Acta Medica et Biologica
Vol. 42, No. 1, 19-24, 1994

Cholera Toxin B Subunit Shows More Intense Affinity to Schwann Cells than Neurons: *In Situ* and *In Vivo* Staining

Toshihiko IWANAGA and **Hiroyuki OHTSUKA**

Department of Anatomy, Niigata University School of Medicine, Asahimachi 1, Niigata 951, Japan

Received January 14, 1994

Summary. **Conjugates of horseradish peroxidase (HRP) with cholera toxin (CT) are known to be more effective as a probe for studies of neuronal connectivity than HRP only, because of the affinity of CT for GM1 ganglioside which may be richly expressed on neuronal plasma membranes. The present *in situ* and *in vivo* staining using CT-fluorescein isothiocyanate (CT-FITC) and CT-HRP demonstrated their intense binding to Schwann cells rather than neurons. Exogenous GM1 successfully competed for the CT binding sites on Schwann cells. This finding suggests the rich existence of GM1 on the Schwann cell surface.**

INTRODUCTION

The B subunit of cholera toxin (CT) selectively binds to cell surfaces through its receptor, GM1 ganglioside (GM1), and the A subunit exhibits biological activities through activation of adenyl cyclase in a variety of cells.[1,2] Numerous biochemical analyses have revealed that the central nervous tissue is the richest source of gangliosides, including GM1.[3-6] Therefore, a binding assay study of CT using homogenates obtained from various tissues has shown that brain homogenate is the most intense in affinity.[7] GM1, like other gangliosides, is believed to be primarily a neuronal plasma membrane constituent.[3,4,8] This is in agreement to the finding that horseradish peroxidase (HRP) is more effective when conjugated with CT (CT-HRP) as a probe for studies of neuronal connectivity and axoplasmic transport.[9,10] However, no studies are available offering morphological evidence of the direct binding of CT on the neuronal plasma membrane, except for some investigations using cultured neurons and related cells.[1,11-16]

In the course of our cytochemical study concerning the affinity of cholera toxin B subunit, we found a selective binding of CT-FITC or CT-HRP to Schwann cells and satellite cells in the peripheral nervous system. The present study deals with the morphological demonstration of binding sites for CT in the peripheral nervous system of rabbits at the light and electron microscopic levels.

MATERIALS AND METHODS

Seven male albino rabbits, weighing 1.0-1.5 kg, were used in this study. Animals were killed by an intravenous injection of an excess dose of pentobarbiturate (40 mg/kg). The sciatic nerve, the trigeminal nerve and ganglion, the dorsal root ganglion, the lip and tongue were dissected out and rapidly frozen in liquid nitrogen. Cryostat sections, 20 μm in thickness, were prepared with Coldtome CM-41 (Sakura, Japan) and incubated in a conjugate of cholera toxin B subunit with fluorescein isothiocyanate (FITC) (CT-FITC, List Biological Laboratories, California, U.S.A.) diluted 1 : 20, for 15 min at room temperature. After staining, the sections were thoroughly rinsed in a phosphate buffered saline (PBS) and covered with glass slips using glycerine. *In vivo* staining using CT-FITC was carried out in rabbits anesthetized with halothane. CT-FITC diluted at 1 : 10 (50 μl) was directly injected into the sciatic nerve using microsyringes. The nerve, 2 cm proximal to the injected portion, was dissected out 1 h after injection and processed for cryostat sections. The preparations stained *in situ* and *in vivo* were observed and photographed under a Leitz Ortholux (Leitz, Germany) equipped with a fluorescence vertical illuminator (Ploemopak 2.2).

