

Fate of Tetanus Toxin Bound to the Surface of Primary Neurons in Culture: Evidence for Rapid Internalization

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ABSTRACT We examined the nature of the tetanus toxin receptor in primary cultures of mouse spinal cord by ligand blotting techniques. Membrane components were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets, which were overlaid with ¹²⁵I-labeled tetanus toxin. The toxin bound only to material at or near the dye front, which was lost when the cells were delipidated before electrophoresis. Gangliosides purified from the lipid extract were separated by thin-layer chromatography and the chromatogram was overlaid with ¹²⁵I-toxin. The toxin bound to gangliosides corresponding to G_{D1b} and G_{T1b}. Similar results were obtained with brain membranes; thus, gangliosides rather than glycoproteins appear to be the toxin receptors both in vivo and in neuronal cell cultures.

To follow the fate of tetanus toxin bound to cultured neurons, we developed an assay to measure cell-surface and internalized toxin. Cells were incubated with tetanus toxin at 0°C, washed, and sequentially exposed to antitoxin and ¹²⁵I-labeled protein A. Using this assay, we found that much of the toxin initially bound to cell surface disappeared rapidly when the temperature was raised to 37°C but not when the cells were kept at 0°C. Some of the toxin was internalized and could only be detected by our treating the cells with Triton X-100 before adding anti-toxin. Experiments with ¹²⁵I-tetanus toxin showed that a substantial amount of the toxin bound at 0°C dissociated into the medium upon warming of the cells. Using immunofluorescence, we confirmed that some of the bound toxin was internalized within 15 min and accumulated in discrete structures. These structures did not appear to be lysosomes, as the cell-associated toxin had a long half-life and 90% of the radioactivity released into the medium was precipitated by trichloroacetic acid. The rapid internalization of tetanus toxin into a subcellular compartment where it escapes degradation may be important for its mechanism of action.

Tetanus toxin is a neurotoxic protein of 150,000 mol wt, made up of two polypeptides linked by a disulfide bond (1). It is produced by the anaerobic bacterium *Clostridium tetani*, enters the body through wounds infected by the bacterium, and causes both local and general spastic paralysis. Interaction of the toxin with a receptor in peripheral nerve endings, internalization into nerve axons, and subsequent retrograde transport to the spinal cord are processes considered fundamental to its mode of action (2–4). Tetanus toxin then apparently crosses the synaptic cleft and accumulates in presynaptic nerve ending (5) where it is thought to block release of

neurotransmitters, primarily those involved in regulating the activity of inhibitory pathways (2–4).

Although there is considerable evidence implicating gangliosides as receptors for tetanus toxin (6–10), there is little information about the process by which the toxin is internalized into the nerve axon. When tetanus toxin labeled with colloidal gold is injected into the anterior eye chamber of rats, toxin-gold particles are found within axons as early as 1–2 h later (11). It is interesting that the particles are contained within smooth membranous elements within the axon, and only a few particles are found in lysosomes. Toxin-gold

particles also have been used to study internalization by cultured cells (12). In the study by Montesano et al., particles bound to the cells at 0°C were rapidly internalized when the temperature was raised to 37°C. The toxin again was found enclosed in smooth vesicles, and the authors found no evidence for involvement of coated pits. Internalization by coated pits, however, is a rapid process (13) and might not have been detected, as 10 min was the earliest time examined after the cells were warmed (12). In addition, the neurotoxic activity of the tetanus toxin-gold particles was not established, very high concentrations (1 mg/ml) were used, and cells of nonneuronal origin (liver cells) were employed in this study (12).

Primary cultures of mouse spinal cord have been shown previously to bind tetanus toxin (14–16), an event followed by changes in membrane electrical activity (16). Therefore, we used these cells and techniques previously developed for cholera toxin (17–19) to investigate the nature of the tetanus toxin receptor and the characteristics of internalization of the surface bound tetanus toxin.

MATERIALS AND METHODS

Cell Culture: Primary mouse spinal cord cell cultures were prepared from 12- to 14-d-old mouse embryos (C57 BL) as described previously (16, 20). The cells were maintained in medium containing 10% horse serum for 2–3 wk and then were adapted over 3–4 d to serum-free medium in order to remove antibodies to tetanus toxin present in horse serum (16). The somatic hybrid cell line NCB20 subclone C (N18TG2 × Chinese hamster fetal brain cells) was kindly provided by Dr. M. Nirenburg (National Institutes of Health, Bethesda, MD) and grown in Dulbecco's modified Eagle's medium (DME)¹ containing 0.45% glucose, 10% fetal calf serum, 100 μM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine.

Tetanus Toxin: Tetanus toxin was purified to homogeneity as previously outlined (7) and had ~2 × 10⁷ mouse lethal doses/mg protein. The toxin was iodinated with Bolton and Hunter reagent to a specific activity of 1–2 mCi/mg and retained >75% of the original neurotoxicity (21). The characteristics of ¹²⁵I-tetanus toxin (21) and its binding to primary mouse spinal cord cells (15) have been described previously.

Identification of Tetanus Toxin Receptors in Primary Mouse Neurons: We used established methods (17, 18)² to characterize the tetanus toxin receptor in primary cultures of mouse spinal cord. In brief, cellular components were separated on 10% SDS-polyacrylamide slab gels and transferred electrophoretically to nitrocellulose sheets, but methanol was omitted from the transfer buffer to prevent loss of lipid components. The transfers were overlaid with 1.3 nM ¹²⁵I-tetanus toxin in 25 mM Tris-acetate buffer (pH 6.0) containing 1% bovine serum albumin for 3 h at 4°C. After washing with the same buffer was done, bound toxin was detected by autoradiography. Cells were delipidated by extraction twice with chloroform/methanol (1:2, vol/vol) for 15 min at 25°C. Gangliosides were purified from the lipid extracts as described previously (22) and separated by thin-layer chromatography on aluminum-backed silica gel sheets by use of chloroform/methanol/0.2% CaCl₂ (5:4:1, vol/vol). The chromatograms were dried, treated with polyisobutyl-methacrylate (23), dried, and overlaid with ¹²⁵I-tetanus toxin as outlined above. Gangliosides that bound toxin were detected by autoradiography.

