

ENDOCYTOSIS OF CHOLERA TOXIN IN GERL-LIKE STRUCTURES OF MURINE NEUROBLASTOMA CELLS PRETREATED WITH GM₁ GANGLIOSIDE

Cholera Toxin Internalization into Neuroblastoma GERL

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ABSTRACT

Cholera toxin (CT), covalently attached to horseradish peroxidase (HRP), is a specific cytochemical marker for GM₁ ganglioside (GM₁) and retains the ability of the native toxin to raise levels of cyclic AMP in avian erythrocytes.

Using a cytochemical stain for HRP, we found that 9% of control cultured murine neuroblastoma cells bound cholera toxin-horseradish peroxidase conjugates (CT-HRP) on their surfaces after incubations for 1 h at 4°C. Exogenous GM₁, the natural receptor of CT, becomes associated in the culture medium with the plasma membranes of these cells so that 96% of cells are stained.

Cells preincubated with GM₁ at 4°C were exposed to CT-HRP for 1 h at 4°C. After washing, cells were incubated at 37°C for 30 min–24 h. Endocytosis of CT-HRP occurred within 30 min and CT-HRP remained, throughout the 24-h period, in tubules, vesicles, and cisternae often found near the Golgi apparatus; this aggregate of peroxidase-positive elements probably corresponds to Golgi apparatus-endoplasmic reticulum-lysosomes (GERL) of neurons. In metaphase cells, CT-HRP was observed in aggregates of vesicles and tubules clustered near the centriole. Conjugates of HRP with subunit B, the GM₁ binding component of CT, were internalized by cells pretreated with GM₁ as was CT-HRP. The 9% of neuroblastoma cells binding CT-HRP in the absence of exogenous GM₁ internalized the ligand in a manner indistinguishable from that of the treated cells.

These findings indicate that, in neuroblastoma cells, a system of vesicles, tubules, and cisternae, analogous to GERL of neurons, is the primary recipient of adsorptive endocytosis of CT bound to endogenous or exogenously introduced GM₁.

KEY WORDS cholera toxin · endocytosis · neuroblastoma · GERL

Antiimmunoglobulin antibodies, lectins, and various other ligands which bind to plasma membrane

receptors undergo endocytosis (adsorptive endocytosis) in a quantitatively, and possibly qualitatively, different fashion than do molecules which lack affinities for plasma membrane moieties (fluid phase or bulk endocytosis) (9, 35). We have

previously reported that antiimmunoglobulin antibodies, or lectins conjugated with horseradish peroxidase (HRP), undergo endocytosis into the Golgi apparatus or Golgi apparatus-endoplasmic reticulum-lysosomes (GERL) of plasma cells and of cultured neurons (14, 15, 28). These ligands induce significant redistribution of surface receptors before, or concomitant with, their endocytosis (2).

In a recent publication, we have shown that cholera toxin (CT), covalently coupled to horseradish peroxidase (CT-HRP) by means of glutaraldehyde as a cross-linking reagent, undergoes endocytosis into GERL of cultured neurons (21). CT, a protein of ~80,000 daltons, is composed of two subunits, A and B (4, 11). The B subunit binds to cell surfaces through its receptor, the GM₁ ganglioside (GM₁), and exhibits biological activity through activation of adenylyl cyclase in a variety of cells (13, 38, 39). CT has several properties which make it a useful probe in the study of plasma membrane dynamics: (a) through a ubiquitous activation of adenylyl cyclase, CT produces diverse biological effects in several cells or tissues but is not toxic to several types of cultured cells (reference 4, p. 4); (b) the structure of its receptor, GM₁, is known (4); (c) the receptor introduced exogenously in the medium becomes associated with the membrane of cells which lack endogenous receptor (25); and (d) because of the univalent nature of GM₁, CT molecules are not cross-linked when they are bound to cells, although a single CT molecule can bind to more than one receptor (GM₁) (6).

Neuroblastoma cells offer several advantages over cultured neurons in the study of ligand-receptor interactions, including ease of maintenance and availability. Kinetic and biochemical analyses possible with neuroblastoma cells are not feasible with cultured neurons, because of restrictions of cell numbers and the almost unavoidable presence of non-neuronal cells in cell cultures. Finally, although most neuroblastoma cells contain insufficient GM₁ to bind CT-HRP conjugates, the levels of receptor can be increased by adding GM₁ to the medium until previously nonresponding cells become able to bind the toxin; furthermore, cells otherwise unresponsive to CT become responsive to the toxin and show activation of adenylyl cyclase after preincubation with GM₁ (22, 25).

In this study, we have examined the binding and endocytosis of conjugates of either CT, or its

subunit B, with HRP in cultured murine neuroblastoma cells which were preincubated with GM₁. Also, we have examined the binding and endocytosis of CT-HRP in neuroblastoma cells which were not exposed to exogenously introduced GM₁. We have observed that these conjugates are endocytosed in vesicles, tubules, or cisternae which are similar to components of GERL of normal neurons (21). Identical patterns of binding and endocytosis of CT-HRP were noted in all positive cells, with or without exogenous GM₁.

MATERIALS AND METHODS

Cell Cultures

Murine neuroblastoma cells (line 2A [CCI-131] obtained from the American Type Culture Collection, Rockville, Md.) were grown either in Falcon bottles (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) or in suspension in Dulbecco's Modified Eagle's Medium (Grand Island Biological Co., Grand Island, N. Y.), with 2 mM glutamine and 10% fetal calf serum. Cells grown on plastic strips (Aclar 33C, 5 mil, Allied Chemical Corp., Specialty Chemicals Div., Morristown, N. J.) were used for growing cells destined for ultrastructural studies.

Cholera Toxin

CT was purchased from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.). The A and B subunits were separated by chromatography on a Biogel P-60 column (0.8 × 50 cm) developed with 1.0 M urea in 0.1 M glycine buffer, pH 3.2 (11). Resolution was complete. After dialysis against 0.1 M phosphate-buffered saline, pH 7.3, both subunits reacted with antitoxin as judged by Ouchterlony double diffusion and precipitation, and gave reactions of nonidentity. Iodination of CT was performed as described previously (21).

