed to result in sprouting of axons from a test root, but other studies reported that sprouting of dorsal root axons either could not be demonstrated or was minimal (*4*). In these studies, however, the numbers of axons were not determined (*5*) because the light microscope cannot resolve unmyelinated axons even when they are darkened by the silver stains (*6*). Thus, to count all axons in the dorsal root, it is at present necessary to use the electron microscope. This study is a quantitative electron microscopic assessment of axonal sprouting in rat thoracic spinal roots 3 months after a spinal hemisection done at birth.

Four newborn rats were anesthetized in icy water for 3 to 5 minutes. The skin of the back was opened and the sixth thoracic spinal process removed. The spinal cord was exposed, making the dorsal vein apparent. Either the right or the left side of the cord was then hemisected with iridectomy scissors. After surgery, the completeness of the lesion was assessed by passing a pin to the floor of the spinal canal in the midline and passing it laterally. The dorsal vein was not damaged. The skin was closed by apposition only. The pups showed no signs of discomfort, and when returned to their mother, nursed within 3 hours.

After 3 months, the young rats were anesthetized with Nembutal and perfused through the heart with a mixture of glutaraldehyde and formaldehyde (*7*). The roots and spinal cord were treated routinely for electron microscopy except for the addition of 1.5 percent potassium ferricyanide to the osmic acid (*7*). The roots were embedded in plastic, sectioned with glass or diamond knives, placed on single-hole grids covered by a Formvar film, and photographed in the electron microscope. After montages were made, all axons, both myelinated and unmyelinated, were counted.

The myelinated axons, which are large and surrounded by an easily stainable sheath, can be counted in either the light or electron microscope. The unmyelinated axons, by contrast, are smaller and have no special sheath and thus cannot be counted in the light microscope. They can be counted in the electron microscope, however (Fig. 1). The axonal counts in dorsal roots cranial and caudal to the hemisection are presented in Table 1. The number of unmyelinated axons was greater in the roots on the sectioned side of the cord than in the roots on the normal side [repeated measures analysis of variance, $F(1, 9) = 10.64$, $P < .01$]. The number of myelinated axons was not affected by the surgery.

Since the area of denervation is in the spinal cord, this sprouting of axons in the dorsal root is at a considerable distance

Table 1. Numbers of myelinated (M) and unmyelinated (U) axons in the dorsal roots for four rats. Each rat had a spinal hemisection at the level of thoracic vertebra 7 or 8, or both. The counted roots were classified as being from segments anterior (A) or posterior (P) to the lesion on the normal (N) or sectioned (S) sides; S.D., standard deviation.

Segment	Rat									
	1		2		3		4		Mean ± S.D.	
	S	N	S	N	S	N	S	N	S	N
3A—M	1685	1722	1570	1411	1299	1140	1343	1582	1474±184	1464±250
U	4422	4338	3891	3062	3246	3007	4250	4221	3952±520	3657±721
2A—M	1565	1434	1587	1603	1184	1343	1417	1304	1438±186	1421±133
U	4821	3544	4197	3773	3298	3055	3584	3127	3975±677	3375±342
1A—M	1395	1144	1195	1514	1417	1185	1323	1269	1333±100	1278±166
U	3495	2608	3865	3920	4055	3278	3771	3381	3797±233	3297±539
Lesion	T7		T8		T8		T7-T8			
1P—M	1340	1411	1605	1506			1257	1322	1401±182	1413± 92
U	4406	3464	6347	4144			4228	4004	4994±999	3870±359
2P—M	1464	1331	1619	1733	1297	1405	1516	1459	1474±134	1482±175
U	5003	4628	5338	5452	4320	4220	4404	3356	4766±488	4414±872
3P—M	1373	1522	1665	1683	1536	1407	1509	1315	1521±120	1482±159
U	4373	4514	5538	4247	4576	4186	4600	3376	4772±521	4081±491