Internalization of Tetanus Toxin: Tetanus toxin bound to the surface of spinal cord cells was assayed by use of a modification of a procedure developed for cholera toxin (19). Cells grown in 35-mm plastic dishes were incubated with 33–66 nM tetanus toxin in DME containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 1% fetal calf serum for 1 h at 0°C. We removed free toxin by rapidly washing the cells three times with ice-cold DME/HEPES. The cells then were incubated at 0 or 37°C in 2 ml of DME/HEPES/1% fetal calf serum for various times, washed twice with phosphate-buffered saline, pH 7.4 (PBS) containing 50 mM glucose, and fixed in 3.7% formaldehyde in PBS containing 5% sucrose for 15 min at 25°C

(as were all subsequent steps). The fixed cells then were washed twice with PBS, incubated for 10 min in PBS containing 0.1% bovine serum albumin, and incubated in 1 ml of the same solution containing a 1:2,000 dilution of human anti-tetanus toxin (Tetanus Immune Globulin, 460 U/ml; Travenol Laboratories, Inc., Glendale, CA). After 45 min, the cells were washed three times with PBS and incubated with 100,000 cpm/ml of ¹²⁵I-labeled protein A (80–100 μCi/μg; New England Nuclear, Boston, MA) in PBS containing 0.1% bovine serum albumin. The cells then were washed three times with PBS, dissolved in 1 M NaOH, and counted for radioactivity in a Beckman Gamma 4000 (Beckman Instruments Inc., Palo Alto, CA). We determined total cell-associated toxin by incubating the fixed cells with 0.1% Triton X-100 in PBS before adding the antitoxin.

Immunofluorescent Localization of Tetanus Toxin: Cells were incubated with tetanus toxin and fixed as described above. Surface bound toxin was detected by incubation of the fixed cells for 45 min at room temperature with 1 ml of a mouse monoclonal antibody to tetanus toxin (18.2.12.6, described in reference 24) diluted 1:100 in PBS containing 0.1% bovine serum albumin. After the cells were washed three times with PBS over a period of 20 min, the cells were incubated for 30 min with rhodamine-labeled affinity purified goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., Avondale, PA) diluted to 25 μg/ml in the same solution. We then washed the cells three times in PBS and viewed them after overlaying with 50% glycerol in PBS and a 25-mm-diam glass coverslip (thickness No. 1). Internalized toxin was detected by incubation of the fixed cells with 0.1% Triton X-100 in PBS for 10 min before addition of the antibody. The cells were examined with a Zeiss standard 16 microscope equipped for epifluorescence and with a 63X Phase 3 oil-immersion objective and filters for rhodamine fluorescence. Photomicrographs were taken with Kodak Tri-X film uprated during development to 800 ASA.

RESULTS

Receptors for Tetanus Toxin in Primary Mouse Spinal Cord Cells

Cellular components were separated by SDS PAGE and transferred to nitrocellulose sheets, which were overlaid with ¹²⁵I-tetanus toxin. Any bound toxin was detected by autoradiography (Fig. 1). Toxin binding was restricted to material that migrated at the front of the electrophoretogram with the tracking dye (Fig. 1, lane *c*) and was well separated from the bulk of the proteins (Fig. 1, lane *a*). This material appeared to be lipid, as extraction of the cells with organic solvents before electrophoresis eliminated the toxin binding (Fig. 1, lane *d*) without any obvious loss of cellular proteins (Fig. 1, lane *b*). Binding of ¹²⁵I-toxin to this material appeared to be specific as it was quantitatively inhibited by an excess (210 nM) of unlabeled toxin (data not shown).

Because gangliosides have been implicated as receptors for tetanus toxin (6–10), we purified the gangliosides from the lipid extract and separated them by thin-layer chromatography. The ganglioside composition of primary mouse spinal cord cells was similar to that of rat brain membranes;² the major gangliosides were G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}. To detect toxin-binding gangliosides, the chromatogram was overlaid with ¹²⁵I-tetanus toxin and bound toxin was detected by autoradiography (Fig. 1, lane *e*). Toxin binding was predominantly to gangliosides with the mobility of G_{D1b} and G_{T1b} although there was some binding in the region of G_{D1a} and G_{M1}. Binding was blocked by bovine brain gangliosides or excess unlabeled toxin (not shown). These results are similar to those recently obtained with rat brain membranes² and are consistent with gangliosides' being the receptors for tetanus toxin in neurons.

Kinetics of Internalization of Tetanus Toxin

To follow the fate of tetanus toxin bound to the neuronal cells, we developed an assay that measures both cell surface and total cell-associated toxin. We quantified cell surface

¹ Abbreviation used in this paper: DME, Dulbecco's modified Eagle's medium.

² Ligand blotting techniques indicate that tetanus toxin bound only to gangliosides isolated from rat brain synaptosomal membranes and not to proteins or glycoproteins (Critchley, D. R., W. H. Habig, and P. H. Fishman, manuscript in preparation).

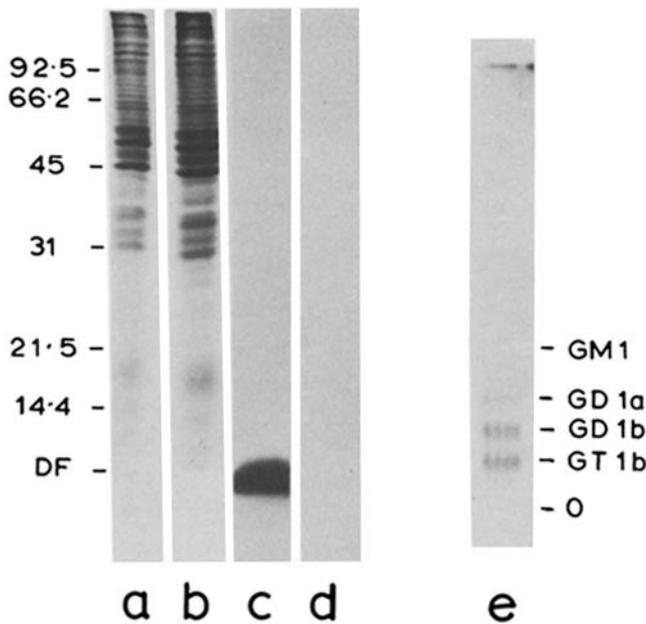


FIGURE 1 Identification of ^{125}I -tetanus toxin binding components in primary mouse spinal cord cells by ligand blotting. Cell components were separated by SDS-polyacrylamide gel electrophoresis before (a and c) and after (b and d) delipidation of the cells. Protein and lipids were transferred electrophoretically from the slab gels to nitrocellulose sheets which were then overlaid with ^{125}I -tetanus toxin as described in Materials and Methods (c and d). Gangliosides were isolated from the lipid extracts and separated by thin-layer chromatography; the chromatograms then were overlaid with ^{125}I -tetanus toxin as described in Materials and Methods (e). The slab gels were stained with Coomassie Blue (a and b), and toxin bound to the transfers (c and d) and chromatogram (e) was detected by autoradiography. In a–d, cell components equivalent to 45 μg protein were applied to each track of the slab gel; in e, gangliosides isolated from the equivalent of 15 μg cell protein were applied to the chromatogram. The mobilities and molecular weights ($\times 10^3$) of standard proteins are indicated on the left. DF, dye front. The mobilities of known gangliosides are indicated on the right; O, origin.

tetanus toxin by adapting a procedure used previously for cholera toxin (19). The cells were exposed to antibodies to tetanus toxin and the amount of antitoxin bound was measured by use of ^{125}I -protein A. Preliminary experiments to establish the efficacy of the method were carried out with a neural hybrid cell line (NCB20 subclone C) previously found to bind tetanus toxin (25). The results are shown in Table I. The ratio of ^{125}I -protein A bound in the presence and absence of toxin was maximal at antibody dilutions of $\sim 1:2,000$. At lower antibody dilutions, this ratio was dramatically reduced. In the absence of antibody, the amount of ^{125}I -protein A bound to control and toxin-treated cells was very low.