Conjugation of CT or Subunit B to HRP (CT-HRP, B-HRP)

Coupling was performed by the two-step method of Avrameas and Ternynck with glutaraldehyde as the cross-linking agent (3, 24). With this method, HRP is treated with glutaraldehyde, and the excess glutaraldehyde is removed through chromatography on a Sephadex G-25 column. During the second step of the conjugation procedure, the glutaraldehyde-treated HRP is exposed to the CT. This mixture does not contain the free cross-linking agent, and thus conjugations between CT molecules are not possible. Finally, through elution of the CT-HRP conjugate on a Sephadex G-200 column, calibrated with standards of various molecular weights, CT-HRP conjugates with molecular ratios of 1 CT/2 HRP were obtained (21).

Glycolipids

Gangliosides were isolated from bovine brain by the method of Lauter and Trams (23). GM₁ was isolated from bovine brain gangliosides by thin-layer chromatography. To prepare asialo GM₁, the gangliosides were treated with 0.1 N H₂SO₄ for 3 h at room temperature, neutralized, and partitioned by the method of Folch et al. (12). This procedure results in the production of various neutral glycolipids, which are then separated by thin-layer chromatography. Asialo GM₁ is obtained by elution of the appropriate area of the chromatograph.

Adsorption Studies

Neuroblastoma cells grown in spinner flasks were washed twice in Earle's balanced salt solution (Microbiological Associates, Walkersville, Md.) buffered with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; Schwarz/Mann), pH 7.4 (Earle's-HEPES), and counted. GM₁ was adsorbed on cells at 10 μg/ml/5 × 10⁸ cells under gentle agitation for 20 min at 4°C. After two washes with Earle's-HEPES, aliquots of 25,000 cells (0.1 ml) were pipetted into separate tubes; 0.1 ml of [¹²⁵I]CT-HRP at 10 ng/ml (10,000 cpm) was added, and tubes were shaken for periods of up to 40 min at 4°C. 5 ml of ice-cold Earle's-HEPES was added, and the cells were pelleted at 2,000 rpm for 5 min. After several washings in cold Earle's-HEPES, the supernates were discarded and the pellets counted. For inhibition studies, CT-HRP (30 mg CT/ml) was treated for 20 min at 4°C with 10 μg/ml of either asialo GM₁ or GM₁ before incubation with cells.

cAMP Assay

To test the biological potency of CT-HRP, cAMP levels were measured in chicken erythrocytes which had been treated with either native toxin or CT-HRP. Freshly drawn heparinized chicken blood was washed twice in Earle's-HEPES, and the buffy coat was removed. Cells were then exposed to exogenous GM₁ (10 μg/ml) as previously described. After washing, cells were suspended in buffer at a hematocrit of 5%. Incubations were performed for 2 h at 37°C with 1.8 μg/ml toxin and CT-HRP in amounts containing 1.8 μg/ml toxin (33). Aliquots were withdrawn, and cAMP was measured with a radioimmune assay kit obtained from New England Nuclear, Boston, Mass. In this assay, free cAMP inhibits the binding of a succinyl tyrosine [¹²⁵I]methyl ester derivative of cAMP to rabbit antisera prepared against a complex of this derivative with bovine serum albumin. After precipitation with a sheep antiserum to rabbit globulin, the ¹²⁵I in the pellet varies inversely with the amount of free cAMP in the biological sample.

Cytochemistry

HRP was detected by the method of Graham and Karnovsky (16) by utilizing diaminobenzidine tetrahy-

drochloride (DAB) at substrate. Acid phosphatase was detected by the method of Novikoff with disodium salt of cytidylic acid (CMP) as substrate (27).

Ultrastructural Studies for Binding and Internalization of CT-HRP or B-HRP

Neuroblastoma cells were plated onto Aclar strips and grown for 48-72 h to confluence before addition of GM₁ and CT-HRP or B-HRP. Cells were washed three times in Earle's-HEPES and incubated with GM₁ (10 μg/ml) in the same buffer for 15 min at 4°C. Subsequently, cells were washed with cold (4°C) Earle's-HEPES and incubated with CT-HRP or B-HRP at 30 μg toxin/ml or 30 μg B subunit/ml for 1 h at 4°C. Strips were then washed in the Earle's-HEPES and either fixed immediately for electron microscopy or placed in full culture medium at 37°C for periods for 30 min, 1, 2, 4, and 24 h. Cells were then washed in the Earle's-HEPES and fixed for 30 min with 2.5% glutaraldehyde, 1% paraformaldehyde, in 0.2 M sodium cacodylate buffer, pH 7.35, containing 0.02% CaCl₂. After fixation, Aclar strips were washed in Earle's-HEPES and stained with DAB. Cells were postfixed in 1% OsO₄ in distilled water containing 1.5% potassium ferrocyanide. The Aclar strips were infiltrated with Araldite and cells were embedded with the use of inverted gelatin capsules filled with the embedding medium (32). Cells were then processed by standard methods of sectioning and staining with uranyl and lead salts (15) and examined in a Siemens IA electron microscope.

Neuroblastoma cells grown under identical conditions except for preincubation with GM₁ were also used for the study of binding and endocytosis of CT-HRP. These cells were treated with CT-HRP, at 30 μg toxin/ml, for 1 h at 4°C before fixation for electron microscopy; for internalization studies of CT-HRP, cells were incubated for 1 h with CT-HRP at 4°C, washed with Earle's-HEPES, and placed in a full culture medium at 37°C for 30 or 90 min before fixing and processing for electron microscopy.

Light Microscope Examination of

Adsorption of CT-HRP Conjugates

Cells grown in spinner bottles were washed twice in Earle's-HEPES and resuspended at a concentration of 10⁸ cells/ml. Cells to be treated with GM₁ were incubated with 10 μg/ml GM₁ for 20 min at 4°C with occasional shaking. After washing twice with Earle's-HEPES, cells were again resuspended at 10⁸ cells/ml and incubated with CT-HRP for 1 h at 4°C. For glycolipid inhibition of CT-HRP binding, GM₁ or asialo GM₁ (10 μg/ml) was incubated with 0.1 mg/ml CT-HRP for 15 min at 4°C before addition to cells. Cells were subsequently incubated for 1 h at room temperature in Earle's-HEPES, pH 7.4, and then deposited on a glass slide with a Cytospin (Shandon Southern Products Inc., Sewickley, Pa.). Equal volumes of cell suspension (10⁶/

ml) and a 10 mg/ml solution of ovalbumin in Earle's-HEPES were mixed thoroughly, and aggregated cells were disrupted with aspirations through a 27-gauge needle; 0.2-ml aliquots of the cell suspension were centrifuged for 7 min at 1,000 rpm. Slides were dried overnight, fixed with the fixative used for electron microscopy, and stained for peroxidase as described above.

