SELECTIVE BINDING, UPTAKE, AND RETROGRADE TRANSPORT OF TETANUS TOXIN BY NERVE TERMINALS IN THE RAT IRIS

An Electron Microscope Study Using Colloidal Gold as a Tracer

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ABSTRACT
A series of specific macromolecules (tetanus toxin, cholera toxin, nerve growth factor [NGF], and several lectins) have been shown to be transported retrogradely with high selectivity from terminals to cell bodies in various types of neurons. Under identical experimental conditions (low protein concentrations injected), most other macromolecules, e.g. horseradish peroxidase (HRP), albumin, ferritin, are not transported in detectable amounts. In the present EM study, we demonstrate selective binding of tetanus toxin to the surface membrane of nerve terminals, followed by uptake and subsequent retrograde axonal transport.

Tetanus toxin or albumin was adsorbed to colloidal gold particles (diam 200 Å). The complex was shown to be stable and well suited as an EM tracer. 1–4 h after injection into the anterior eye chamber of adult rats, tetanus toxin-gold particles were found to be selectively associated with membranes of nerve terminals and preterminal axons. Inside terminals and axons, the tracer was localized mainly in smooth endoplasmic reticulum (SER)-like membrane compartments. In contrast, association of albumin-gold complexes with nervous structures was never observed, in spite of extensive uptake into fibroblasts.

Electron microscope and biochemical experiments showed selective retrograde transport of tetanus toxin-gold complexes to the superior cervical ganglion.

Specific binding to membrane components at nerve terminals and subsequent internalization and retrograde transport may represent an important pathway for macromolecules carrying information from target organs to the perikarya of their innervating neurons.

KEY WORDS tetanus toxin · membrane receptors · axonal transport · pinocytosis · EM tracer

Cell surface membranes contain a large variety of specific macromolecules which serve as binding sites for many endogenous and exogenous ligands, such as hormones, neurotransmitters, lectins, and toxins. The surface membranes of nerve terminals are highly specialized structures involved in forming contacts with corresponding target cells and in the release and inactivation of neurotransmitters.
Since ribosomes are totally absent from axons and nerve terminals, all structural components have to be supplied by the nerve cell body by means of fast and slow axonal transport. A large part of the material reaching the nerve terminals by fast axonal transport consists of macromolecules destined for surface and intracellular membranes (9, 38). A considerable part of this rapidly transported material subsequently returns to the cell body by retrograde transport (2).

Certain macromolecules, such as nerve growth factor (NGF), tetanus toxin, cholera toxin, and several lectins, have been shown to be taken up by nerve terminals and transported retrogradely with high selectivity, i.e., in relatively large quantities compared with other macromolecules of similar molecular weight and similar general physicochemical properties (e.g., horseradish peroxidase, albumin, ferritin) (11, 36, 37, 42-45). The amount of protein that must be injected in order to detect retrograde transport in the case of NGF, tetanus and cholera toxin, or several lectins is several hundred times less than that necessary in the case of horseradish peroxidase (HRP) or albumin (39, 42). Specific binding sites on the cell membrane have been characterized for all macromolecules showing selective retrograde transport (1, 13, 18, 22, 25, 26, 32, 44, 46).

To study the initial binding and uptake at the nerve terminal membrane, we chose tetanus toxin (mol wt 150,000) (3, 21, 25) which binds with high affinity to di- and trisialogangliosides (GD_{1b}, GT_{1}) (22, 26). EM autoradiography cannot be used for fine structural localization because of its limited resolution. Furthermore, methods involving the use of ferritin are unsuitable because of the poor visibility of ferritin in tissues (in contrast to isolated cells or membranes). We therefore have devised a new method (described previously for antibody labeling) in which colloidal gold is used (12, 27). By reducing tetrachlor-gold solutions with citric acid, colloidal gold particles of a variable but defined size can be prepared (14). The particles used in the present study had a diameter of ~200 Å. Such particles carry high negative charges on their surface. Thus, when macromolecules of adequate size (12, 27) are added to the gold suspension, they are tightly adsorbed to the gold particles without undergoing chemical modification as is the case with ferritin, peroxidase coupling, or iodination. These gold-protein particles are highly electron-dense and are thus easily visible in the EM, even at low magnifications.