Similar results were obtained with primary spinal cord cells (Table I). More than 88% of the ^{125}I -protein A bound to toxin-treated neurons appeared to be specific, as this binding was not detected with untreated neurons. The levels of cell surface tetanus toxin detected in association with the neurons was about sixfold greater than that bound to the NCB20 cells. This ratio is in good agreement with that found by assay of ^{125}I -tetanus toxin binding to cell membranes.³ To measure

³ The neuronal and NCB20 membranes bound 87 and 14 pmol toxin/mg protein, respectively. Membranes were incubated with 0.46 nM ^{125}I -tetanus toxin in 25 mM Tris-acetate buffer (pH 6.0) containing

TABLE I
Detection of Cell-Surface and Total Cell-associated Tetanus Toxin using Antitoxin and ^{125}I -Protein A

Cells	Anti-toxin	Triton X-100	^{125}I -Protein A bound			Ratio +/– toxin
			+Toxin	–Toxin	Specific	
<i>cpm</i>						
NCB20	None	–	289	306	–	–
	1:100	–	6,170	2,810	3,360	2.2
	1:2,000	–	4,980	630	4,350	7.9
Neurons	1:1,000	–	31,260	3,640	27,620	8.6
	1:1,000	+	35,660	9,460	26,200	3.8

Dishes (35-mm diam) containing approximately equivalent numbers of either NCB 20 cells or primary mouse spinal cord neurons were incubated with or without tetanus toxin (10 $\mu\text{g}/\text{ml}$) for 1 h at 0°C, washed, fixed, and assayed for bound toxin by use of antitoxin and ^{125}I -protein A as described in Materials and Methods. In addition, spinal cord cells were treated with 0.1% Triton X-100 after fixation but before addition of the antitoxin. Values are the mean of triplicate determinations, which varied <10%.

total cell-associated tetanus toxin, the cells were treated with Triton X-100 after fixation but before addition of the antitoxin.⁴ Permeabilizing the cells led to a marked increase in binding of ^{125}I -protein A to control neurons (Table I). When the total counts bound to toxin-treated cells were corrected for the appropriate nonspecific binding, the amount of tetanus toxin detected associated with cells before and after permeabilization was essentially identical (Table I). Thus, all of the tetanus toxin appeared to remain on the cell surface, as expected for cells incubated at 0°C.

We next followed the time course of loss of tetanus toxin from surface of primary mouse spinal cord cells (Fig. 2a). There was extensive loss of surface bound toxin 1 h after the cells had been shifted from 0 to 37°C, with little additional loss over the next 3 h. The amount of total cell-associated toxin also was assayed using the permeabilized cells in order to determine if any of the toxin had become internalized. There was no difference between cell-surface and total cell-associated toxin after 1 h at 0°C. When the cells were shifted to 37°C, substantially more toxin was detected when the cells were treated with Triton X-100, and the difference increased with time at 37°C, which suggests that some of the toxin was being internalized.

The kinetics of these processes were followed more closely (Fig. 2b). Reduction of surface-bound tetanus toxin was evident as early as 15 min after the cells were warmed to 37°C, and appeared to reach a plateau by 30 min. In contrast, there was no significant loss of toxin from the cell surface when the cells were maintained at 0°C. Internalization also appeared to be a rapid process at 37°C as there was a substantial and reproducible increase in the difference between total and cell surface toxin within 15 min at 37°C.

Release of Tetanus Toxin into the Medium

Although internalization clearly accounts for some of the loss of cell-surface tetanus toxin, the amount of total cell-

1% bovine serum albumin at 0°C for 1 h and filtered under vacuum on 0.5 μm Millipore EHWP filters (Millipore Corp., Bedford, MA); the filters were washed with the same buffer.

⁴ Pilot experiments with ^{125}I -tetanus toxin showed that little or no toxin was extracted during permeabilization of the cells with Triton X-100 at 0°C. To stabilize the cells, however, and to avoid any antibody-induced loss of bound toxin, the cells were routinely fixed before detergent treatment or addition of antitoxin.

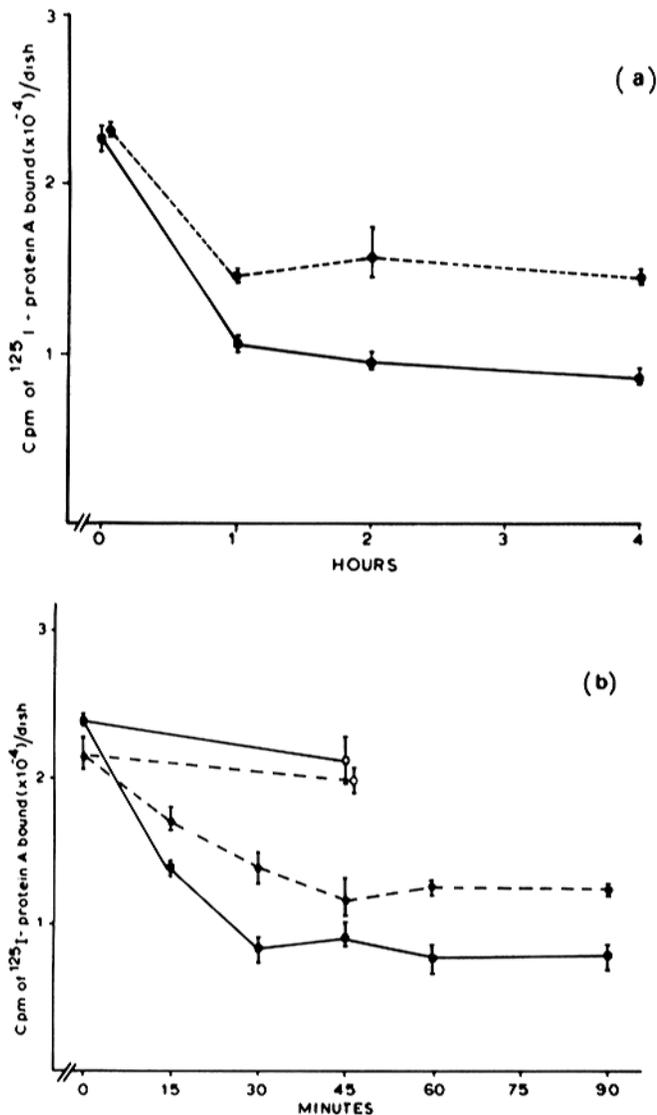


FIGURE 2 Fate of tetanus toxin bound to the surface of primary mouse neuronal cultures. (a) Cells were incubated with 66 nM tetanus toxin for 1 h at 0°C, washed, incubated at 37°C for the indicated times, and fixed as described in Materials and Methods. The cells then were assayed for surface bound toxin (—■—) or permeabilized with Triton X-100 and assayed for total cell-associated toxin (---○---) using the anti-toxin and ¹²⁵I-protein A procedure. The incubations with tetanus toxin were staggered so that the cells were fixed and assayed all at the same time. (b) Same as in a except some of the cultures were maintained at 0°C for 45 min after incubation with tetanus toxin and before assay for surface (—□—) and total cell-associated (---○---) toxin. Each value represents the mean ± SE of triplicate determinations and has been corrected for non-specific binding to cells not treated with tetanus toxin as described in Table I. Similar results were obtained in a total of five separate experiments.