Electron Microscope Examination of Cells which Bind CT-HRP

To compare the number of neuroblastoma cells which bind CT-HRP after preincubation with GM₁ with the number which bind CT-HRP without exogenous GM₁, cells grown on Aclar strips were used. 48-72 h after cell plating, the Aclar strips were removed from the culture medium and cut in two; one-half of each of three Aclar strips was incubated with CT-HRP for 1 h at 4°C as described above, while the other half of the strip was preincubated with GM₁ before the addition of CT-HRP. Cell counts were performed on thin sections which were stained for HRP with DAB. We examined only one section per cell. 131 cells exposed to GM₁, and 149 cells not exposed to GM₁, were examined.

RESULTS

[¹²⁵I]CT-HRP that was treated with GM₁ before it was introduced into the suspension cultures did not bind to the cells. Asialo GM₁ did not prevent binding of CT-HRP to cells (Fig. 1). Preincubation of these neuroblastoma cells with GM₁ increased the time-dependent binding of [¹²⁵I]CT-HRP. This assay is more sensitive than the mor-

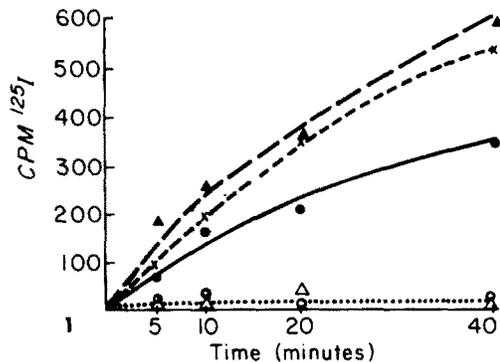


FIGURE 1 Cells in spinner flasks were incubated with 10,000 cpm [¹²⁵I]CT-HRP for times indicated and then washed. cpm of ¹²⁵I in the cell pellets were determined. ▲, cells preincubated with GM₁. ×, cells preincubated with GM₁; CT-HRP preincubated with asialo GM₁. ●, untreated cells. △, cells preincubated with GM₁; CT-HRP also preincubated with GM₁. ○, untreated cells; CT-HRP preincubated with GM₁.

phologic demonstration of CT-HRP by the peroxidase reaction with DAB as substrate, and hence lower levels of [¹²⁵I]CT-HRP were used.

Free CT as well as CT-HRP conjugate was able to induce significant increases in the levels of cAMP in avian (chicken) erythrocytes, whereas B-HRP did not (Table I).

Light microscope studies of CT-HRP uptake by cells grown in spinner culture and not treated with GM₁ showed absence of peroxidase stain (Fig. 2). In contrast, cells incubated with GM₁ showed intense stain (Fig. 3). This stain was abolished when CT-HRP was incubated with GM₁ before its use with cells; however, when CT-HRP was preincubated with the asialo derivative of GM₁, the cellular stain was intensely positive (Fig. 4).

Studies with the electron microscope: (a) Most control neuroblastoma cells did not bind CT-HRP (Fig. 5b). Of 149 sections from different cells photographed and counted, only 14, or ~9%, showed a surface stain for HRP, indicative of binding of CT-HRP. In contrast, virtually all cells pretreated with GM₁ bound CT-HRP (Fig. 5a). Of 131 sections from different cells exposed to GM₁, 126, or ~96%, bound CT-HRP. When the surface stain was present, it was similar in cells whether or not pretreated with GM₁. This stain appeared as a continuous or coarsely granular layer of oxidized DAB, 200- to 300-Å thick. This cytochemical procedure did not reveal any native, endogenous peroxidase activity in neuroblastoma cells. (b) Endocytosis. Similar sites of endocytosis of CT-HRP were observed in neuroblastoma cells, with or without exogenous GM₁ (Figs. 6 and 7). Of cells pretreated with GM₁, those with surface or endocytosed CT-HRP were abundant and were easily found in the electron microscope in random

TABLE I
Effect of Cholera Toxin and Toxin Conjugates upon cAMP Levels of Chicken Erythrocytes

Additions	Time	
	0 h	2 h
CT (1.3 μg/ml)	0.25	0.21
CT-HRP (1.8 μg CT/ml)	0.41	2.20
CT-HRP (1.8 μg Sub B/ml)	0.21	1.50
B-HRP (1.8 μg Sub B/ml)	0.22	0.25

Chicken erythrocytes were incubated for 2 h as indicated. Aliquots were withdrawn and cAMP was measured as described in Materials and Methods. Numbers are the average of four determinations. Units are nm cAMP/ml of packed cells.

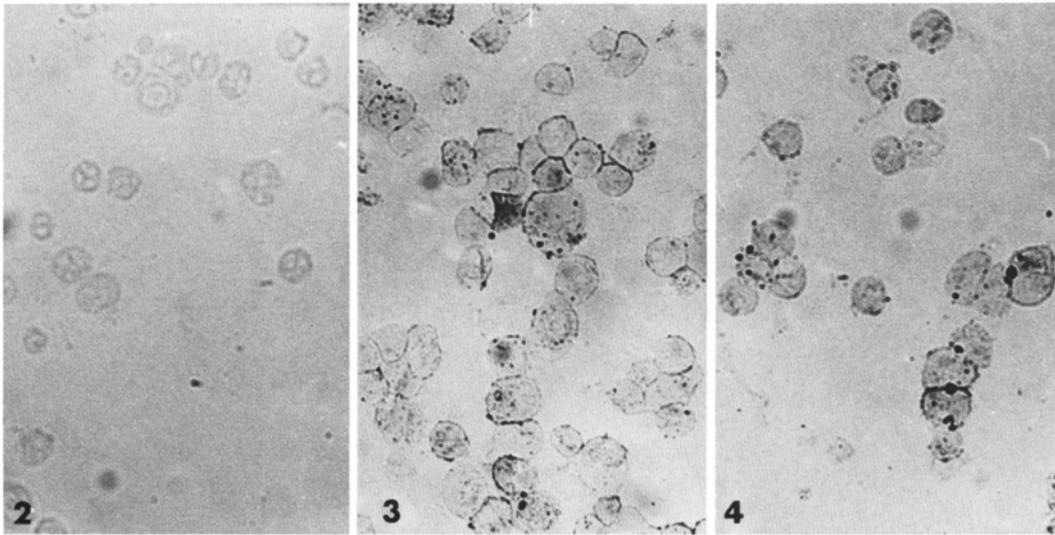


FIGURE 2 Spinner cultures of murine neuroblastoma cells incubated with 30 $\mu\text{g/ml}$ CT-HRP for 1 h at 4°C, washed, and incubated for 1 h at room temperature before cyto centrifugation, fixation, and staining with DAB. Note absence of surface and intracytoplasmic staining. $\times 75$.

