Proc. Natl. Acad. Sci. USA
Vol. 75, No. 6, pp. 2815–2819, June 1978
Cell Biology

Endocytosis of cholera toxin into neuronal GERL

(neurons/Golgi)

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Communicated by Alex B. Novikoff, March 13, 1978

ABSTRACT **Cholera toxin linked covalently by glutaraldehyde to horseradish peroxidase was incubated with cultured chicken sympathetic neurons at 4°. Cells were washed and brought to 37° to permit endocytosis of bound toxin on plasma membranes. Massive internalization of the ligand into vesicles and cisterns of the *G*olgi–*e*ndoplasmic *r*eticulum–*l*ysosome (GERL) system was demonstrated by the cytochemical reaction for the enzyme. Surface binding and subsequent endocytosis of the cholera toxin–enzyme conjugate was inhibited when conjugate and monosialoganglioside (GM_1) were simultaneously applied to cells at 4°. Cholera toxin is not toxic to neurons at the levels used. These results indicate that GERL is the primary site of endocytosis of presumed complexes of cholera toxin with its plasma membrane receptor (GM_1 ganglioside-containing moieties). It is suggested that, in neurons, plasma-membrane bound ligands are taken up primarily into GERL.**

Significant information on the structure and function of mammalian cell plasma membranes has been obtained by the use of antibodies or lectins conjugated with marker molecules such as fluorescein, ferritin, or horseradish peroxidase (1–4). Subsequent to their binding to plasma membranes, lectins are taken into the general area of the Golgi apparatus and specifically into vesicles, tubules, or cisterns corresponding to the *G*olgi–*e*ndoplasmic *r*eticulum–*l*ysosome system (GERL) of cultured neurons (5–7); we have also shown that anti-immunoglobulin antibody is taken into the Golgi cisterns of plasma cells (8). By binding to their plasma membrane receptors, lectins and cholera toxin (neurons) or anti-immunoglobulin antibodies (plasma cells) may provide a more accurate view of "membrane flow" than do soluble markers (7, 9, 10). The significance of this unusual internalization of plasma membrane bound moieties (adsorptive endocytosis) into the Golgi apparatus or GERL is unknown (10).

In the present study, conjugates of cholera toxin with horseradish peroxidase (toxin–HRP complex) were used for a cytological study of its binding and subsequent endocytosis in cultured chicken embryo sympathetic neurons. Cholera toxin appears to bind ganglioside GM_1 irreversibly as its natural receptor (11, 12). The binding property of the toxin resides only in one of its two major subunits (B subunit). The other subunit (A) presumably dissociates from the binding subunit B upon interaction with GM_1 at the cell plasma membrane and activates adenyl cyclase by an unknown mechanism (13). Although our experiments were not designed to study the mechanism of toxin action upon whole cells, the findings may be of possible relevance to such a question. The toxin–HRP complex demonstrated the same specificity for GM_1 ganglioside as did the free toxin and was taken up into vesicles, tubules, or cisterns of GERL.

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MATERIALS AND METHODS

Lumbar sympathetic chains or dorsal root ganglia from 10- to 12-day chicken embryos were dissociated into single cells by trypsinization and transferred into Eagle's medium supplemented with glucose (5 mg/ml), 20% fetal calf serum, and nerve growth factor (20 ng/ml). Chains of ganglia were dissociated by pipetting (14). Neurons were cultured either on poly(L-lysine) or on collagen-coated Aclar coverslips (15).