associated toxin after incubation of the cells at 37°C was markedly less than that initially bound (Fig. 2). A substantial amount of the toxin might be released into the medium after the cells are warmed to 37°C. This possibility was confirmed in studies using ¹²⁵I-tetanus toxin (Fig. 3). After the cells were incubated with the iodotoxin at 0°C for 1 h, they were washed free of unbound toxin and warmed to 37°C. There was a rapid loss of cell-associated ¹²⁵I-tetanus toxin into the medium during the first 40 min (Fig. 3a). The half-life of this fraction

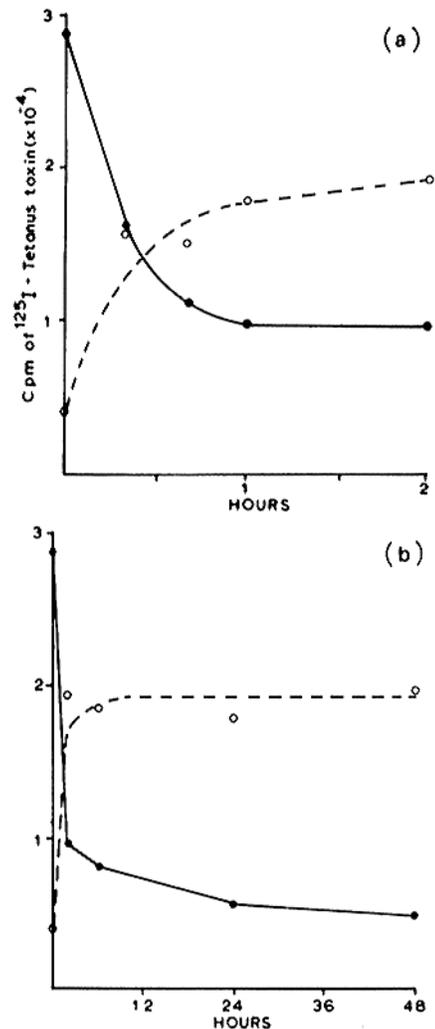


FIGURE 3 Fate of ¹²⁵I-tetanus toxin bound to primary mouse spinal cord cell cultures. Cells were incubated with 0.2 nM ¹²⁵I-tetanus toxin (895 cpm/fmol) in DME/HEPES/1% bovine serum albumin for 1 h at 0°C and washed three times with ice-cold PBS. The cells then were incubated in complete growth medium at 37°C. At the indicated times, the medium (○) was assayed for 10% trichloroacetic acid-precipitable radioactivity and the cells (●) were dissolved in 1 M NaOH and counted for radioactivity.

of bound toxin was 15 min. A further small reduction in bound toxin occurred over the next 24 h, with little further loss between 24 and 48 h (Fig. 3b). Thus, the toxin that remains cell associated after 24 h at 37°C appears to have a very long half-life.⁵ Most of the ¹²⁵I-tetanus toxin released into the medium was precipitated by trichloroacetic acid (98% after 6 h and 91% after 48 h), which suggests that there was little degradation of the toxin by the cells.

Immunofluorescence Studies on the Fate of Tetanus Toxin

The fate of tetanus toxin bound to spinal cord cells also was investigated by use of immunofluorescence. Fig. 4 confirms that binding of tetanus toxin to primary mouse spinal cord cultures is predominantly to cells with a typical neuronal

⁵ More extensive studies indicated that the half-life of tetanus toxin in these cultures was 6 d (W. H. Habig, unpublished observations).

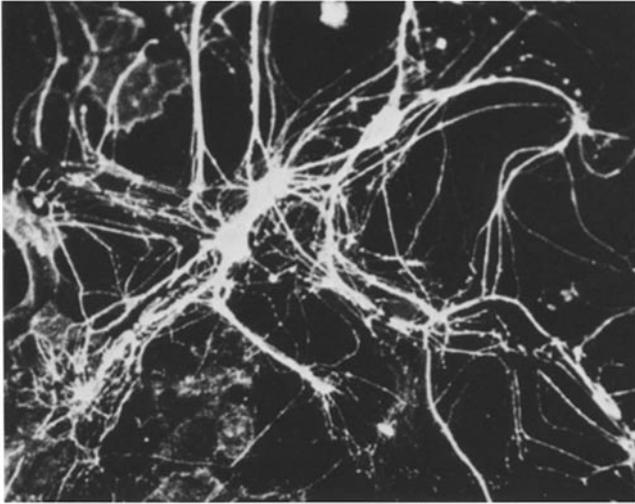


FIGURE 4 Binding of tetanus toxin to primary mouse neurons as detected by indirect immunofluorescence. Cells were incubated with tetanus toxin (20 $\mu\text{g/ml}$) in DME/HEPES/1% fetal calf serum for 1 h at 0°C. After the cells were washed, they were incubated with goat anti-tetanus toxin antiserum (1:40 dilution), washed, and stained with fluorescein isothiocyanate-labeled rabbit anti-goat antibodies (1:50 dilution from Miles-Yeda, Rehovot, Israel) both diluted in DME/HEPES/5% fetal calf serum. $\times 140$.

morphology; the underlying fibroblasts bind little, if any, toxin. When the cells were warmed to 37°C, much of the surface bound toxin was rapidly lost within 1 h (Fig. 5), in agreement with the studies described above that used the ^{125}I -protein A. The profile of fluorescent staining of cells incubated with tetanus toxin for 1 h at 0°C was essentially identical without (Fig. 5*b*) and with (Fig. 6*a*) Triton X-100 treatment before addition of the antibody. Within 15 min at 37°C, the permeabilized cells displayed a pattern of bright punctate staining (Fig. 6*b*) not observed in cells not treated with the detergent (Fig. 5*d*). This pattern of intracellular staining for tetanus toxin was stable for up to at least 8 h of incubation at 37°C (Fig. 6) although there appeared to be some reduction in fluorescence intensity after 24 h (not shown).