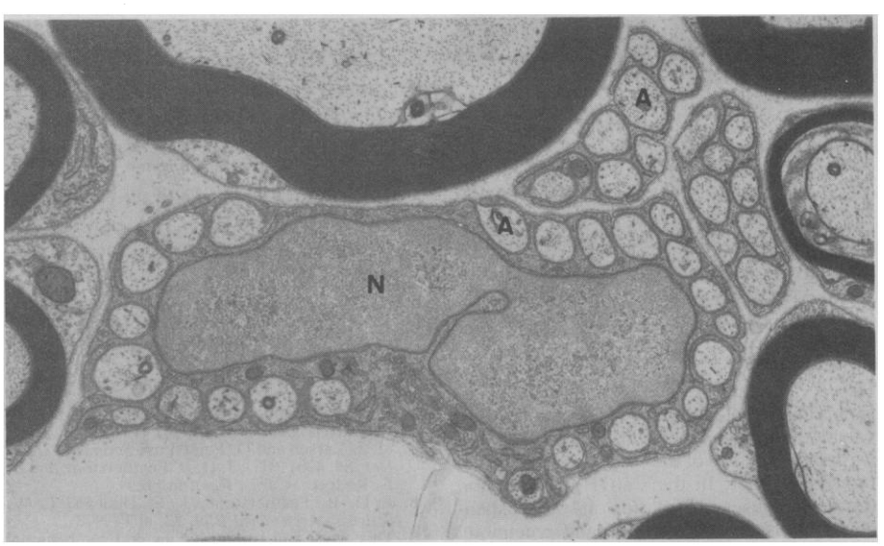

Fig. 1. Electron micrograph of three groups of unmyelinated axons. Note the nucleus of the Schwann cell (*N*) and the relatively clear unmyelinated axons (*A*) located in troughs of Schwann cell cytoplasm (×7000).

0036-8075/81/0828-1020$01.00/0

from the site of denervation. The increase of unmyelinated axons is approximately 22 percent (15 and 29 percent) one segment from the lesion and approximately 13 percent (8 to 18 percent) in roots two and three segments from the lesion. Since the sprouting can be quantified, it will be possible to determine the effects of age on the amount of sprouting and to see if different types of spinal denervation produce different amounts of sprouting. Such studies may lead to a greater understanding of the various determinants of sprouting of dorsal root axons in response to spinal injury.

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5. The neural components of the dorsal root are myelinated and unmyelinated axons. Sprouting is an increase in the number of myelinated or unmyelinated axons, or both, in a test pathway, in this case the dorsal root. The studies (*2, 3*) demonstrated sprouting in the following way. An area of spinal cord into which a test root projected was partially denervated—for example, by hemisection or by cutting other dorsal roots. Enough time was allowed for products of degeneration to disappear and sprouting to occur. Then the test root and its mate on the intact side of the cord were cut. When axonal degeneration was maximal, the animal was killed, the spinal cord was sectioned histologically, and a silver stain was applied. The usual finding was that more silver was deposited on the sectioned side of the cord, and the conclusion was that the increased staining on the denervated side represented an increased number of axons. Some alternate explanations are that silver staining is capricious, that shrinkage of neural tissue leads to an illusory enhancement of the staining, or that axons on the damaged side change—by an increase in neurofilaments, for example—so that more silver is deposited per nerve fiber. Thus, a technique that allows all axons to be counted is to be preferred over the use of silver stains or other light microscopic techniques that do not permit the unmyelinated axons to be resolved. At present, therefore, electron microscopic examination of the tissue is necessary.
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16 December 1980; revised 23 February 1981

In vitro Cultivation of the Exoerythrocytic Stage of *Plasmodium berghei* from Sporozoites

Abstract. *When inoculated with sporozoites of* Plasmodium berghei, *the human embryonic lung cell line WI38 supports the complete asexual developmental cycle of the exoerythrocytic stage. Cultured parasites were sensitive to primaquine, were shown to resemble parasites in living hosts by immunofluorescent antibody tests, and on subinoculation into mice induced a red blood cell infection, the gametocytes of which produced sporozoites in anopheline mosquitoes.*