The present report shows the selective uptake of colloidal gold particles coated with tetanus toxin by nerve terminals in the rat iris. Within the nerve terminals and during subsequent retrograde transport in the axons, the toxin-gold complex is preferentially located in membrane-bounded, SER-like compartments. In contrast, no uptake of albumin-coated gold particles was observed.

**MATERIALS AND METHODS**

**Preparation of Colloidal Gold-Protein Complexes**

Colloidal gold was prepared according to the method described by Frens (14). 100 ml of an aqueous H\(_2\)AuCl\(_4\) (Merck Chemical Div., Merck & Co., Inc., Rahway, N. J.) solution (0.01%) was heated to boiling, then 2.5 ml of 2% aqueous sodium citrate was added and the mixture was kept boiling for an additional 10-15 min. Soon after the addition of citrate, the color of the solution changed from slightly yellow to violet and then to purple and dark red. The final size of the gold particles is homogeneous but can be varied according to the relative amount of citrate added, and is reflected by the final color of the gold suspension (14). In the present experiments the diameter of the gold particles was about 200 Å. Colloidal gold is kept in suspension by electric repulsion of the negatively charged particles. Therefore, to prevent precipitation, care has to be taken that no cations (salts) are added in large amounts together with the protein. Very basic proteins can also precipitate the colloid.

Highly purified tetanus toxin (Dr. B. Bizzinni, Institut Pasteur, Paris, France) was dialyzed for 2 h against water and 100 μg was added to 10 ml of the gold suspension. The mixture was stirred for 1 min and then stabilized by the addition of 0.1 ml of carbowax 1% (27). Finally, the suspension was centrifuged for 30 min at 10,000 g. The pellet was resuspended for washing and centrifuged again. The final pellet (~100 μl) was diluted with 200 μl of water and injected. Bovine serum albumin was purchased from Sigma Chemical Co. (St. Louis, Mo.) and coupled to colloidal gold in an identical manner.

**Stability of the Tetanus Toxin-Gold Complex**

Colloidal gold was coupled to radioactive \(^{125}\text{I}\)tetanus toxin (sp act 5.6 μCi/μg) as described above. The radioactivity remaining in the supernate after the first centrifugation amounted to 7.4% of the total radioactivity. The supernate resulting from the second centrifugation contained only 0.4% of the total radioactivity.

The stability of the complex was checked by incubation of 20 μl (74 μCi) of \(^{125}\text{I}\)tetanus toxin-gold (pellet) with 1 ml of rat serum. During 1 h at 37°C, 4% of the
total radioactivity was found in the supernate after centrifugation (30 min at 10,000 g). After 14 h of incubation at room temperature under nonsterile conditions, 9% of the total radioactivity was found in the supernate.

These experiments demonstrate tight binding of tetanus toxin to the colloidal gold. Thus, stability under in vivo conditions is expected, in agreement with the high stability of gold-antibody complexes (12).

Injection and Fixation

**Iris** Female Sprague-Dawley rats of 200-250 g body weight under ether anaesthesia were injected with 10 µl of toxin-gold complex, by means of a Hamilton syringe (Hamilton Co., Reno, Nev.) into the anterior eye chamber. Due to the intense red color of the solution, it was easy to ascertain whether the injected material stayed within the anterior eye chamber or escaped into the vitreous body or through the needle hole in the sclera. Only animals with injections well in place were used for fixation. After 1, 2, 4, or 6 h the rats were anaesthetized and perfused through the heart with a short prerinse of Ringer’s solution containing 1,000 U of heparin (USP) (Liquemine, Hoffman-La Roche Inc., Basle, Switzerland) and 0.1% procaine followed by a mixture of 2.5% glutaraldehyde and 1% formaldehyde in 0.1 M phosphate buffer, pH 7.4, containing 5% sucrose, for 10-15 min. After perfusion, the iris was carefully exposed and small blocks were cut from the dilator region. These blocks were immersed in the same fixative for an additional 2 h. They were then washed overnight in 0.1 M phosphate buffer, pH 7.4, with 5% sucrose, postfixed in 1.33% OsO4 for 2 h, washed in 0.1 M cacodylate buffer, dehydrated, block-stained with 2% uranyl acetate in 95% ethanol, and embedded in Epon 812. Thin sections were stained with 2% uranyl acetate in 95% ethanol, and stained with 2% uranyl acetate (sp act at the time of injection: 0.35 mCi/ml; concn 0.5 mg/ml) as it is used for radiotherapy, was injected in the same manner. Radioactivity in the ganglia was determined 14 h after injections.