DISCUSSION

As described in the present study, we have developed a simple immunochemical assay to distinguish surface-bound from total cell-associated tetanus toxin, and have used the assay to establish the fate of tetanus toxin bound to primary cultures of mouse spinal cord cells. We have found that much of the toxin bound to cells at 0°C rapidly disappeared from the cell surface when they warmed to 37°C, but not if the cells were kept at 0°C. Identical experiments with ^{125}I -tetanus toxin indicated that a substantial amount of the bound toxin was released into the culture medium at 37°C without being degraded. These observations are consistent with previous reports that toxin binding is optimal at 0–4°C and that release of bound toxin increases with temperature (7, 25, 26). The results with tetanus toxin contrast markedly with those obtained with cholera toxin. The latter toxin, when bound to cells at 0°C, is not released into the medium upon warming of the cells but is internalized and degraded (19, 27).

We also have found that some of the tetanus toxin bound to the cells at 0°C was internalized upon warming the cells to 37°C. To detect total cell-associated toxin, we permeabilized

the cells with detergent before adding the antitoxin. Under these conditions, additional toxin was detected in cells warmed to 37°C but not in cells maintained at 0°C. Internalization of the toxin was rapid, and a substantial increase in the ratio of total to cell-surface toxin was detected after 15 min at 37°C. There appeared to be no further internalization of tetanus toxin after 30 min, and little or no further decrease in surface toxin beyond this time. The significance of this finding is unclear. It is possible that the remaining surface-bound toxin is associated with receptors that either do not undergo internalization or do, but very slowly. The concept of effective and ineffective binding of tetanus toxin has been proposed already (28). Thus, productive binding may be associated with rapid internalization of the toxin. Alternatively, internalization may depend on metabolic processes that are sensitive to the experimental protocol, i.e., prolonged incubation at 0°C and numerous washes.

Our observation that tetanus toxin is internalized within 15 min by primary mouse spinal cord neurons may have biological significance as the toxin altered the membrane electrical activity in these cells after a lag period of 15–20 min (16). Similarly, the inhibition of Ca^{2+} -dependent action potentials in mouse neuroblastoma NIE-115 cells by tetanus toxin exhibited a lag of 10–20 min (29). A lag phase is also characteristic of the activation of adenylate cyclase in intact cells by cholera toxin (30, 31). During this lag period, the A_1 peptide, which is an ADP-ribosyltransferase (32), is generated from the bound cholera toxin (31). Whether tetanus toxin, like cholera and diphtheria toxin, elicits its intracellular effects through some enzymatic activity remains to be elucidated.

The results using immunofluorescence confirm and extend those obtained using ^{125}I -protein A. Thus, the immunofluorescent staining for tetanus toxin on cells exposed to the toxin at 0°C was initially extremely bright but was dramatically reduced within 30 min after the cells were warmed at 37°C. Some of the staining was recovered when the cells were permeabilized with detergent. In addition to surface staining, there was considerable intracellular fluorescence, confirming that some of the toxin had been internalized. The intracellular tetanus toxin exhibited a punctate distribution, which suggests that the toxin was associated with discrete subcellular structures rather than diffusely distributed throughout the cytoplasm. Appearance of toxin in these structures occurred within 15 min at 37°C, was maximal after 30 to 60 min at this temperature, and was still clearly visible after 24 h. The precise nature of these structures is not known, but we presume that they are not lysosomes because (a) they were not phase dense; (b) the half-life of the toxin stably associated with the cells was several days;⁵ and (c) little if any degraded toxin was released from cells incubated with ^{125}I -tetanus toxin. As we indicated in the introduction, studies with colloidal gold-labeled tetanus toxin *in vivo* (11) showed that most of the internalized toxin was contained within smooth membranous structures and not within lysosomes.

Precisely how the toxin avoids degradation by lysosomal enzymes is unclear. Recent studies on transferrin, which is rapidly internalized through receptor-mediated endocytosis, show that it too avoids the lysosomal compartment (33, 34). Transferrin is known to bind to its receptor more strongly at an acid pH. Thus, during acidification in endosomes, transferrin does not dissociate from its receptor and is recycled with its receptor back to the cell surface where it encounters a neutral pH and dissociates into the extracellular milieu.