FIGURE 3 Same as in Fig. 2, except that cells were preincubated with 10 $\mu\text{g/ml}$ GM_1 . Note surface and probably intracytoplasmic stain. $\times 75$.

FIGURE 4 Same as in Fig. 3, except that CT-HRP was incubated with asialo GM_1 (10 $\mu\text{g/ml}$) before it was added to cultures. Treatment of CT-HRP with asialo GM_1 did not inhibit staining. $\times 75$.

thin sections. Cells exposed to CT-HRP without previous treatment with GM_1 were few in number. Their study in the electron microscope required preselection from semi-thin sections. Cells incubated at 37°C for 30 min after initial labeling with CT-HRP showed a continuous peripheral stain of oxidized DAB and a significant endocytosis of CT-HRP (Fig. 7). The endocytosed CT-HRP was often localized in one juxtannuclear area of the cell, measuring $\sim 8\text{--}10 \mu\text{m}^2$, while most of the peripheral cytoplasm was free of structures containing peroxidase reaction product (Fig. 7). The endocytosed CT-HRP was located in round vesicles, cisternae, and occasionally in round or oval bodies. (Fig. 8). The membrane surrounding the endocytosed CT-HRP was smooth, and radial densities, characteristic of coated vesicles, were observed infrequently; coated vesicles containing CT-HRP were seen occasionally near GERL-like formations (21). Patching or capping of the surface stain was not seen. There are at least two plausible explanations for the absence of capping of CT-HRP: (a) the endogenous or exogenously introduced CT-HRP receptor (GM_1) is not suffi-

ciently large to span the entire thickness of the plasma membrane and to interact with subsurface cytoskeletal or motile systems which may be instrumental in capping (7), and (b) because of the univalent nature of the receptor GM_1 , CT-HRP molecules cannot be cross-linked with each other.

Patterns of endocytosis were essentially unchanged by prolonged incubations at 37°C, and surface staining was still present, though diminished, after 24 h. This suggests that there is either a very slow clearance of the plasma membrane-bound CT-HRP, or possibly a reinsertion (recycling?) of the ligand from the interior of the cell back to the plasma membrane. Prolonged periods of culture (1, 2, 4, and 24 h) did not significantly alter the distribution of CT-HRP, although round bodies (lysosomes?) did appear more strongly stained after longer periods of exposure to CT-HRP. However, even after 24 h at 37°C, the prominent sites of endocytosed HRP were smooth vesicles and tubules, $\sim 200\text{--}700 \text{ \AA}$ in diam, or cisternae of indeterminate length (Fig. 8). Neuroblastoma cells with a well-developed Golgi apparatus had endocytosed CT-HRP in vesicles or

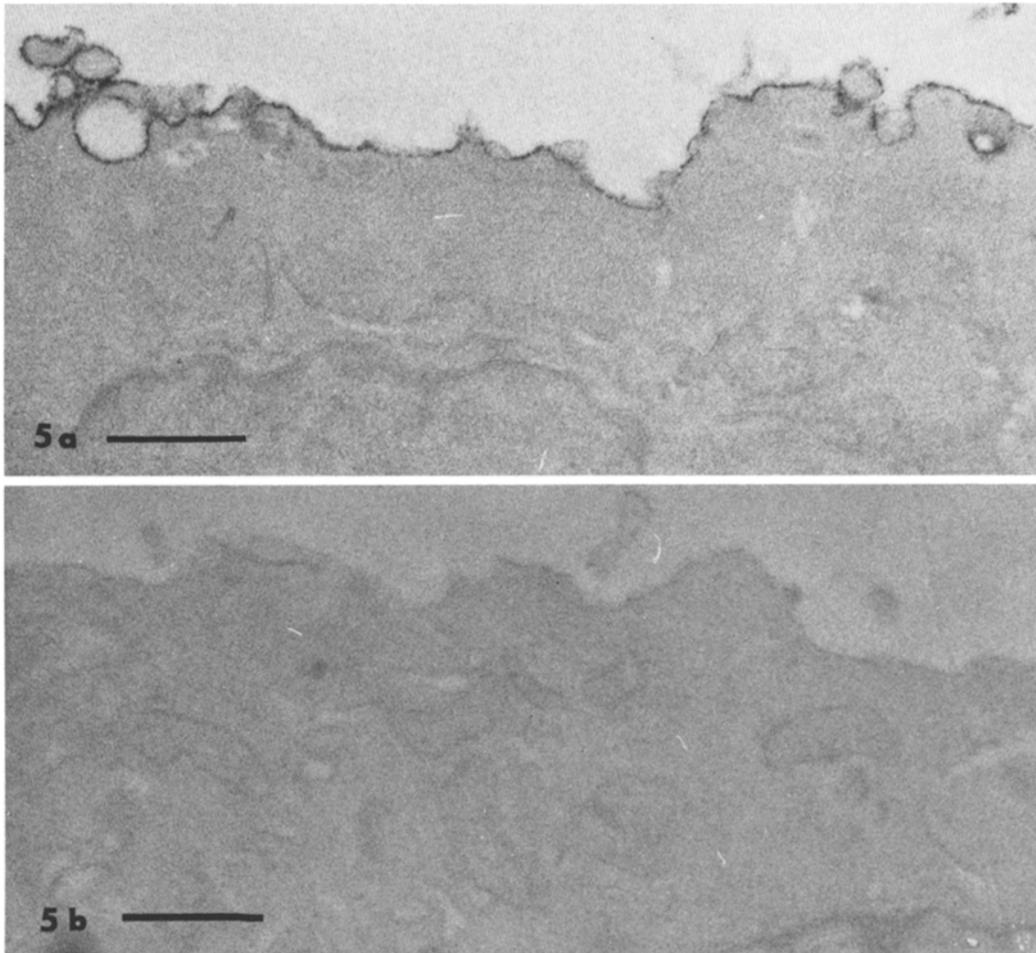


FIGURE 5 (a) Thin section of cell preadsorbed with GM_1 , labeled with CT-HRP for 1 h at $4^\circ C$, and stained with DAB. $\times 18,000$. (b) Thin section of cell labeled with CT-HRP for 1 h at $4^\circ C$ and stained with DAB. Bar, $1 \mu m$. $\times 18,000$.

tubules near one aspect of the Golgi apparatus; this arrangement of organelles resembles GERL of normal neurons (Fig. 8) (15, 21). When stained for acid phosphatase, neuroblastoma cells which had endocytosed CT-HRP, or neuroblastoma cells which were not exposed to CT-HRP, did not show the typical network of GERL of normal neurons as revealed by the acid phosphatase reaction (15). Only lysosomes in neuroblastoma cells were positive for acid phosphatase.