Cholera toxin was purchased from Schwarz/Mann (Orangeburg, NY). Toxin was labeled with ^{125}I according to Cuatrecasas (16). The specific activity of the undiluted preparations was 1600 μCi/mol. Covalent coupling of the toxin to horseradish peroxidase via the two-step method utilizing glutaraldehyde as the crosslinking agent was performed according to Avrameas and Ternynck (17). For "activation" of peroxidase, 5 mg of enzyme was incubated overnight at room temperature with 1.25% glutaraldehyde in 0.1 M sodium phosphate (pH 6.8). The peroxidase was then separated from free glutaraldehyde on a Sephadex G-25 column (0.9 × 40 cm). Elution was performed with 0.15 M NaCl. The activated peroxidase was coupled to cholera toxin at pH 9.5 for 24 hr at 4°; the pH was adjusted with 1 M carbonate pH 9.5 buffer. The conjugate was dialyzed against buffered physiologic saline at 4°. Uncoupled peroxidase was neutralized by the addition of L-lysine hydrochloride to a final concentration of 0.1 M.

Coupling of ^{125}I-labeled cholera toxin to horseradish peroxidase could be demonstrated by Sephadex G-200 chromatography (Fig. 1). A peak at molecular weight 140,000 seen after coupling probably represents a complex of two peroxidase molecules per molecule of toxin. The molecular weight of the free toxin is about 80,000 but it runs on gel filtration as 60,000 (18). The molecular weight of the peroxidase is 40,000 and it runs accordingly on gel filtration. Toxin–HRP complex was separated from free toxin by gel filtration on Bio-Gel A 0.5 (0.9 × 100 cm). Polyacrylamide gel electrophoresis of the coupling reaction mixture before and after Bio-Gel separation demonstrated coupling and removal of free toxin from the coupled product (Fig. 2). This conjugate was used for surface binding or endocytosis (internalization). The yield of coupled toxin as judged by the amount of ^{125}I in the conjugate was 25%. Ouchterlony analysis of the coupled product yielded strong precipitation lines with both goat anti-horseradish peroxidase and rabbit anti-cholera toxin antisera.

Antitoxin was prepared by subcutaneous injections of 0.15 mg of toxin and complete Freund's adjuvant into rabbits once every 10 days for a total of three injections. One week later, the animals were bled out and antibody was revealed by Ouchterlony double-diffusion and precipitation. Gangliosides were

Abbreviations: GERL, Golgi–endoplasmic reticulum–lysosome system; toxin–HRP complex, conjugate of cholera toxin with horseradish peroxidase.

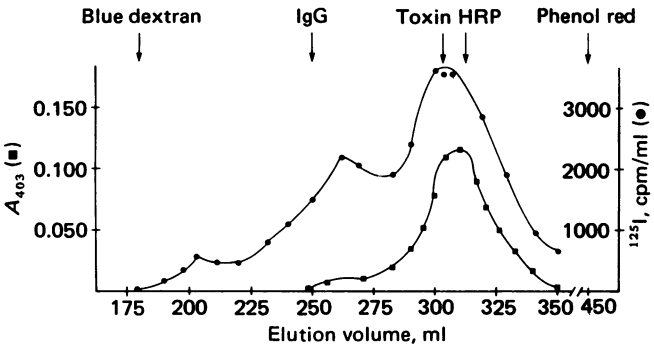

FIG. 1. Gel filtration on Sephadex G-200 (80 × 2.5 cm) of a coupling mixture of ^{125}I-labeled toxin and horseradish peroxidase (HRP). Elution volume from 200 to 265 ml represents coupling. Column was standardized with the following molecular weight standards: blue dextran (>10^6), IgG (160,000), toxin (80,000), and horseradish peroxidase (HRP) (40,000).

isolated from bovine brain by the method of Lauter and Trams (19). GM_1 was isolated from this mixture by thin-layer chromatography.