For electron microscopy, cryostat sections of fresh

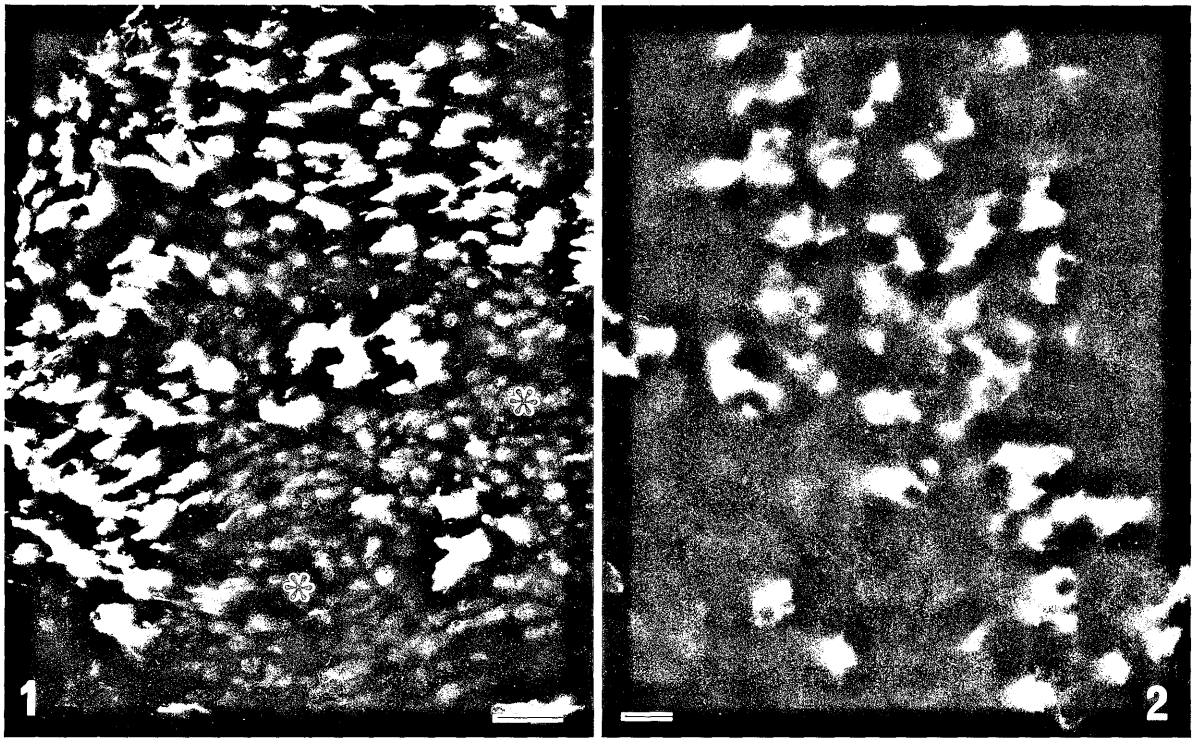


Fig. 1. The sciatic nerve of a rabbit. Intense fluorescence indicating the binding sites of the chorela toxin B subunit is recognizable in approximately half of the nerve fibers. Asterisks show unlabeled nerve fibers. Bar= 50 μm

Fig. 2. Closer view of the cross-sectioned sciatic nerve. Ring structures are selectively positive in reaction, leaving a nonreactive space in the center. Bar=20 μm

sciatic nerves were fixed in periodate-lysine-paraformaldehyde (PLP) fixative for 30 min and incubated overnight with the cholera toxin B subunit-HRP conjugate (CT-HRP, List Biological Laboratories) diluted at 1 : 100. The binding ability of CT-HRP and also CT-FITC on the tissue sections was influenced by fixation: It remarkably decreased in intensity on the cryostat sections obtained from materials fixed with conventional fluids including paraformaldehyde. However, the rapid fixation of fresh cryostat sections by PLP did not significantly influence the binding ability. After incubation with CT-HRP, the enzyme reaction was developed with a mixture of diaminobenzidine hydrochloride (0.02%) and H_2O_2 (0.01%). The sections were post-fixed in 1% OsO_4 for 30 min, dehydrated through a graded series of ethanol, and embedded in Araldite. Ultrathin sections were stained shortly with uranyl acetate and observed in a Hitachi H-7000 electron microscope.

