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Tetanus Toxin: Direct Evidence for Retrograde Intraaxonal Transport

Abstract. *The neurotoxin tetanospasmin causes tetanus when it reaches the central nervous system. In this autoradiographic study, ^{125}I-labeled tetanospasmin was injected into the leg muscles of rodents, and the nerves supplying these muscles were crushed. The labeled toxin accumulated within axons on the distal side of the crush. This study provides direct evidence for retrograde axonal transport of a macromolecular toxin that acts at synapses in the central nervous system.*

In the time of Hippocrates, tetanus was known by the triad of wounding, lockjaw, and death (*1*); even now the disease continues to be a worldwide health problem of major proportions (*2*). Tetanus results when tetanospasmin, the protein neurotoxin produced in contaminated wounds by *Clostridium tetani*, reaches the central nervous system (CNS) (*2*). In the CNS, tetanospasmin interferes with inhibitory synaptic inputs on spinal motor neurons (*3*). The route by which the toxin reaches the CNS has been the subject of study for many years (*4*). Three pathways of entry have been proposed: intraaxonal transport from peripheral nerve endings to the CNS; passage through intraneural, extraaxonal tissue spaces; or vascular and lymphatic spread. In this study, autoradiography was used to localize [^{125}I]tetanospasmin in nerves after intramuscular and intraneural injection of toxin. The results provide direct evidence for retrograde intraaxonal transport of tetanospasmin.

Purified tetanus toxin was used in these studies; intramuscular injection of 0.1 μg of toxin (*5*) killed all mice at 24 hours. A portion of the toxin was iodinated by using chloramine T (*5*) followed by chromatography on a Sephadex (Pharmacia) G75 column equilibrated with 0.1*M* phosphate buffer (*p*H 7.0) with 0.1 percent bovine serum albumin. The final preparation of ^{125}I-labeled toxin had a specific activity of 3.19 μc/μg [7.027×10^6 disintegrations per minute (dpm) per microgram]. The labeled toxin retained toxicity in mice, producing tetanus followed by death 24 to 36 hours after intraperitoneal injection of 140 ng of toxin (40 μl containing 1×10^6 dpm). Before and after iodination, electrophoresis of the toxin on 7 percent polyacrylamide disc gels showed one major protein component (*5*).

This [^{125}I]tetanospasmin was used in a series of experiments designed to look for distal-proximal migration of labeled toxin within nerves. The strategy consisted of injecting labeled toxin into muscle or nerve, crushing the appropriate nerve proximally, and, after an interval, removing the nerves for autoradiographic studies (Table 1). After fixation, the nerve segments above and below the crush or injection were embedded in Epon, sectioned (1.5 μm), and processed for autoradiography (*6*). The use of a crush allowed collection of migrating material at a single site, so small amounts of radioactivity could be detected (*7*). The systems investigated are summarized in Table 1. We examined mouse and rat sciatic nerves after injection into the distal muscles of hind limb, and rat sciatic nerve segments proximal and distal to a direct intraneural microinjection (*8*).

In the experiments in which ^{125}I-labeled toxin was injected into rat or mouse

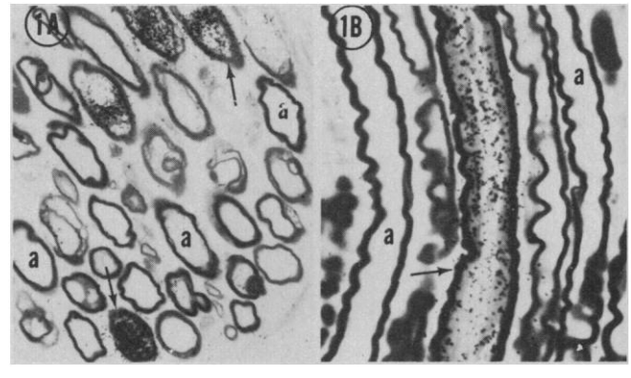

Fig. 1. Autoradiograms of sciatic nerves distal to crush. Sections cut at 1.5 μm are stained with toluidine blue. (A) Axons and myelin sheaths in cross section. The abnormal appearance of axons and myelin is typical of the alterations commonly present in proximity to a crush. Silver grains are present within four axons. Arrows point to two labeled axons. Three unlabeled axons are indicated by *a* (× 400). (B) Axons and myelin sheaths in longitudinal section. Silver grains are present within the axon in center of field. The label is heaviest over dark-stained material rather than over the clear central core. Electron microscopy showed that the basophilic material within the axon represents mitochondria, dense bodies, and accumulations of membranous and vesicular organelles. The central core contains neurofilaments. Unlabeled axons are indicated by *a* (× 900).