Cells were incubated with toxin–HRP complex as described (7). Toxin–HRP complex (30 μg of toxin per ml) was incubated with cells for 1 hr at 4°. Cells were then either fixed immediately (surface labeling) or after repeated washings for 1½ hr in Eagle's medium brought to 37° (endocytosis). Cultures were fixed with 1% paraformaldehyde/2.5% glutaraldehyde/0.2 M sodium cacodylate, pH 7.35, containing 0.4 ml of 0.5% $CaCl_2$ per 100 ml of fixative. Cytochemical peroxidase activity was revealed by the method of Graham and Karnovsky with diaminobenzidine tetrahydrochloride as substrate (7, 20). Short incubations (10 min) at room temperature were used. After cytochemical staining, cultures were postfixed in 2% OsO_4/1% potassium ferrocyanide and dehydrated in alcohol for Araldite embedding. When cells were simultaneously incubated with toxin–HRP complex and free GM_1 (10 μg/ml), binding of the toxin–HRP complex, as judged by staining for peroxidase, did not occur. Manuelidis and Manuelidis (21) have demonstrated the same high specificity of the toxin–HRP complex for GM_1. Both thick (1 μm) and thin (500–600 Å) sections stained only with peroxidase and thin sections stained with peroxidase, uranyl acetate, and lead citrate were examined in a Siemens Elmiskop I electron microscope (7).

RESULTS AND DISCUSSION

After incubation of toxin–HRP complex with cells for 1 hr at 4°, a strong reaction product at the plasma membrane was seen. Intense peroxidase reaction product was also seen on the plasma membranes of neurites but cell processes as well as growth cone areas were not studied. The dense reaction product representing oxidized diaminobenzidine–osmium complexes was in the form of a continuous amorphous or granular band 200–300 Å thick. Diffuse or focal staining of the neuronal cytoplasm adjacent to the plasma membrane (diffusion of reaction product) was not seen. Incubation of cells with free horseradish peroxidase or with glutaraldehyde-treated peroxidase ("activated") under these conditions yielded no surface staining. The lack of internalization of the toxin–HRP complex after incubation only at 4° parallels previous results with lectins and antibodies (6–8). After incubations at 37°, the plasma membrane was not cleared of toxin–HRP complex and distinct patching or capping of toxin–HRP complex was not observed; this may relate to the univalent nature of GM_1.

Endogenous peroxidase activity in the form of vesicles, tubules, cisterns, or round bodies was not seen. Thick sections (1 μm) of neurons fixed after initial incubation at 4° and subsequent incubation in culture medium for 1.5 hr at 37° showed extensive, massive endocytosis of toxin-HRP complex (Fig. 3) that exceeded by far the endocytosis of lectins reported previously (7). Perinuclear areas of peroxidase staining measuring 3–5 μm in their greatest diameter were observed. These areas were filled with linear and curvilinear or vesicular profiles. Occasionally, round profiles 0.5–1 μm in diameter appeared to be stained. However, because of the inherent density of the round bodies (dense bodies? lysosomes?), their positive reaction for peroxidase activity could not be determined with certainty.

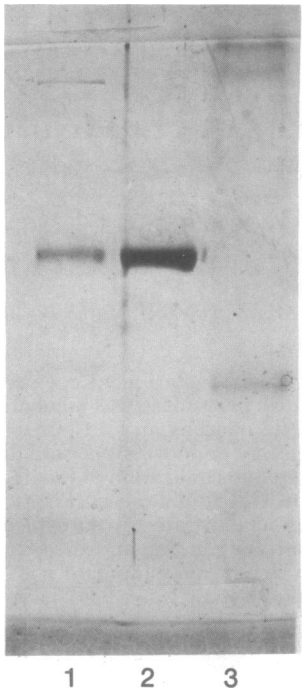

FIG. 2. Electrophoresis on 8% polyacrylamide gel. Lanes: 1, conjugate mixture of toxin and peroxidase; 2, free toxin; 3, conjugate after separation on Bio-Gel A 0.5. Note absence of free toxin in lane 3 and presence of high molecular weight conjugate (top).