The specificity in the binding of CT-FITC was confirmed by preincubation of CT-FITC with GM1 ganglioside (1, 10, 100, 1,000 μg/ml diluted solution, List Biological Laboratories) for 15 min. Moreover, the sections were pretreated with the cholera toxin B subunit (0.5, 5, 50, 500 μg/ml, List Biological Laboratories) for 2 h at room temperature before incubation with CT-FITC.

RESULTS

Intense fluorescence indicating the binding sites of CT-FITC was recognized in approximately half of the nerve fibers in the sciatic nerve (Figs. 1 and 2), although populations of the positive nerve fibers in individual nerve bundles encircled by the perineurium varied. There were some nerve bundles totally lacking in fluorescent nerve elements. In the trigeminal nerve, in contrast, virtually all nerve fibers were associated with fluorescent cellular elements (Fig. 3). Closer views of cross-sectioned nerves revealed that in both nerves, ring structures were selectively positive in reaction, leaving a nonreactive space in the center (Fig. 2). It was obvious in longitudinally cut

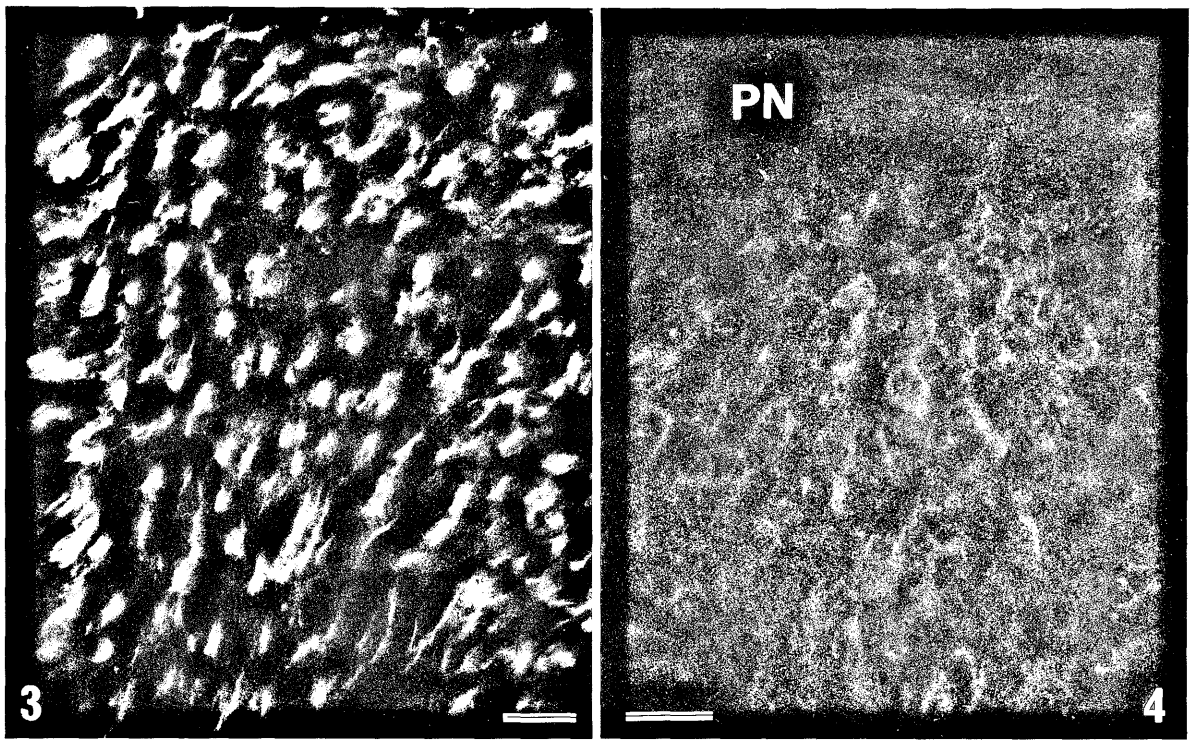


Fig. 3. In the trigeminal nerve, virtually all nerve fibers are associated with fluorescent cellular elements. Bar= 50 μm