Infection of the mammalian host with malarial parasites begins with the bite of an infected *Anopheles* mosquito and injection of sporozoites, which invade liver parenchymal cells and undergo schizogony, a cycle of asexual multiplication. These exoerythrocytic schizonts rupture to release merozoites, which may invade erythrocytes and initiate repeated cycles of asexual development with which are associated the symptoms of malarial infection. Some merozoites enter erythrocytes and differentiate into sexual forms called gametocytes. When a mosquito again bites, gametocytes are ingested, undergo sexual development, and ultimately produce sporozoites. In the rodent malarial parasite *Plasmodium berghei* only a single exoerythrocytic cycle is believed to occur and does not cause recognizable disease. However, blood infections with this parasite are usually lethal (*1*).

Although the exoerythrocytic stages of avian malarial parasites have been in continuous tissue culture since 1966, in vitro cultivation of the exoerythrocytic stages of a mammalian malarial parasite was not accomplished until recently. In 1979 Strome *et al.* (*2*) reported that sporozoites of *P. berghei* successfully entered rat embryonic liver and brain cells and developed into multinucleated schizonts. However, only 10 percent of the cultures became infected, the number of exoerythrocytic parasites was low, and complete cytoplasmic division did not occur. We now report the consistent development of large numbers of exoerythrocytic forms of *P. berghei* and the completion of schizogony in these parasites, with release of merozoites. Thus the complete asexual cycle of the exoerythrocytic stage of a mammalian malarial parasite is now available in vitro.

Human embryonic lung cells (line WI38) were obtained from the American Type Culture Collection and grown in Falcon 25-cm^2 flasks in NCTC-135 medium supplemented with 10 percent heat-inactivated fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 μg/ml) at 37°C in air containing 5 percent CO_2. When confluent, the cells were removed by trypsinization (0.25 percent trypsin in Hanks balanced salt solution) and subcultured onto 1-cm^2 round cover slips in vials with 0.5 ml of medium. They were grown for 2 to 3 days under the above conditions until nearly confluent. It was found that allowing up to 3 days of growth before the sporozoites are added greatly increases the susceptibility of cells to infection.

Anopheles stephensi mosquitoes were infected by allowing them to feed on NIH/NMRI mice infected with *P. berghei* (ANKA strain). The insects' salivary glands were aseptically dissected and collected in heat-inactivated mouse serum. Five sets of salivary glands were pooled, gently disrupted by passage through an 18-gauge needle, and added to the cultures. Cultures were periodically removed and stained with Giemsa; some were also fixed with methanol at 4°C for 5 minutes, rinsed with Hanks balanced salt solution, and subjected to an indirect immunofluorescence assay (*3*). The assay was run with mouse antiserum to *P. berghei* sporozoites, prepared by injecting mice three times with 30,000 ^{60}Co-irradiated sporozoites, and reactive with air-dried sporozoites; with mouse antiserum to erythrocytic-stage parasites, prepared by giving mice repeated red blood cell (RBC) infections cured by chloroquine, and reactive with both RBC stages and sporozoites; and with a commercially prepared rabbit antiserum to mouse immunoglobulin G conjugated with fluorescein (Miles Laboratories). Exoerythrocytic parasites located by immunofluorescence assay were also examined by phase microscopy.

Over 80 percent of the cultures were infected, with an average range of 100 to 500 exoerythrocytic parasites per culture. As many as 2000 parasites were counted in a single culture, representing an infection of 5 percent of the cells. Treatment of infected cultures with primaquine diphosphate (10 μg/ml), an antimalarial drug known to affect the exoerythrocytic stage in vivo, completely destroyed the parasites in vitro. Uninucleate exoerythrocytic trophozoites 5 μm in diameter were detected by Giemsa staining and immunofluorescence as early as 16 hours after the addition of sporozoites. By 24 hours the exoerythrocytic

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