Thin sections (500–600 Å) of neurons stained only for peroxidase confirmed the vesicular, tubular, or cisternal nature of the structures containing the toxin-HRP complex with its presumed receptors (Fig. 4). The diameter of the cisterns or tubules was 0.1–0.3 μm. Often, clusters of peroxidase-positive tubules, vesicles, or cisterns were noted at the trans aspect of the Golgi apparatus, the cisterns of which were unstained (Fig. 5). These thin sections of neurons containing toxin-HRP complex and counterstained with lead citrate and uranyl acetate revealed that peroxidase-positive vesicles or cisterns were bounded by smooth membranes 70–90 Å thick. Frequently, peroxidase-positive vesicles appeared in rows, and peroxidase-positive cisterns or tubules may have been produced from the coalescence of vesicles. Peroxidase-positive vesicles were also seen directly adjacent to the plasma membrane after 1.5 hr of endocytosis; this observation suggests either a continuous endocytosis or possible reinsertion (recycling) of toxin or toxin–receptor complexes (Fig. 6). In another series of experiments, toxin-HRP complex was internalized for 24 hr. These cells were normal by electron microscopy, and peroxidase reaction product was identified in GERL and on the plasma membrane. Because cholera toxin is not toxic to these cells, we think that

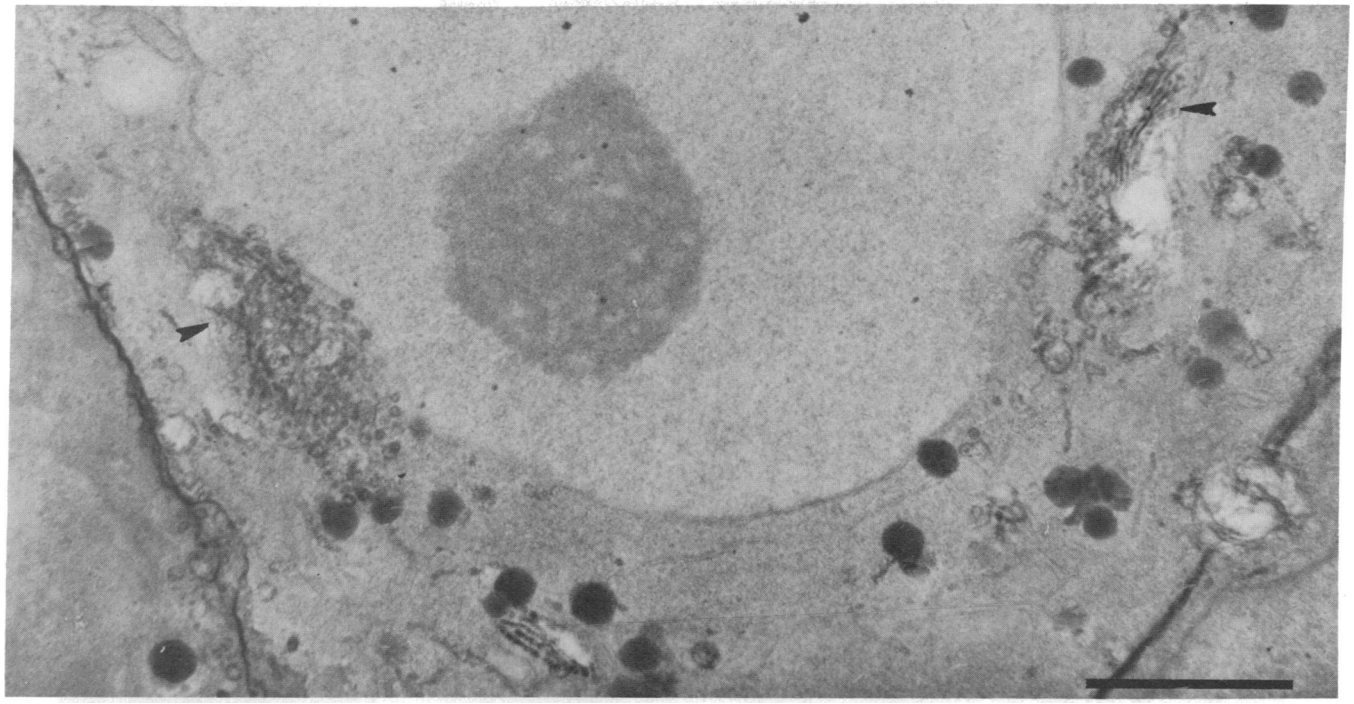

FIG. 3. Thick section (1 μm) of sympathetic neuron incubated with toxin–HRP complex for 1 hr at 4°, washed, and incubated in culture medium at 37° for 90 min. Arrowheads, vesicular or cisternal endocytosis of toxin–HRP complex. (Stained only for peroxidase; ×27,000.)