**RESULTS**

**Binding and Uptake of Tetanus Toxin-Gold Complex by Nerve Terminals**

Good penetration of the protein-gold complexes into the iris after injection into the anterior eye chamber could be observed as soon as 1 h after injection. The loose structure of the iris allowed penetration in spite of the relatively large size of the complex (diameter of the gold grains 200 Å, plus protein coat). In addition to being influenced by the size of the complex and the type of tissue, the penetration could be influenced by the properties of the macromolecule bound to the colloidal gold. In the case of tetanus toxin, the gold complexes tended to form short rows of three to five particles and to show a high degree of association with collagen fibers. Thus, penetration was markedly less than with the albumin-gold complex which was monodisperse and showed less association with collagen. The steep gradient in the extracellular concentration of toxin-gold complexes within the iris made it almost impossible to carry out quantitative assessment and a comparison with experiments done in the presence of excess unlabeled toxin.

1 and 2 h after injection into the anterior eye chamber, the toxin-gold complexes were associated with the surface and were within SER-like compartments of nerve terminals and preterminal axons (Figs. 1 and 2). Association with surface membranes of other cells was rarely observed, although fibroblasts occasionally contained large amounts of gold in their lysosomes (Fig. 4a). Toxin-gold complexes bound to neuronal membranes were found even in the complete absence of gold in the surrounding extracellular space (Figs. 1 and 2b). An accumulation of gold particles around collagen fibrils was observed mainly at sites of high toxin-gold concentrations, supporting the interpretation of this phenomenon as nonspecific binding.

Toxin-gold complexes bound to membranes of nervous structures were present as single grains or in rows and clusters (Figs. 1 and 2b). However, as there seemed to be a tendency for tetanus...
toxin-gold to line up also along collagen fibrils, no definite conclusions can be drawn from this clustered appearance.

Labeled coated vesicles could not be observed although pinocytotic figures involving coated vesicles were frequent in nerve terminals and, occasionally, also preterminal axons. It could not be decided unequivocally whether the uptake of the toxin-gold complexes by nerve terminals occurred by means of coated vesicles or smooth membrane structures.

**Retrograde Transport within Axons**

Internalized toxin-gold complexes in axons and nerve terminals were observed 1 and 2 h after injection (Figs. 1 and 2). In every case, tracer was found within a membrane-bounded compartment. As can be seen from Table I, these compartments were preferentially SER-like structures in axons and nerve terminals. In addition, a small percentage of toxin-gold was found in lysosomal structures (small or large dense bodies, elongated multivesicular bodies). Occasionally in nerve terminals, structures indistinguishable from synaptic vesicles contained a single gold particle.

4 and 6 h after injection, less toxin-gold complexes were found in the SER of axons and nerve terminals. This observation suggests that labeled material was removed from the iris by retrograde axonal transport, in agreement with the appearance of gold particles in the superior cervical ganglion 14-16 h after injection. In addi-
tion, infiltration of the tissue with leukocytes as a sign of local inflammation due to the injection was observed after 4–6 h.

In contrast to the situation in axons and nerve terminals, toxin-gold complexes were almost exclusively incorporated into lysosomes by fibroblasts and, rarely, also into Schwann cells (Figs. 1 and 4a).