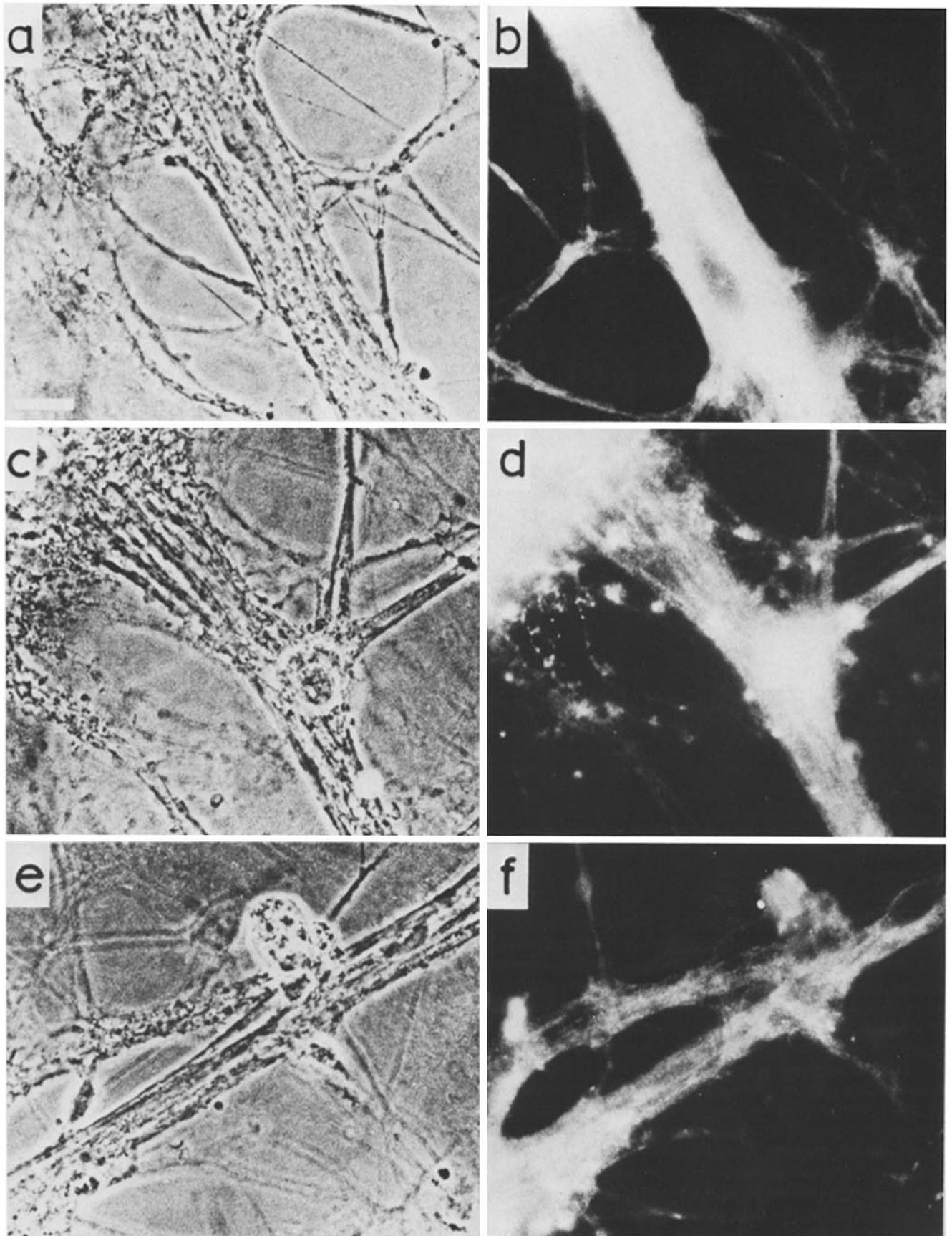


FIGURE 5 (Above and opposite) Fate of tetanus toxin bound to the surface of primary mouse neurons as followed by indirect immunofluorescence. Cells were incubated with tetanus toxin ($8 \mu\text{g/ml}$) for 1 h at 4°C , washed, and incubated in complete medium at 37°C for the indicated times. The cells then were fixed and stained for surface bound toxin using mouse monoclonal anti-toxin and rhodamine-labeled anti-mouse IgG as described in Materials and Methods. Cells were examined under both phase contrast (a, c, e, g, and i) and fluorescence (b, d, f, h, and j) microscopy. (a and b) Cells fixed immediately after incubation at 4°C ; (c and d) 15 min at 37°C ; (e and f) 30 min; (g and h) 60 min; (i and j) cells not exposed to tetanus toxin. Bar (shown in a), $20 \mu\text{m}$.

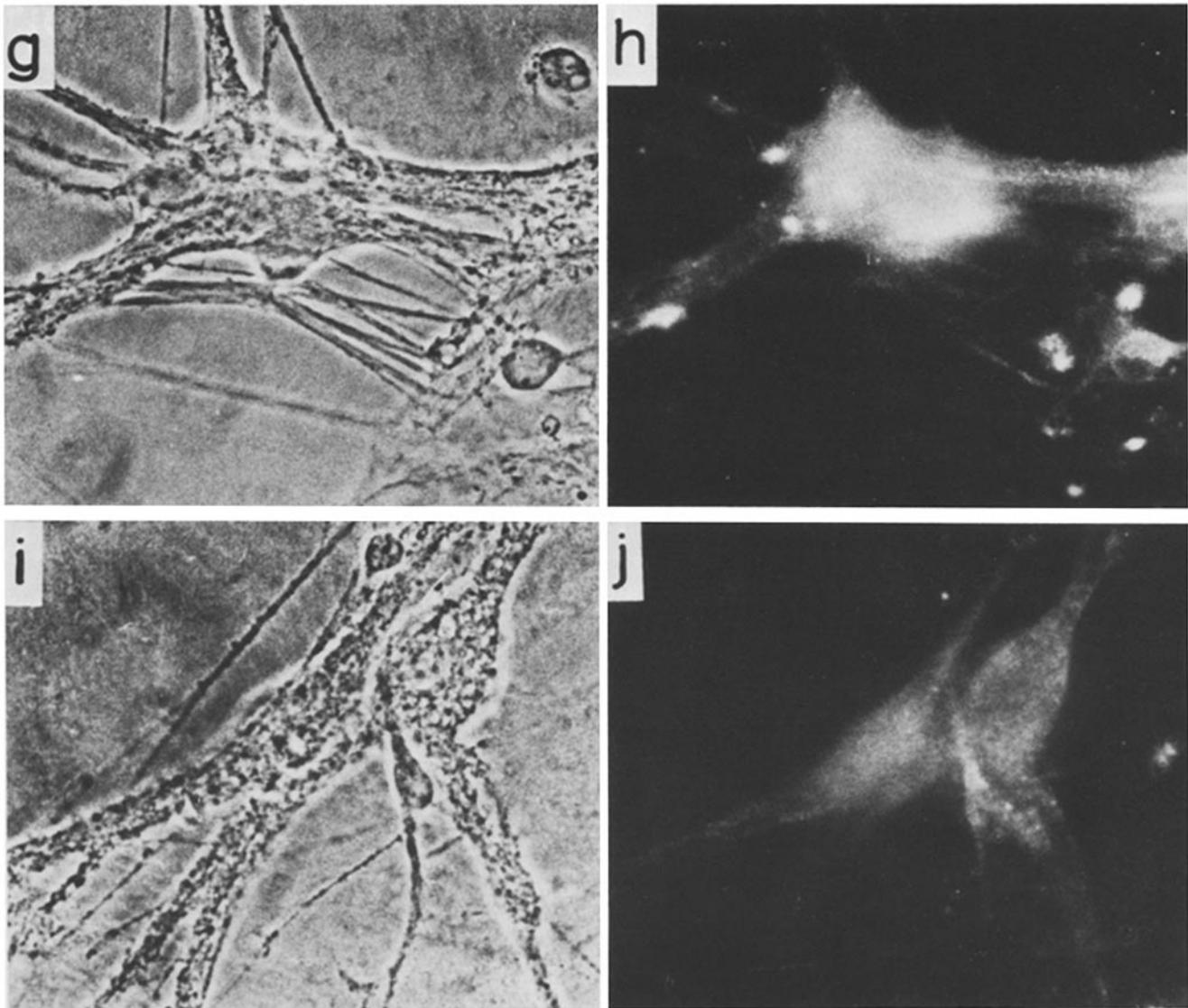


FIGURE 5 continued

Other ligands that dissociate from their receptors at an acid pH are apparently targeted to lysosomes for degradation (35, 36). As the optimum pH for tetanus toxin binding to its membrane receptor is pH 4.5–5.0 (7),² it is tempting to speculate that a mechanism analogous to that used by transferrin might explain why tetanus toxin not only avoids degradation but also is exported from the axon, perhaps into the synaptic cleft (5). In this regard, the observation that tetanus toxin fragment B forms channels in lipid vesicles at low pH may be relevant (37). It is not yet clear, however, as to whether tetanus toxin enters the cell through coated pits via receptor-mediated endocytosis. In the one study in which this point was addressed, no evidence was found for internalization of the toxin by a coated pit mechanism although cells of non-neuronal origin that bind little toxin were used in the study (12). We should point out that the punctate distribution of internalized tetanus toxin as revealed by immunofluorescence is very reminiscent of that observed with ligands internalized via coated pits (38).

It is also unclear whether ligands that bind to gangliosides

can be internalized through this pathway. Gangliosides G_{D1b} and G_{T1b} have been implicated as receptors for tetanus toxin (6–10).² In the present study, we found that tetanus toxin will directly bind to these gangliosides present in neuronal cells but not to other membrane components such as proteins and glycoproteins. The receptor for cholera toxin also is a ganglioside, namely G_{M1} (39). Several electron micrographic studies on the endocytosis of cholera toxin suggest that it is not internalized through coated pits (12, 40, 41).