its internalization may represent a naturally occurring "membrane flow" of significant magnitude. Whether this phenomenon is connected with the "lipid flow" that Bretscher (22) has hypothetized is unknown at present.

This study clearly demonstrates the usefulness of toxin–HRP complex in internalization studies of glycolipids specifically and plasma membranes in general. The specificity of binding, lack of cytotoxicity, and high degree of internalization are the major reasons for this. It is not known if the internalization of toxin–HRP complex relates to the toxin-induced activation of the

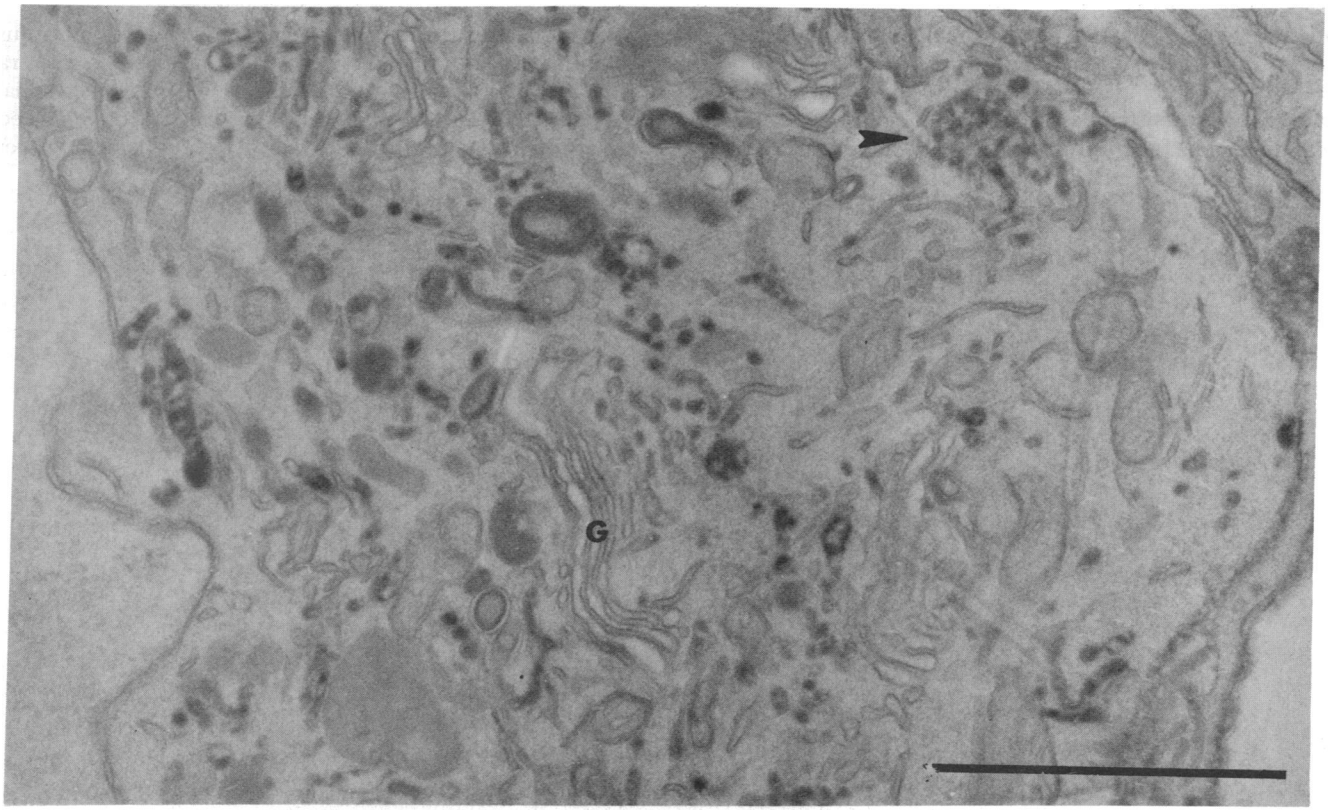

FIG. 4. Thin section (500–600 Å) of sympathetic neuron treated as in Fig. 3. G, Golgi apparatus; arrowhead, vesicles and tubules positive for peroxidase adjacent to the plasma membrane. (Stained only for peroxidase; ×47,000.)