**Localization in Nerve Cell Bodies**

14–16 h after injection of tetanus toxin-gold complex into the anterior eye chamber and the submandibular gland, neurons containing gold particles were discernible in sections of the superior cervical ganglion. The lower labeling intensity in case of the toxin-gold complex compared with \[^{125}\text{I}]\text{tetanus toxin} (41) corresponds to the much lower amount of toxin-gold complexes reaching the nerve terminals because of the limited penetration and the size of the complex.

Retrograde transport of tetanus toxin-gold was also confirmed by counting the radioactivity in superior cervical ganglia 14 h after unilateral injection of \[^{125}\text{I}]\text{tetanus toxin-gold complex into the eye and into the submandibular gland}. A low but consistent accumulation of radioactivity was seen on the injected side (mean ± SEM: 560.5 ± 115.9 cpm, }\text{n} = 6), and only background activity was seen on the noninjected side (141.0 ± 22.1 cpm, equals background of counter and tubes).

**Localization of Albumin-Gold Complexes**

Albumin-gold complexes were never observed to be associated with the surface membrane or internalized within axons or nerve endings in spite of high extracellular concentrations (Fig. 3). A single exception was uptake by a pinocytic-coated vesicle at a nerve terminal. In contrast, uptake of albumin-gold complexes by fibroblasts and Schwann cells occurred as frequently as uptake of the toxin-gold complex.

In addition, there was no accumulation of radioactivity in the ganglion after injection of radioactive Au\(^{198}\) coated with gelatine, used as a control for nonspecific retrograde transport.

**DISCUSSION**

The present results with the use of protein-coated colloidal gold as a novel EM tracer show selective binding of tetanus toxin-gold complexes to the membranes of autonomic nerve terminals and preterminal axons in the rat iris. These findings confirm the autoradiographic data of Price et al. (37), who showed binding and uptake of \[^{125}\text{I}]\text{tetanus toxin by neuromuscular and spinal cord synapses}. The binding was followed by uptake and incorporation of the toxin-gold complex into SER-like membrane compartments. Subsequently, the toxin-gold complex was transported retrogradely within the axons and reached the adrenergic ganglion cell bodies in the superior cervical ganglion.

**Table I**

|                        | Distribution of Tetanus Toxin-Gold Associated with Nervous Structures in the Rat Iris |
|------------------------|---------------------------------------------------------------------------------------|
|                        | 1 or 2 h After injection                                                                 |
|                        | Gold grains | Percent | Gold grains | Percent |
| Iris:                  |             |         |             |         |
| Axons                  | 207         | 32.75   | 42          | 43.3    |
| Nerve terminals        | 425         | 67.25   | 55          | 56.7    |
| Axons:                 |             |         |             |         |
| Surface membrane       | 145         | 70.0    | 15          | 35.7    |
| Internalized:          | 62          | 30.0    | 27          | 64.3    |
| SER                    | 59          | 95.2    | 19          | 70.3    |
| Lysosomes              | 3           | 4.8     | 8           | 29.7    |
| Nerve terminals:       |             |         |             |         |
| Surface membrane       | 346         | 81.4    | 47          | 85.5    |
| Internalized:          | 79          | 18.6    | 8           | 14.5    |
| SER                    | 68          | 86.1    | 6           | 75      |
| Lysosomes              | 7           | 8.8     | 0           | -       |
| Synaptic vesicles      | 4           | 5.1     | 2           | 25      |

Electron microscope sections of the dilator region of the rat iris 1 or 2 h after injection (\text{n} = 10) and 4 h after injection (\text{n} = 3) of 10 \(\mu\)l of tetanus toxin-gold were carefully screened. Every gold particle associated with an axon or nerve terminal was photographed at a primary magnification of 10,000, 20,000, and 31,000.
The localization of toxin-gold complexes in SER-like membrane compartments during retrograde transport corresponds to previous findings with \[^{125}I\]tetanus toxin, \[^{125}I\]NGF, and NGF-HRP coupling product in the same neurons (39, 41), and for HRP in various central and peripheral neurons (5, 30, 31). However, in the case of HRP, lysosomes seem also to be involved in addition to the SER (50). In any case, SER-like compartments seem to represent an important part of the transport machinery for retrograde as well as orthograde transport (9). Apart from the fact that retrograde transport of NGF and tetanus toxin is blocked by colchicine (34, 43, 44), nothing is known of the possible interactions of SER-like compartments with microtubules and of the localization of the force-generating mechanism (20).