Endocytosis of Subunit B of CT

Surface labeling and endocytic sites of the conjugate of HRP with subunit B of CT were essentially similar to the results obtained with the HRP

conjugate with the entire toxin (Fig. 9). Abundant endocytosis of B-HRP in vesicles or tubules was seen after 30 min in $37^\circ C$. In addition, larger round or oval endocytic sites of B-HRP, $\sim 0.75\text{-}\mu m$ in diam, were seen (Fig. 9). These endocytic vacuoles had often a lucent center and a peroxidase-positive rim of varying thickness.

Mitotic Cells

Cells in metaphase showed endocytosis of CT-HRP in predominantly tubular or vesicular structures which displayed characteristic polarity (Fig. 10). The endocytic vesicles and tubules were clustered near the centriole from which spindle tubules radiated towards the chromosomes (Fig.

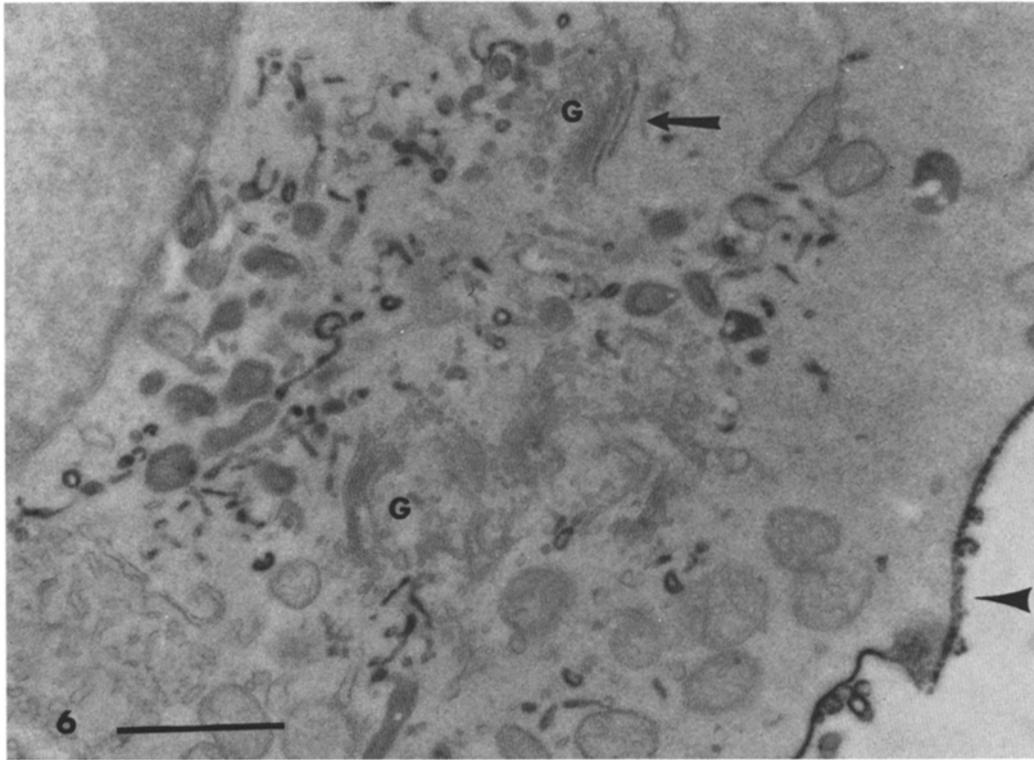


FIGURE 6 In this preparation, cells were incubated with CT-HRP for 1 h at 4°C, washed, and incubated for 90 min in full culture medium before fixation and staining for HRP with DAB. *G*, unstained Golgi apparatus. Arrow, HRP-stained cisterns near Golgi apparatus. Arrowhead, surface stain. Bar, 1 μ m. \times 22,000.

10). The limiting membrane of the peroxidase-positive vacuole was smooth. These cells were incubated at 37°C for 2 h after their initial labeling with CT-HRP at 4°C; therefore, most likely, the endocytosed CT-HRP was bound by either late interphase or early prophase cells.

DISCUSSION

Previous studies from this laboratory utilized conjugates of toxic lectins with HRP; the absorptive endocytosis of those conjugates into GERL is probably not related to a pathological effect because CT is similarly endocytosed but has no apparent cellular toxicity (4). In our cell system, conjugates of CT with HRP had no apparent toxic effect on the viability of cells as judged by morphologic criteria. A role for activated adenyl cyclase in the internalization process can be discounted because the conjugate of the inactive subunit B of CT and HRP was endocytosed the

same way as the complete toxin-HRP conjugate. This is consistent with the results of Stossel et al., who did not find changes in cAMP levels in actively phagocytic cells (37).

Recently, we reported that 12.5–15.0% of neuroblastoma cells in suspension were adsorbed onto magnetic microspheres coated with CT (21). In the present study, 9% of neuroblastoma cells grown on Aclar strips and exposed to CT-HRP bound CT-HRP, as judged by the peroxidase reaction. However, almost all neuroblastoma cells grown as described above and preincubated with GM₁ bound CT-HRP as shown by a strong surface peroxidase reaction of 96% of GM₁-treated cells. The apparent slight discrepancy between a previous study in which neuroblastoma cells in suspension were exposed to CT-coated magnetic microspheres and this study in which adherent cells exposed to CT-HRP were used is probably a result of different methods employed for the detection of CT binding and to different culture