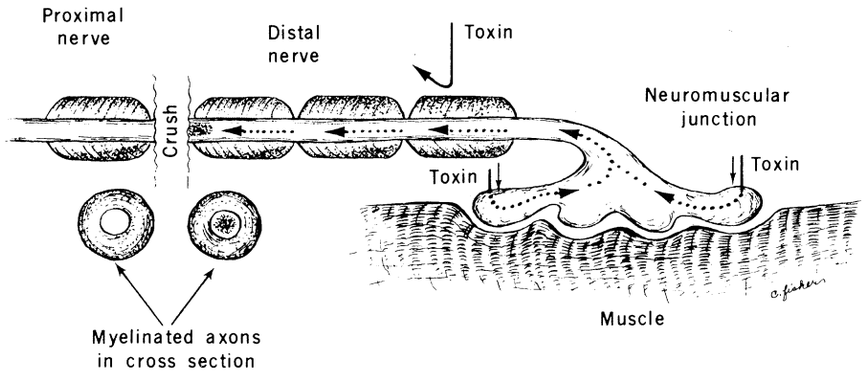

Fig. 2. Schematic drawing of experimental model with interpretation of results. [^{125}I]-Tetanospasmin, injected into muscle (solid arrows), is presumably taken up at nerve terminals, rather than along axon, and carried by retrograde axonal transport (dotted arrows) to the crush where it accumulates in distal segment. Grains are depicted as dots in distal segment of crushed axon; grain distribution is based on Fig. 1. Radioactivity was not demonstrable in nerve segments proximal to the crush.

muscle, light microscopic autoradiograms of the nerve at the level of the crush showed dense accumulations of silver grains in the axons in the segment distal to the crush (Fig. 1, A and B). At this level, some axons contained high concentrations of radioactivity; these concentrations overlie more darkly staining axoplasm (*7*). The remaining axons were unlabeled. In the nerve samples from below the crush, there was a population of axons containing definite but much less label. It is likely that the labeled axons are those innervating the inoculated muscles; the unlabeled axons presumably are either sensory or they innervate uninjected muscles. The intraaxonal localization of radioactivity deserves emphasis; there was no significant label in extraaxonal compartments (Schwann cells, endoneurium, perineurium, epineurium, or blood vessels). After intraneural injection, there was no intraaxonal label even at the site of injection; neither was there accumulation of label around the proximal or distal crush. Therefore, the toxin only appeared to be taken up when inoculated into muscle (that is, around nerve endings) and not when injected along the course of the nerve. Our interpretation of these findings is diagrammatically represented in Fig. 2.

These results provide direct evidence that [^{125}I]tetanospasmin is transported retrograde within axons. This finding has implications for the long-standing controversy: How does tetanus toxin gain access to the CNS? Recent studies have shown radioactivity appearing in nerves following systemic injection of labeled tetanospasmin (*9*). Following local injection of [^{125}I]tetanospasmin into muscles, the radioactivity increased in a centripetal direction along the nerve with time, and ligature of nerves resulted in decreased radioactivity reaching the ipsilateral side of the spinal cord. However, these studies were not designed to distinguish between intraaxonal and intraneural extraaxonal spread. Several lines of evidence favored the latter hypothesis. It was suggested that the toxin might move through intraneural, extraaxonal tissue spaces because the ascent of toxin could be blocked by making nerves sclerotic (*10*), the toxin could not be demonstrated in axon terminals by use of antitoxin labeled with fluorescein or horseradish peroxidase (*11*), and fluorescein- and tritium-labeled toxin appeared to be present in the intraneural tissue spaces (*12*). In a review (*1*) of this problem, the authors thought it unlikely that the toxin was absorbed at the neuromuscular junction and carried centripetally within the nerve fiber. On the basis of our autoradiographic studies, we think it likely that tetanospasmin can reach the CNS by retrograde intraaxonal transport, but we cannot exclude some contribution by the circulatory system.

Our observations are in accord with the recent recognition that retrograde intraaxonal transport provides a major route for moving substances from the periphery to the CNS. A wide variety of macromolecules, including proteins exogenous to the neuron as well as viruses, accumulate in the nerve cell bodies following inoculation into the periphery (*13*). The exogenous tracers Evans blue–conjugated albumin and horseradish peroxidase have been extensively used for studies of retrograde transport. Although in most of these studies the evidence for intraaxonal spread is indirect, it has recently been shown that horseradish peroxidase is taken up by pinocytosis into axon terminals (*14*) and that its centripetal passage is intraaxonal (*15*). Within the axon, the enzyme has been localized in membrane-bound vesicles, agranular reticulum, and multivesicular bodies. Although direct measure of the rate of transport is not available, the time of appearance of tracer in the nerve cell body implies a rate in excess of 75 mm per day. It is not known to what extent the uptake and intraaxonal transport of tetanospasmin has relative specificity for the toxin compared to these other macromolecules.

In contrast to the other macromolecular tracers, which are without significant electrophysiologic effect on neurons, tetanospasmin is thought to act presynaptically on inhibitory terminals (*16*) that use glycine as a neurotransmitter (*17*). If tetanospasmin does act at these presynaptic sites

Table 1. Summary of [^{125}I]tetanospasmin injection experiments. The ^{125}I-labeled toxin was injected into muscle or nerve and, hours later, the nerve was crushed. Species, site of injection, dose of toxin, site of nerve crush, and survival time interval after injection are indicated for each experiment.