A fundamental difference was observed between the distribution of tetanus toxin-gold complexes and that of albumin-gold in the rat iris. Whereas both complexes were taken up by fibro-
blasts, only the toxin-gold complex was bound to nerve terminal membranes and taken up and transported retrogradely. This difference in behavior explains earlier biochemical results, viz., that tetanus toxin, NGF, cholera toxin, and wheat germ agglutinin are transported retrogradely in high amounts even after injection of relatively low protein concentrations, i.e., under conditions where no retrograde transport of albumin, HRP, cytochrome c, or ferritin could be detected (39, 42). It seems that albumin, HRP, dextran, and many other macromolecules, if they are present in the extracellular space in high concentrations, are taken up accidentally together with extracellular fluid by nerve terminals mainly during transmitter release and vesicle recycling (6, 24, 48). In fact, it was shown that uptake of HRP is highly dependent upon the electrical activity of the terminal (6, 24, 47-49). In contrast, tetanus and cholera toxins, NGF, and some lectins seem to bind specifically to the membrane of nerve terminals. Specific membrane constituents have been identified as binding sites for all these macromolecules (1, 13, 18, 22, 25, 26, 32, 44, 46). In fact, retrograde transport of tetanus toxin, which binds to di- and trisialogangliosides (22, 26), and cholera toxin, which binds to monosialogangliosides (13, 26), could be significantly inhibited or
FmUgE 3 Rat iris, 4 h after injection of albumin-gold. In spite of a very high extracellular concentration, no albumin-gold is associated with or taken up by preterminal axons (Fig. 3a, × 24,000) or nerve terminals (Fig. 3b, × 37,000).

blocked by injection of the respective ganglioside (46). Biochemical studies on brain have shown selective enrichment of di- and trisialogangliosides in plasma membranes of neurons and synaptosomes (8, 18, 19, 29). In addition, NGF is transported only in peripheral adrenergic and sensory neurons, which are known to possess specific NGF receptors, but not in motoneurons from which such receptors seem to be absent (44).

In various cell types, binding of ligand (lectins, cholera toxin) to a specific membrane-binding site induces redistribution of these binding sites on the cell surface, eventually followed by pinocytosis (see references 10 and 32 for review). Pinocytic uptake of a ricin-HRP coupling product by sympathetic and dorsal root ganglion cell bodies in culture occurs selectively compared with uptake of HRP alone (15, 16). NGF-coated erythrocytes bind to specific clones of neuroblastoma cells and are subsequently phagocytosed (4). These findings are consistent with the hypothesis of specific bind-

FigURE 3 Rat iris, 4 h after injection of albumin-gold. In spite of a very high extracellular concentration, no albumin-gold is associated with or taken up by preterminal axons (Fig. 3a, × 24,000) or nerve terminals (Fig. 3b, × 37,000).
ing of certain macromolecules, e.g. tetanus toxin or NGF, to receptors on the nerve terminal membrane, triggering the removal of occupied receptors together with the ligand by internalization and subsequent retrograde transport. The ligand reaches the cell body in an undegraded form (17, 45) and is able to trigger specific effects (NGF) (33, 34) or to leave the neuron again and to migrate trans-synaptically to presynaptic terminals of second-order neurons (tetanus toxin) (40, 41).

There may be specific receptors for endogenous molecules on nerve terminals. The quality and quantity of such molecules reaching the cell body by retrograde transport may represent important signals for the cell regarding its survival and differentiation during development, the reaction to lesions of axons or terminals, or the regulation of the synthesis or activity of specific enzymes (7, 23, 28, 33, 35).

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FIGURE 4a Tetanus toxin-gold present in secondary lysosomes of a fibroblast in the rat iris, 2 h after injection. × 27,000.
Figure 4b Gold particles (arrow) in an adrenergic neuron of the superior cervical ganglion after retrograde transport from the anterior eye chamber (iris) and the submandibular gland. × 22,000.
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