Fig. 4. For checking the specificity of positive staining, cholera toxin-FITC solution was pretreated with an excess dose of GM1 molecule (10 μg/ml). The positive staining of the sciatic nerve seen in Fig. 1 is completely inhibited. **PN:** perineurium Bar=50 μm

sections that slender cells with long processes projecting straight in opposite directions were labeled by CT-FITC. These findings mean that CT-FITC binds to the Schwann sheath and the cell bodies rather than to axons. When the tongue and lip were stained, numerous nerve fibers were selectively and intensely labeled (Fig. 5). An extremely dense distribution of the positive nerve elements was seen around the taste buds in the circumvallate papillae. Here again, the fluorescence was localized at the Schwann sheath and the cell bodies. The selective binding of CT-FITC to Schwann cells was supported by a finding that several satellite cells in the trigeminal ganglion were labeled (Fig. 6).

The trigeminal ganglion and dorsal root ganglion contained several fluorescent cell bodies of neurons in addition to positive Schwann cells and satellite cells. The fluorescent neuronal somata in the trigeminal ganglion, less than 10% of all somata, were small in size and dispersed throughout the ganglion (Fig. 7). In the dorsal root ganglion, the positive neuronal somata were more numerous, counting approximately 30% of all cell bodies. The positive neuronal somata in both ganglia were always enveloped by intensely positive satellite cells; satellite cells surrounding one positive neuronal soma were all positive for CT-FITC (Fig. 8).

The absorption test using CT-FITC pretreated with excess doses of GM1 molecule (more than 10 μg/ml) showed that the reaction of the Schwann cells and neuronal somata was completely inhibited (Fig. 4). Furthermore, when tissue sections were preincubated with the B subunit of CT at concentrations of 50 and 500 μg/ml, the CT-FITC probe could be prevented from binding to the cell surface.

Electron microscopic observation of materials treated with CT-HRP demonstrated subcellular localization of binding sites for CT-HRP. Electron-dense reaction products were found in the outer leaf of myelinated nerve fibers which were comparatively small in diameter (Fig. 9). Both the cytoplasm and cell membrane of Schwann cells appeared to stain positively. No reaction products were recognized in the myelin matrix or axon. Myelinated nerve fibers of