Species	Injection site	Dose		Site of nerve crush	Survival after injection (hours)
		μg	dpm		
Sprague-Dawley female rat, 200 g	Intrinsic hind foot muscles	0.14	1×10^6	Tibial nerve	6
CBA female mouse	Biceps femoris muscles, tibial nerve	0.14	1×10^6	Sciatic nerve, midthigh	20
Sprague-Dawley female rat, 200 g	Sciatic nerve	0.035	2.5×10^5	2 cm proximal to injection site in mid-sciatic nerve	6
Sprague-Dawley female rat, 200 g	Sciatic nerve	0.035	2.5×10^5	2 cm proximal and 2 cm distal to injection site in mid-sciatic nerve	6

(*18*), then it must be postulated that the toxin can pass retrograde across the synapse from motor neurons to inhibitory nerve terminals. This study demonstrates that [^{125}I]tetanospasmin is carried retrograde within axons, providing direct evidence for axonal transport of a macromolecule that interferes with synaptic transmission in the CNS.

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6. The nerves were fixed with 3 percent glutaraldehyde in 0.1*M* cacodylate buffer with 3 percent sucrose, washed in the same buffer with 10 percent sucrose, postfixed in OsO_4, dehydrated in alcohol, and embedded in Epon 812. For light autoradiography, 1.5-μm sections were dipped in Kodak NTB-2, developed in Kodak D-19, stained with toluidine blue, and examined in a Zeiss photomicroscope II.
7. Although nerve crush ultimately results in Wallerian degeneration of the distal stump, previous studies have shown that retrograde transport persists for hours following axotom→ [L. Lubinska and S. Niemierko, *Brain Res.* **27**, 329 (1970)]. Dark-staining axoplasm is commonly present in proximity to the crush; ultrastructural study shows that the axoplasm consists of mitochondria and membranous and vesicular organelles. The central light-stained portion represents a core of neurofilame→ [J. Zelena, L. Lubinska, E. Gutmann, *Z. Zellforsch. Mikrosk. Anat.* **91**, 200 (1968)].
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Cell Surface Differences in Ducts from Cancerous and Noncancerous Human Breasts

Abstract. *The scanning electron microscope reveals structural differences between the apical microvilli of duct cells from cancerous and noncancerous human breasts. The alterations in the microvilli from carcinomatous breasts appear to be highly specific, to extend throughout the affected breast, and may be pathognomonic for this condition.*

The structure and composition of the plasma membrane seem to vary among different cell types (*1*) and with the functional activities of any given cell (*2*). In addition, it is commonly suspected that cancer cells may have unique or highly specialized plasma membranes which give rise to or facilitate their loss of contact inhibition, accelerated growth, tumorigenicity, and metastatic spread throughout the body (*3*). Recently, Porter *et al.* and others (*4, 5*), using the scanning electron microscope, found several differences between the surface morphology of normal and transformed cells in vitro, while others described differences in the binding of plant lectins to the outer surface of the plasma membranes from normal and cancer cells (*6*).

Since last year we have been studying the surface topography of human breast tissues with the scanning electron microscope. We have observed material obtained at biopsy or mastectomy from 32 women with the following breast abnormalities: 11 dysplasias, 4 fibroadenomas, 2 lobular carcinomas in situ, and 15 infiltrating duct carcinomas. The results to date strongly suggest that the duct epithelium within dysplastic breasts or those containing benign neoplasms is distinctly different from the duct epithelium within carcinomatous breasts. Thus, the purpose of this report is to draw attention to previously undetected differences in the surface morphologies of ducts from cancerous and noncancerous breasts which may be of potential diagnostic or prognostic importance. The surface morphology of mammary carcinoma cells will be described in detail elsewhere (*7*).

The surgical specimens were rinsed briefly in 0.1*M* cacodylate buffer, *p*H 7.4, fixed for 4 days to 2 weeks in a modified Karnovsky's fixative (*8*), dehydrated in alcohol, and frozen in liquid Freon. The frozen tissue was cracked with a precooled scalpel (*9*) and critical-point-dried in liquid CO_2 (*10*). Then the dried tissue was viewed under a dissecting microscope and the upper halves of ducts close to the surface of the specimen were teased away. As a result, the observer obtains an unimpeded view of essentially all cell surfaces bordering one half of the duct lumen. The specimens were mounted on stubs with silver conducting paint, coated with gold about 200 Å thick on a rotating stage in a vacuum evaporator, and viewed at 20 kv in a Kent-Cambridge S_4 scanning electron microscope.

Usually, the apical surface of the glandular epithelial cells within both normal and hyperplastic ducts from noncancerous breasts is covered with minute projections—the microvilli. The diameter of the microvilli is fairly constant (about 0.1 μm). The average microvillus is 1.5 to 2 μm long; however, there is moderate variation in the length of the microvilli on individual cells as well as among adjacent cells. Sometimes the surface contains only small spherical knobs, but it is more common to find knobs intermingled with microvilli. Since the diameter of these knobs is about 0.1 μm and we found no new surface structures on these cells with the transmission electron microscope, they may be rudimentary microvilli or surface blebs which are disappearing from or forming on the surface of the cells during specific portions of the cell cycle or in response to other alterations in their environment [see (*4*) for discussion]. Cells undergoing apocrine