In summary, we have provided clear evidence for the rapid internalization of tetanus toxin by primary mouse spinal cord neurons into a subcellular compartment where it escapes degradation. We are currently attempting to clarify the nature of this compartment and to establish the mechanism by which the toxin enters the cell.

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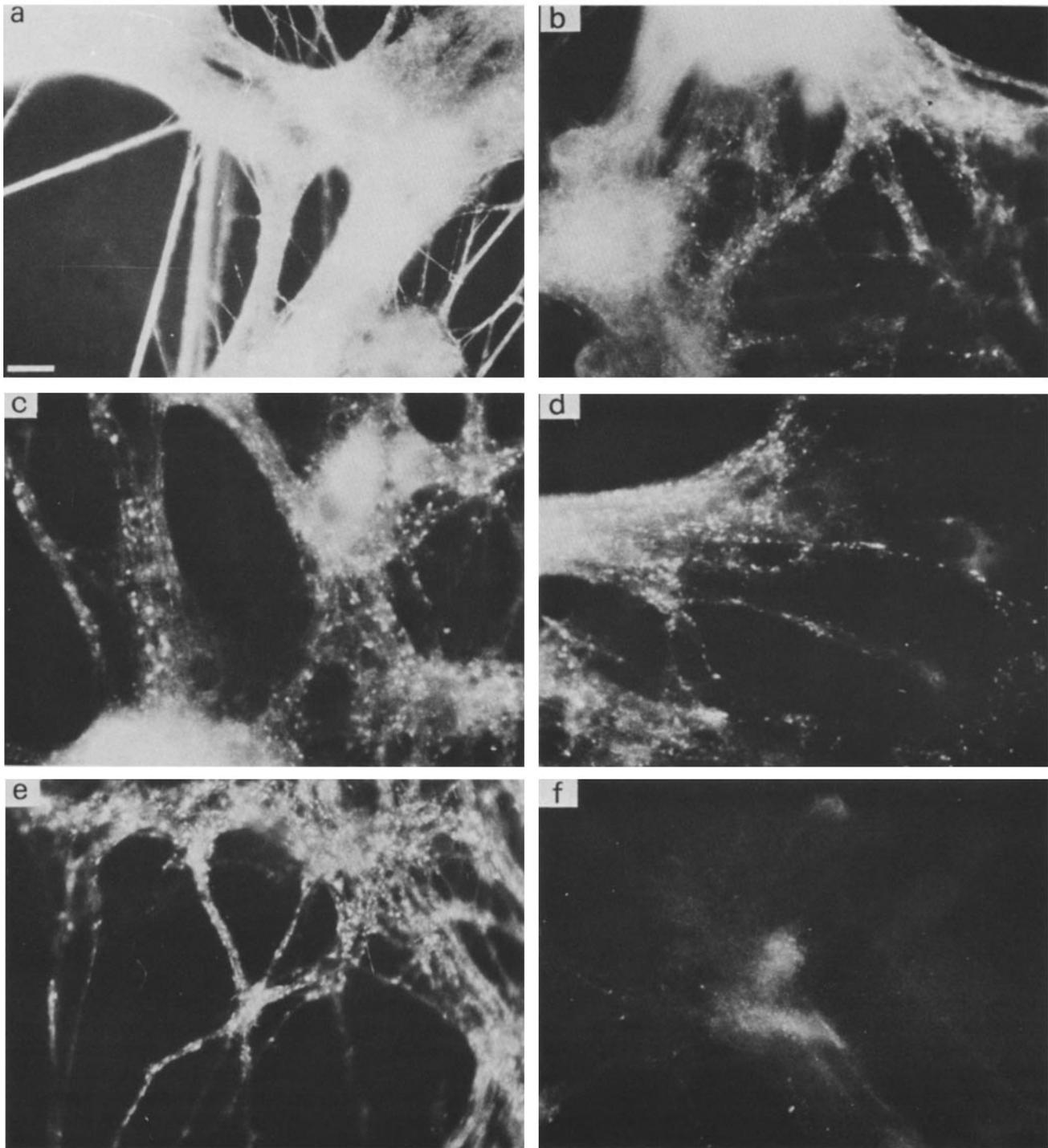


FIGURE 6 Internalization of tetanus toxin bound to primary mouse neurons as detected by immunofluorescence. The experiment was run in parallel to the one described in the legend to Fig. 5. After the cells were fixed, they were treated with 0.1% Triton X-100 before the anti-toxin was added as described in Materials and Methods. (a) Cells fixed immediately after incubation with tetanus toxin at 4°C; (b) after 15 min at 37°C; (c) 30 min; (d) 1 h; (e) 8 h; (f) cells not exposed to tetanus toxin. Bar, 20 μ m.

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REFERENCES

1. Robinson, J. P., and J. H. Hash. 1982. A review of the molecular structure of tetanus toxin. *Mol. Cell. Biochem.* 48:33-44.
2. Mellanby, J., and J. Green. 1981. How does tetanus toxin act? *Neuroscience.* 6:281-300.
3. Wellhoner, N. H. 1982. Tetanus neurotoxin. *Rev. Physiol. Biochem. Pharmacol.* 93:1-68.
4. Bizzini, B. 1979. Tetanus toxin. *Microbiol. Rev.* 43:224-240.
5. Schwab, M. E., K. Suda, and H. Thoenen. 1979. Selective retrograde transsynaptic transfer of a protein, tetanus toxin, subsequent to its retrograde axonal transport. *J. Cell Biol.* 82:798-810.
6. van Heyningen, S. 1980. Tetanus toxin. *Pharmacol. & Ther.* 11:141-157.
7. Ledley, F. D., G. Lee, L. D. Kohn, W. H. Habig, and M. C. Hardegree. 1977. Tetanus toxin interactions with thyroid plasma membranes. Implications for structure and function of tetanus toxin receptors and potential pathophysiological significance. *J. Biol. Chem.* 252:4049-4055.
8. Holmgren, J., H. Elwing, P. Fredman, and L. Svennerholm. 1980. Polystyrene-absorbed