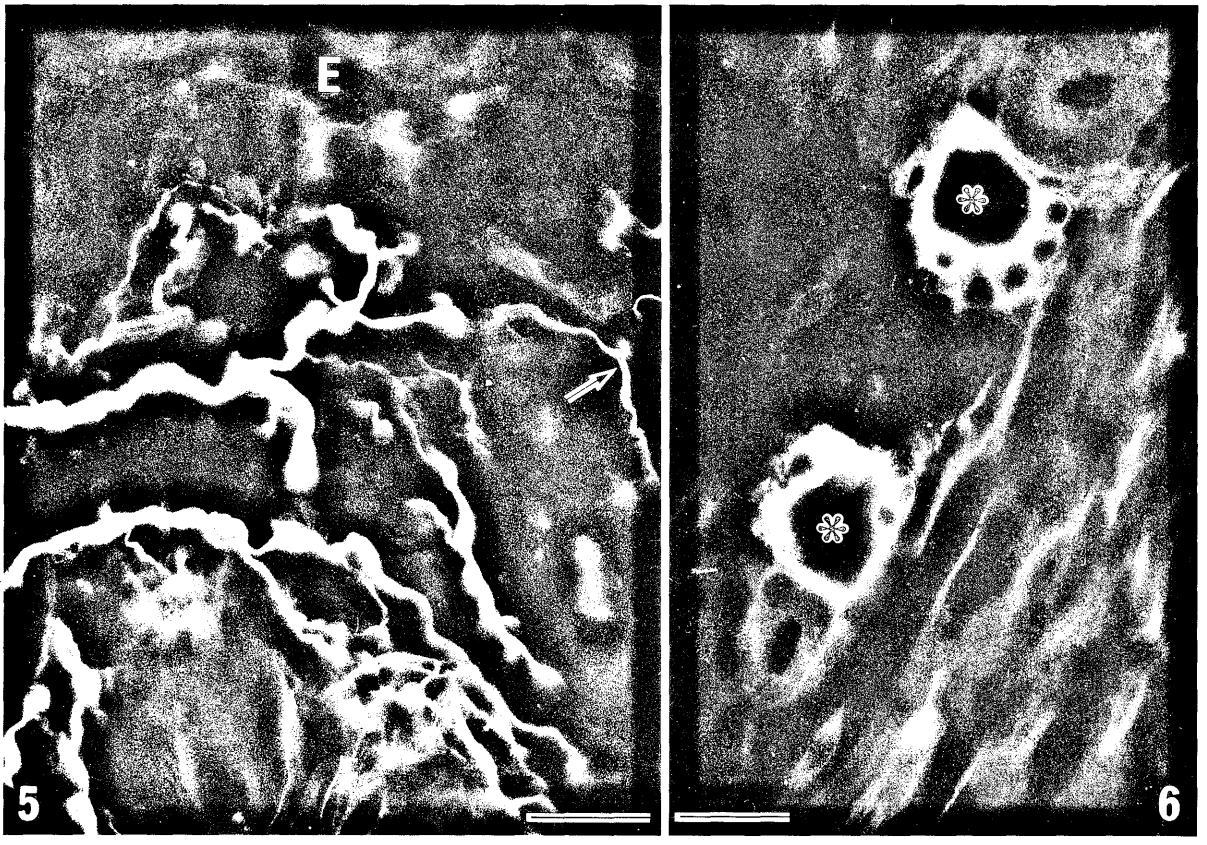


Fig. 5. Numerous nerve bundles in the subepithelial region of the lip are stained positively. Thin positive elements indicated by an arrow sheath a nerve fiber which lacks in the fluoresence. **E:** epithelium Bar=100 μm

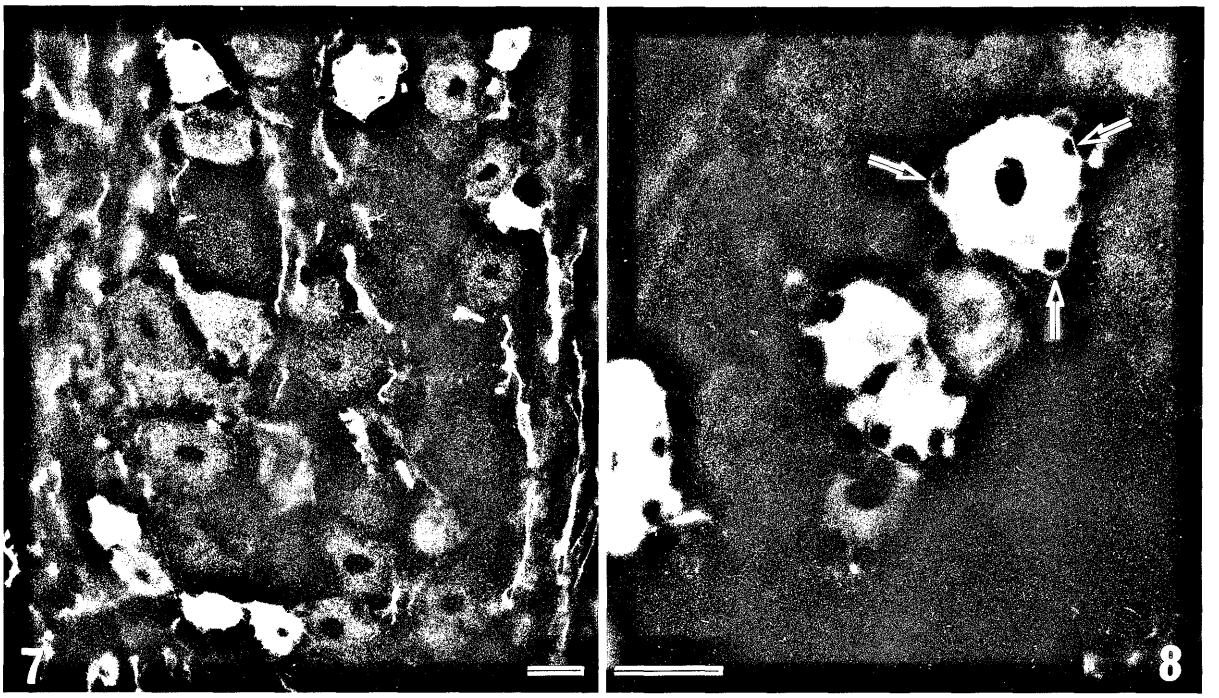
Fig. 6. The trigeminal ganglion. Two neuronal somata (asterisks), which are negative in reaction, are enveloped by fluorescent satellite cells. Bar=50 μm