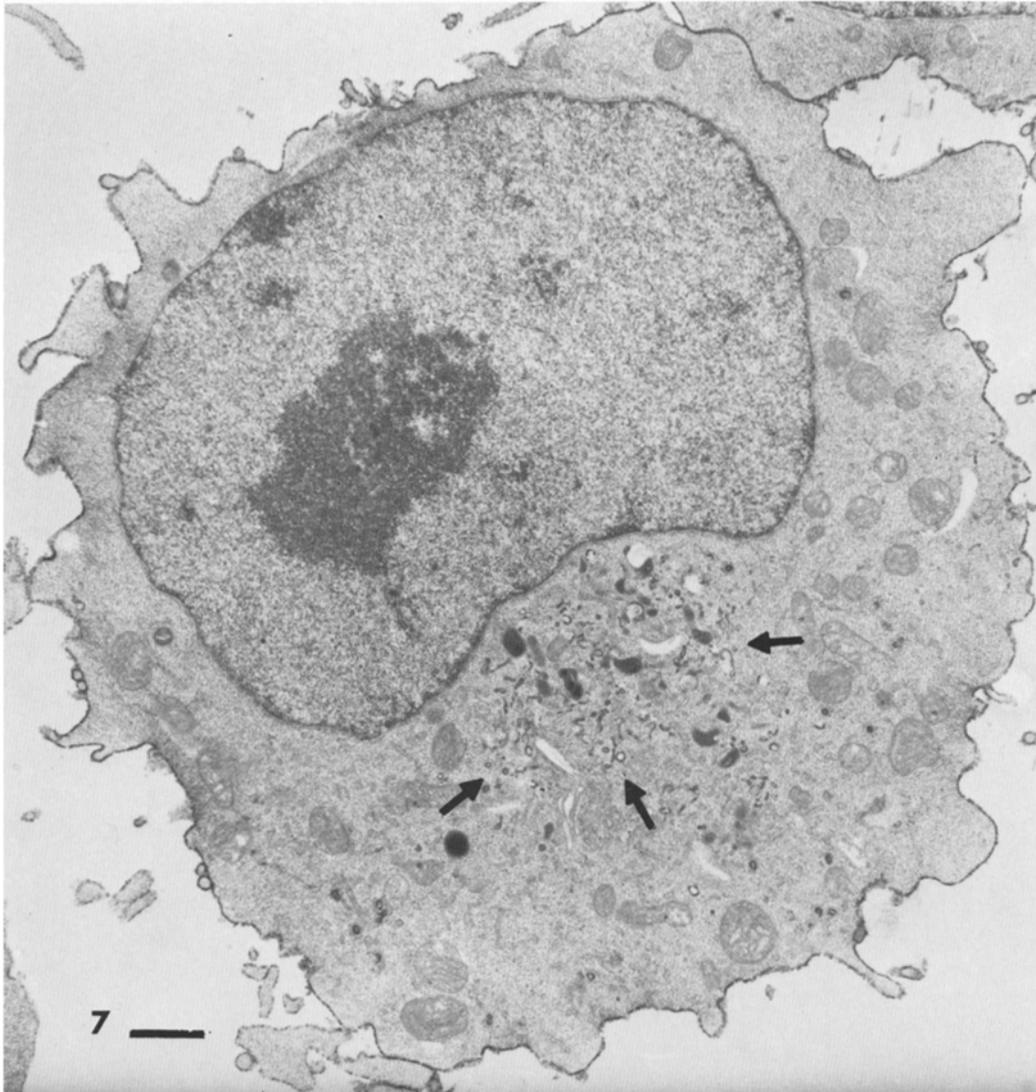


FIGURE 7 Cells were preadsorbed with GM₁, labeled with CT-HRP for 1 h at 4°C, washed, and incubated for 30 min at 37°C in full culture medium before fixation and staining for HRP and DAB. Note continuous surface stain of oxidized DAB. Arrows, focal juxtannuclear endocytosis of CT-HRP in the form of vesicles, tubules, and-possibly-round bodies. Stained with uranyl acetate and lead citrate. Bar, 1 μm. × 10,000.

conditions of the neuroblastoma cells. Similarly, the discrepancy between the significant uptake of [¹²⁵I]CT-HRP by cells not preincubated with GM₁ as judged by radioactivity counts (Fig. 1), and the lack of uptake of sufficient CT-HRP by neuroblastoma cells not preincubated with GM₁, as judged by a positive cytochemical demonstration of HRP (Fig. 3), is probably a result of the higher sensitiv-

ity of radioactivity counting of [¹²⁵I]CT-HRP over the cytochemically demonstrable HRP. While preabsorption with GM₁ was necessary, in most neuroblastoma cells, for the cytochemical demonstration of surface binding and endocytosis of CT-HRP, preabsorption with GM₁ was not needed in normal cultured neurons for a cytochemical demonstration of CT-HRP (21). The described differ-