- gangliosides for investigation of the structure of the tetanus toxin receptor. *Eur. J. Biochem.* 106:371-379.
9. Rogers, T. B., and S. H. Snyder. 1981. High affinity binding of tetanus toxin to mammalian brain membranes. *J. Biol. Chem.* 256:2402-2407.
 10. Yavin, E. 1984. Gangliosides mediate association of tetanus toxin with neural cells in culture. *Arch. Biochem. Biophys.* 230:129-137.
 11. Schwab, M. E., and H. Thoenen. 1978. Selective binding, uptake, and retrograde transport of tetanus toxin by nerve terminals in rat iris: an electron microscope study using colloidal gold as tracer. *J. Cell Biol.* 77:1-3.
 12. Montesano, R., J. Roth, A. Robert, and L. Orci. 1982. Non-coated membrane invaginations are involved in binding and internalization of cholera and tetanus toxin. *Nature (Lond.)*. 296:651-653.
 13. Pastan, I. H., and M. C. Willingham. 1984. Receptor-mediated endocytosis of hormones in cultured cells. *Annu. Rev. Physiol.* 43:239-250.
 14. Dimpfel, W., and E. Habermann. 1977. Binding characteristics of ¹²⁵I-labelled tetanus toxin to primary tissue cultures from mouse embryonic CNS. *J. Neurochem.* 29:1111-1120.
 15. Brenneman, D. E., E. A. Neale, W. H. Habig, L. M. Bowers, and P. G. Nelson. 1983. Developmental and neurochemical specificity of neuronal deficits produced by electrical impulse blockade in dissociated spinal cord cultures. *Dev. Brain Res.* 9:13-27.
 16. Bergey, G. K., R. L. Macdonald, W. H. Habig, M. C. Hardegree, and P. G. Nelson. Tetanus toxin: convulsant action on mouse spinal cord neurons in culture. *J. Neurosci.* 3:2310-2323.
 17. Critchley, D. R., J. L. Magnani, and P. H. Fishman. 1981. Interaction of cholera toxin with rat intestinal brush border membranes. Relative roles of gangliosides and galactoproteins as toxin receptors. *J. Biol. Chem.* 256:8724-8731.
 18. Critchley, D. R., C. H. Steuli, S. Kellie, S. Ansell, and B. Patel. 1982. Characterization of the cholera toxin receptor on Balb/c 3T3 cells as a ganglioside similar to, or identical with, ganglioside GM₁. No evidence for galactoproteins with receptor activity. *Biochem. J.* 204:209-219.
 19. Fishman, P. H. 1982. Internalization and degradation of cholera toxin by cultured cells: relationship to toxin action. *J. Cell Biol.* 93:860-865.
 20. Ransom, B. R., E. A. Neale, M. Henkart, P. N. Bullock, and P. G. Nelson. 1977. Mouse spinal cord cells in culture. I. Morphology and intrinsic neuronal electrophysiological properties. *J. Neurophysiol.* 40:1132-1150.
 21. An Der Lan, B., W. H. Habig, M. C. Hardegree, and A. Chranbach. 1980. Heterogeneity of ¹²⁵I-labeled tetanus toxin in isoelectric focusing on polyacrylamide gel and polyacrylamide gel electrophoresis. *Arch. Biochem. Biophys.* 200:206-215.
 22. Fishman, P. H., J. Moss, and V. C. Manganiello. 1977. Synthesis and uptake of gangliosides by cholera-responsive human fibroblasts. *Biochemistry.* 16:1871-1875.
 23. Mangani, J. L., B. Nilsson, M. Brockhaus, D. Zopf, Z. Steplewski, H. Koprowski, and V. Ginsburg. 1982. A monoclonal antibody-defined antigen associated with gastrointestinal cancer is a ganglioside containing sialylated lacto-N-fucopentaose II. *J. Biol. Chem.* 257:14365-14369.
 24. Kenimer, J. G., W. H. Habig, and M. C. Hardegree. 1983. Monoclonal antibodies as probes of tetanus toxin structure and function. *Infect. Immun.* 42:942-948.
 25. Yavin, E., and W. H. Habig. 1984. Binding of tetanus toxin to somatic neural hybrid cells with varying ganglioside composition. *J. Neurochem.* 42:1313-1320.
 26. Yavin, E., Z. Yavin, and L. D. Kohn. 1983. Temperature-mediated interaction of tetanus toxin with cerebral neuron cultures: characterization of a neuraminidase-insensitive toxin-receptor complex. *J. Neurochem.* 40:1212-1219.
 27. Chang, P. P., P. H. Fishman, N. Ohtomo, and J. Moss. 1983. Degradation of cholera toxin bound to cultured human fibroblasts and mouse neuroblastoma cells. *J. Biol. Chem.* 258:426-430.
 28. Zimmerman, J. M., and J.-Gl. Piffaretti. 1977. Interaction of tetanus toxin and toxoid with cultured neuroblastoma cells. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 296:271-277.
 29. Higashida, H., N. Sugimota, K. Ozutsumi, N. Miki, and M. Matsuda. 1983. Tetanus toxin: a rapid and selective blockade of the calcium, but not sodium component of action potentials in cultured neuroblastoma NIE-115 cells. *Brain Res.* 279:363-368.
 30. Fishman, P. H. 1980. Mechanism of action of cholera toxin: studies on the lag period. *J. Membr. Biol.* 54:61-72.
 31. Kassis, S., J. Haggmann, P. H. Fishman, P. P. Chang, and J. Moss. 1982. Mechanism of action of cholera toxin on intact cells. Generation of A₁ peptide and activation of adenylate cyclase. *J. Biol. Chem.* 257:12148-12152.
 32. Moss, J., and M. Vaughan. 1979. Activation of adenylate cyclase by cholera toxin. *Annu. Rev. Biochem.* 48:581-600.
 33. Klausner, R. D., G. Ashwell, J. van Renswoude, J. B. Harford, and K. R. Bridges. 1983. Binding of apotransferrin to K562 cells: explanation of the transferrin cycle. *Proc. Natl. Acad. Sci. USA.* 80:2263-2266.
 34. Dautry-Varsat, A., A. Ciechanover, and H. F. Lodish. 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA.* 80:2258-2262.
 35. Harford, J., K. R. Bridges, G. Ashwell, and R. D. Klausner. 1983. Intracellular dissociation of receptor-bound asialoglycoprotein in cultured hepatocytes. A pH mediated non-lysosomal event. *J. Biol. Chem.* 258:3191-3197.
 36. Brown, M. S., R. G. W. Anderson, and J. L. Goldstein. 1983. Recycling receptors: the round-trip itinerary of migrant membrane proteins. *Cell.* 32:663-667.
 37. Boquet, P., and E. Duflot. 1982. Tetanus toxin fragment forms channels in lipid vesicles at low pH. *Proc. Natl. Acad. Sci. USA.* 79:7614-7618.
 38. Pastan, I. H., and M. C. Willingham. 1981. Journey to the center of the cell: role of the receptorosome. *Science (Wash. DC)*. 214:504-509.
 39. Fishman, P. H. 1982. Role of membrane gangliosides in the binding and action of bacterial toxins. *J. Membr. Biol.* 69:85-97.
 40. Joseph, K. C., A. Stieber, and N. K. Gonatas. 1979. Endocytosis of cholera toxin in GERL-like structures of murine neuroblastoma cells pretreated with GM₁ ganglioside. *J. Cell Biol.* 81:543-554.
 41. Gonatas, N. K., A. Stieber, J. Gonatas, T. Mommoi, and P. H. Fishman. 1983. Endocytosis of exogenous GM₁ ganglioside and cholera toxin by neuroblastoma cells. *Mol. Cell. Biol.* 3:91-101.