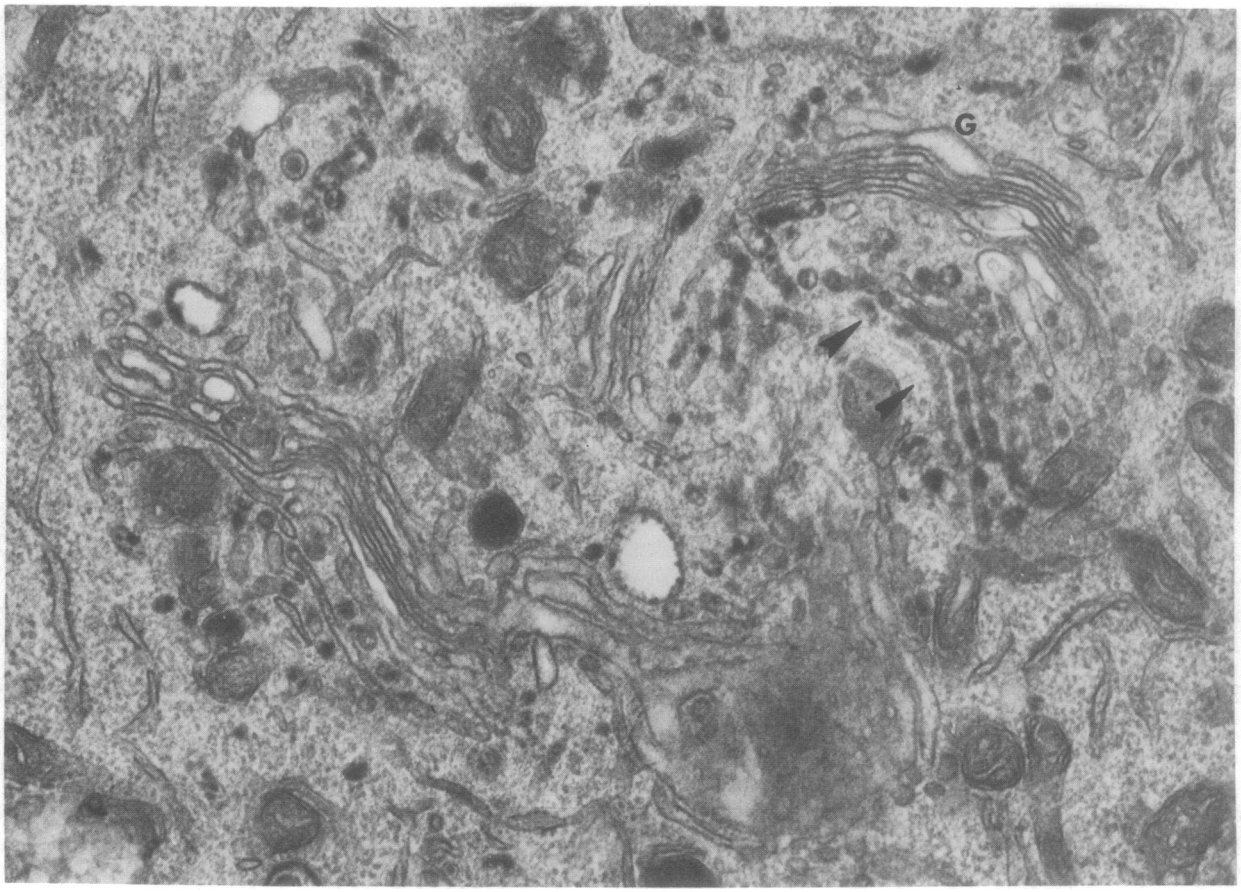

FIG. 5. Same as in Fig. 2, but section has been stained for peroxidase and with uranyl acetate and lead citrate. Arrowheads, endocytosis of toxin–HRP complex into cisterns (tubules?) or vesicles at trans aspect of Golgi apparatus (G). (×58,000.)

adenylate cyclase, but the levels of cholera toxin used are consistent with its activation of the enzyme.

The similarity of identity of the sites of adsorptive endocytosis of these heterogeneous ligands (anti-immunoglobulin antibody, lectins, cholera toxin) strongly suggests that adsorptive endocytosis of plasma membrane moieties into the Golgi apparatus (immunoglobulin of plasma cells) or GERL (lectins, cholera toxin in neurons) represents general phenomena and not special properties of a particular ligand. This conclusion is enhanced by similar or identical sites of endocytosis of colloidal silver