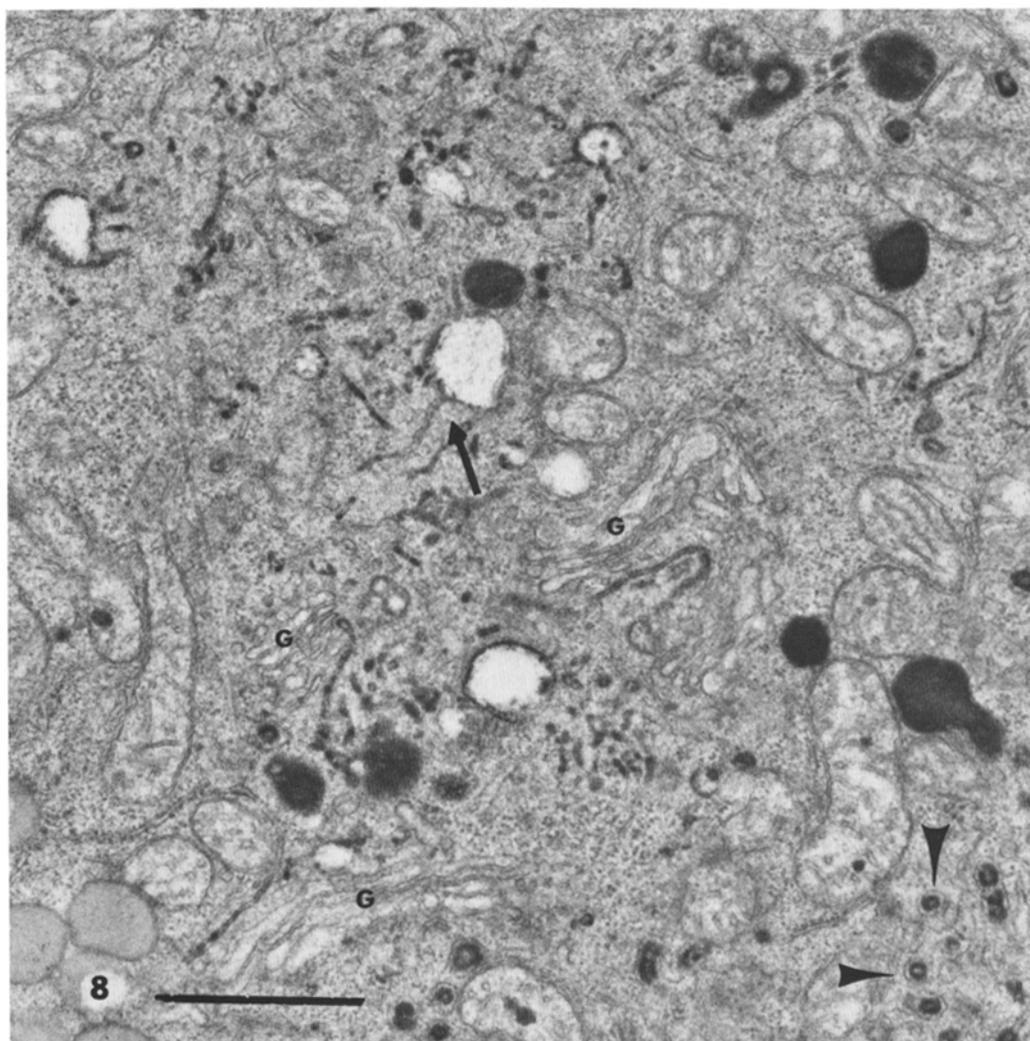


FIGURE 8 Cells preadsorbed with GM₁, incubated with CT-HRP for 1 h at 4°C, washed, and incubated in full culture medium for 24 h at 37°C before fixation and staining with DAB. Endocytic sites are in the form of clusters of tubules or vesicles near unstained cisternae of the Golgi apparatus. G, larger, empty-appearing vacuoles show peripheral stain. Arrow, suggestive fusion of cisterna with large empty vacuole. Arrowheads, intracisternal type A virus particles commonly found in murine neuroblastoma. Bar, 1 μm.

ence of uptake of CT-HRP by neuroblastoma cells and normal neurons is probably related to differences in the ganglioside compositions of plasma membranes of normal and neoplastic cells or to a progressive loss of CT binding by neoplastic cells in culture as shown by Manuelidis and Manuelidis (24).

The increased uptake of CT-HRP by neuroblastoma cells pretreated with GM₁ is consistent with the view expressed by Moss et al. that exogenous

GM₁ can be “functionally integrated into the surface membrane of intact cells and serve as the cholera toxin receptor” (25). In that regard, it should be emphasized that the endocytosis of CT-HRP occurred in the same cytoplasmic sites in both GM₁-treated and untreated cells (Fig. 7 and 8).

During the last years, several investigators have studied, with different marker molecules, fluid-phase (bulk) and adsorptive endocytosis in a num-

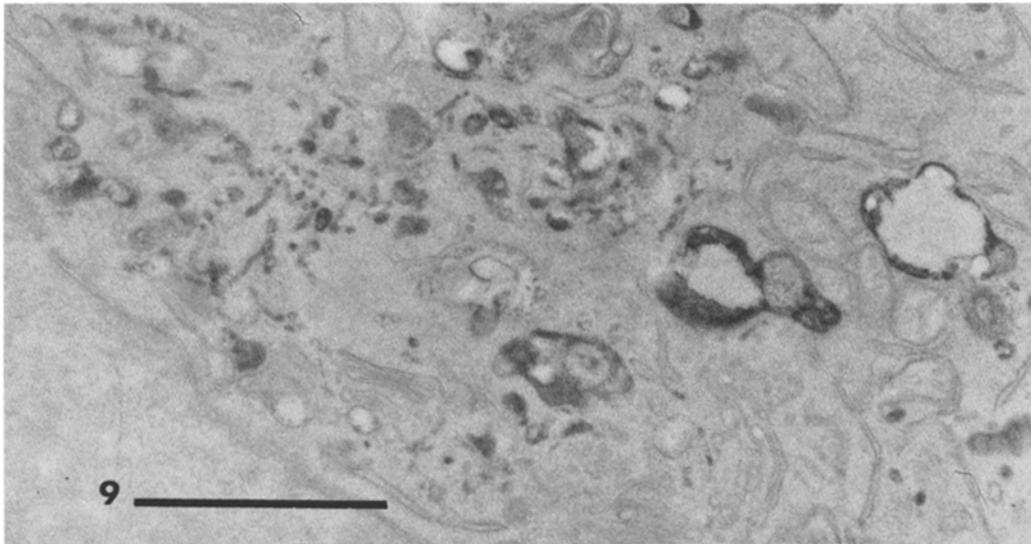


FIGURE 9 Murine neuroblastoma. Preadsorbed with GM₁. Endocytosis of B-HRP for 2 h at 37°C after initial labeling for 1 h at 4°C. Endocytosed conjugates in vesicles or tubules are basically similar to endocytosed CT-HRP. Bar, 1 μm. × 33,000.

ber of *in vitro* and *in vivo* cells or tissues (1, 2, 5, 8–10, 14, 15, 18, 19, 21, 30, 31, 34, 35, 40).

Fluid-phase endocytosis results in uptake of soluble marker molecules into coated vesicles, dense bodies, and multivesicular bodies (9, 19, 30, 35, 36, 40). Fluid-phase endocytosed HRP or reovirus coat protein, for example, appear to be entirely delivered to a degradative compartment within hours (34).

Vesicles with short linear protrusions or “bristles” toward the cytoplasm (coated vesicles) have been implicated in: (a) the uptake of proteins and low density lipoproteins (LDL) by adsorptive endocytosis, (b) in secretion, (c) in the formation of new plasma membrane, and (d) in lysosomal functions (reviewed by Silverstein et al., 35). Coated vesicles did not partake in the adsorptive endocytosis of CT-HRP by neuroblastoma cells; in a previous study of adsorptive endocytosis of lectin-HRP conjugates by normal neurons, coated vesicles, “pinching off” from cisterns of GERL, contained endocytosed lectin-HRP (15). Thus, the contribution of coated vesicles in adsorptive endocytosis probably depends on the kind of ligand and on the cell type.

Peroxidase conjugates with certain ligands binding to plasma membrane receptors of mammalian cells are internalized into the region known as GERL (14, 15, 21); GERL is a cytoplasmic

vesicular and tubular system which contains acid phosphatase (26, 28, 29). The system includes saccules and vesicles near the trans aspect of the Golgi apparatus, but not the Golgi apparatus *per se*. Recently, a role for GERL in cellular secretory processes was suggested by Novikoff et al. (27, 28), who concluded that nascent secretory granules (condensing vacuoles) are extended portions of GERL. No connections between GERL and the Golgi apparatus were seen (27, 28). Hand and Oliver (17) have studied peroxidase-secreting acinar cells of the rat exorbital lacrimal gland and also found secretory granules attached to GERL but not to the Golgi apparatus. In fact, the Golgi apparatus was negative for peroxidase (17). This work may be consistent with the finding by Jamieson and Palade (20) that involvement of the Golgi apparatus in the packaging of secretory proteins appears to be minimal in the nonstimulated state.