large diameter, including their Schwann sheaths, were free from the CT-binding.

DISCUSSION

The specificity of CT for the monosialo ganglioside GM1 and the usefulness of CT-HRP conjugates for the cytochemical detection of GM1 were established by Manuelidis and Manuelidis.[14] The present absorption test by pretreatment of CT-FITC solution with GM1 or of tissue sections with CT confirmed the specificity of the binding of CT-FITC to GM1.

Though the affinity of CT to neurons is apparent, the neuronal labeling by CT-HRP which has been used by many neuroanatomists is a phenomenon observed many hours after the local injection, and cannot be taken to prove its direct binding to neuronal plasma membrane. It was in cultured neurons from rat and chicken embryos and neonates that the direct binding of CT to the surface of neurons was morphologically demonstrated.[1,11,12,14-16] Virtually no studies have morphologically analyzed the binding sites of CT *in vivo*. The present *in situ* and *in vivo* staining demonstrated the selective binding of CT-FITC or CT-HRP to the Schwann sheath of peripheral nerve fibers, but not on the axoplasmic membrane, or at least at rapid phases. Several immunohistochemical experiments aimed at detecting CT receptors on Schwann cells were performed using isolated, cultured cells from the sciatic nerves of newborn rats, but have yielded variable results. Brockes et al.[17] and Raff et al.[16] reported that 30-50% and 50-80% of Schwann cells were labeled with CT, respectively, although all neurons of central and peripheral nervous systems examined were labeled intensely by CT.[16] Okada et al.[15] failed to find specific binding on cultured Schwann cells by the use of CT and a monoclonal antibody against it. When mouse sciatic nerves teased into individual fibers were incubated



Figs. 7 and **8.** The trigeminal ganglion (Fig. 7) and dorsal root ganglion (Fig. 8). Small populations of neuronal somata are selectively labeled in both ganglia. The positive somata are always enveloped by intensely positive satellite cells (Fig. 8, arrows). Bar=50 μm