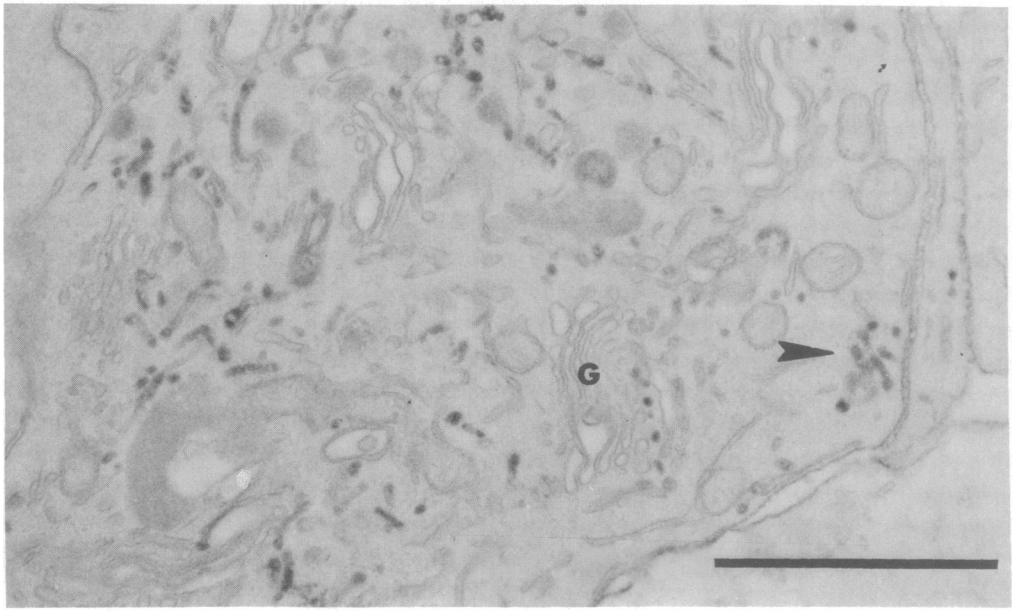

FIG. 6. Vesicular or tubular endocytosis of toxin–HRP complex. G, Golgi apparatus; arrowhead, cluster of peroxidase-positive vesicles and tubules near the plasma membrane. (Stained only for peroxidase; ×39,000.)

(alveolar macrophages), colloidal thorium oxide (chick sensory ganglia), and dextran (lacrimal and parotid gland) that have been described (23–25).

The authors thank Dr. R. A. Angeletti and Mrs. J. Gonatas for help in gel filtration and polyacrylamide electrophoresis. This work was supported by Grants NS05572-13 and GM00092-02 from the National Institutes of Health.

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