While the variety of marker molecules and of cells or tissues used by several investigators in the study of adsorptive or fluid-phase endocytosis precludes any generalizations, certain significant concepts have emerged from these investigations. (a) Molecules which bind on cell surfaces are internalized more quickly and in far greater amounts than molecules such as HRP which lack affinities for cell surfaces (10, 35); (b) The intracellular fate of an endocytosed molecule is influ-

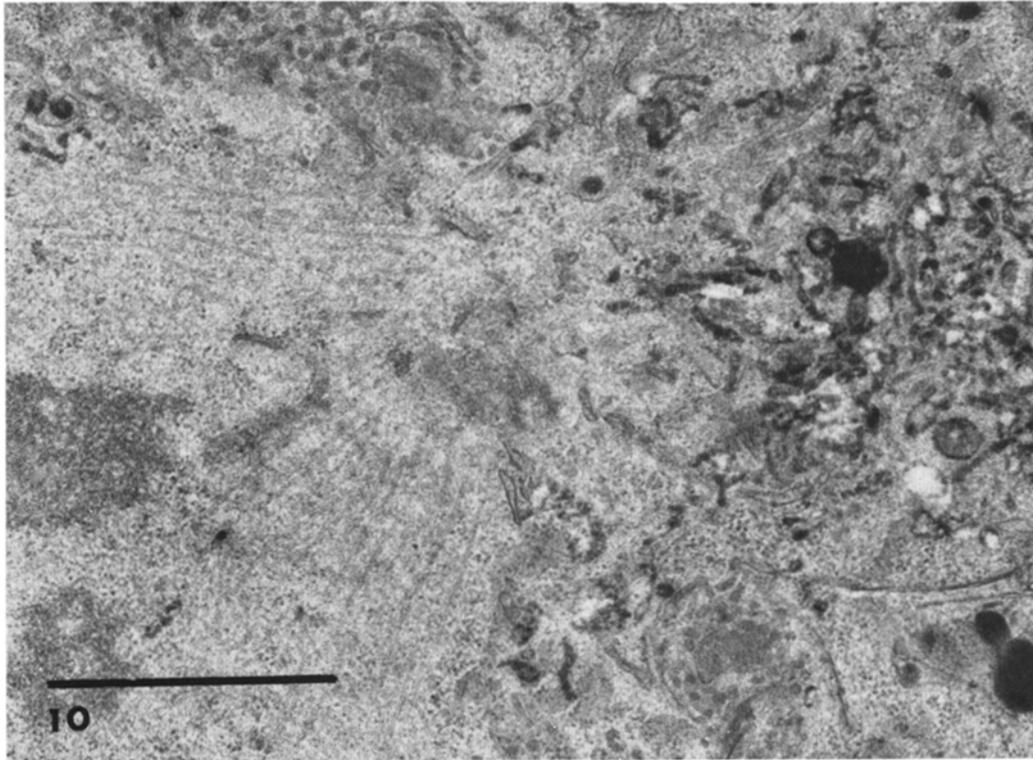


FIGURE 10 From a metaphase cell. The aggregation of endocytosed CT-HRP in vesicles or tubules near the centriole is clearly seen. Note radiating spindle tubules. Stained with uranyl acetate and lead citrate. Bar, 1 μm . $\times 38,000$.

enced by its affinity with plasma membrane receptors, its electric charge, and possibly by other factors such as the functional state of the cells or tissues (9, 18, 19, 30, 31); (c) Cisternae of the Golgi apparatus, or elements of GERL, are recipients of several different molecules in various cell types, both in vivo and in vitro (2, 5, 8, 9, 10, 14, 15, 18, 19, 21, 31, 40).

The functional implications of the endocytosis of various ligands or marker molecules in the Golgi apparatus or GERL are unknown, and the following two hypotheses have been advanced. (a) Novikoff and Novikoff, and Hand and Oliver believe that GERL is involved in the formation of secretory granules (17, 28); Novikoff and Novikoff have suggested that GERL is participating in the degradation of endocytosed plasma membrane moieties (28). (b) Herzog and Farquhar have suggested that some of the endocytosed plasma membrane is "retrieved" in the Golgi cisternae for possible recycling into the membranes of secretory granules (18). In this regard, a probably

relevant finding in the present work and in a previous study is the persistence of CT-HRP not only in GERL of normal and possibly of neoplastic neurons after 24 h at 37°C but also upon the cell surface of these cells. The massive and persistent nature of the internalization of CT-HRP into GERL, coupled with studies implicating GERL in secretory processes (28), tempts one to speculate that if recycling of plasma membrane components does occur, GERL may be the site at which endocytosed material enters the secretory process.

A possible explanation for the accumulation of CT-HRP in GERL-like structures of neuroblastoma cells is the failure of fusion of the endocytic vacuoles with primary or secondary lysosomes. This hypothesis has been previously introduced and may be relevant to the endocytosis of CT-HRP (35). On the other hand, GERL may serve as a central locus for the accumulation of membrane-derived moieties for subsequent reutilization, reassembly, or degradation. The isolation and biochemical study of GERL and the develop-

ment of new specific and sensitive cytochemical stains for various enzyme markers of plasma membranes, Golgi apparatus, lysosomes, and endoplasmic reticulum may further clarify the functional role of GERL.

Endocytosis of CT-HRP in Cells in Metaphase

In an earlier study, we observed a prominent polar aggregation of smooth membrane-bounded polymorphic cisternae in HeLa cells in metaphase (32, Fig. 17). These cisternae correspond strikingly to sites of endocytosis of CT-HRP in metaphase neuroblastoma cells (Fig. 10). These sites of endocytosis of plasma membrane-bound moieties (in this case, probably complexes of GM₁-CT-HRP) may represent a specialized route for internalization of plasma membrane moieties for degradation or reutilization, and possibly for the reassembly of the Golgi apparatus or other cellular membranes in the daughter cells.

This work was supported by grants NS-05572-14 and GM-0092-02 of the National Institutes of Health.

Received for publication 26 June 1978, and in revised form 26 January 1979.

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