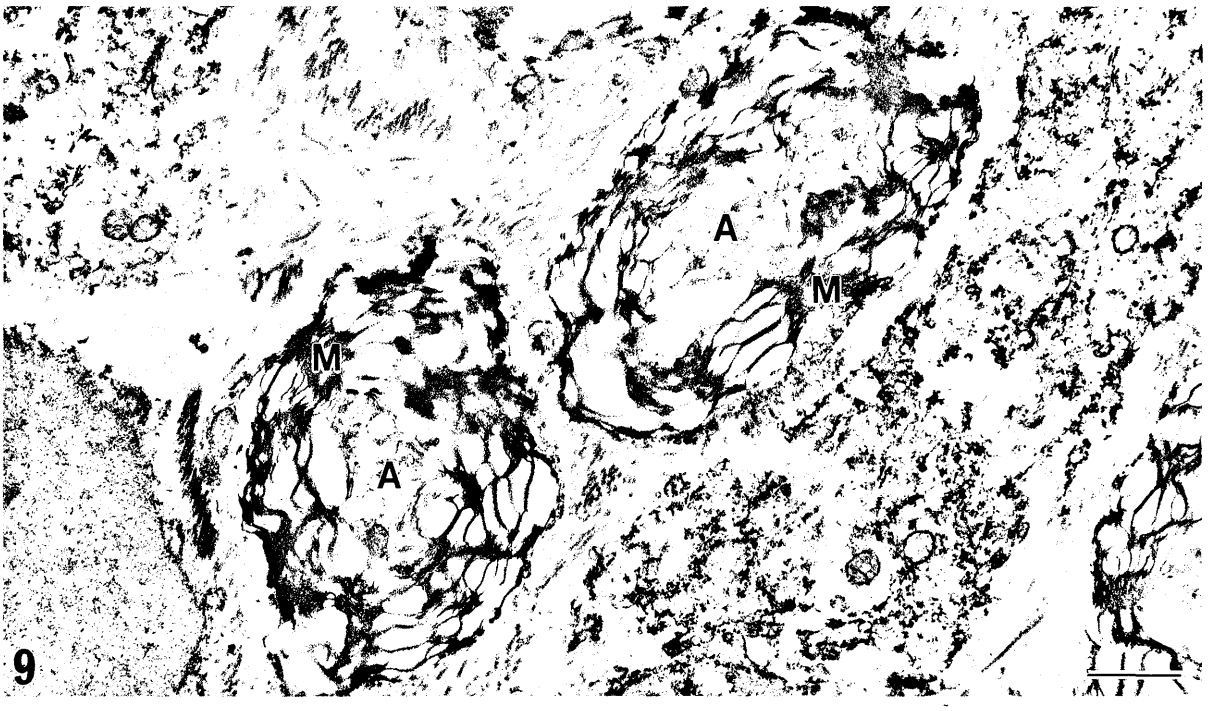


Fig. 9. Electron micrograph showing the localization of binding sites for cholera toxin-HRP. Reaction products are found in the outer leaf of myelinated nerve fibers, comparatively small in diameter. **A:** axon, **M:** Myelin sheath. Bar=1 μm

with CT and then exposed to anti-CT serum, intense labeling was found at the nodes of Ranvier, but not the internodal Schwann cell surfaces.[18] The selective labeling of Ranvier nodes in the teased sciatic nerves was quite different from the present findings obtained from both *in vivo* and *in situ* stainings. This discrepancy could be explained by differences in species used or in detection systems. We believe that our *in vivo* staining is more reliable than staining using teased nerve fibers which might be damaged.

The present study showed that considerable numbers of nerve fibers in the sciatic nerve were not associated with CT-labeled Schwann cells. In the trigeminal nerves, on the other hand, almost all nerve fibers appeared to possess labeled Schwann cells. Our staining by use of CT-FITC also demonstated numerous nerve fibers innervating the circumvallate papillae, which are all sensory in nature. These findings suggest that CT binds to Schwann cells in a special type/s of nerves, possibly sensory neurons.

Intensely stained by CT-FITC in ganglia were satellite cells, which share every glial feature with Schwann cells. Here again, a limited number of satellite cells were selectively labeled. Noteworthily, satellite cells enveloping a neuronal soma showed the same staining attitude, suggesting that the stainability of satellite cells depends on the types of neurons. Another binding of CT-FITC to certain ganglion cells demonstrated in the present study seems to contradict our idea that CT selectively binds to the Schwann/satellite cell surface. It is worthy of note, however, that the positive staining of a ganglion cell was always associated with that of its satellite cells. Therefore, it is still tenable that Schwann cells/satellite cells determine the affinity of cholera toxin to nervous tissue.

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