of 1.0 μ1/10^6 cells is used (*15*), the estimated PRPP concentration in cells incubated with 0.1 m*M* glutamine is in the range of the Michaelis constant (K_M) for PRPP, 7 μM, observed with purified APRT (*16*). Thus, a subsaturating concentration of PRPP could account for the diminished rate of adenine conversion to nucleotide indicated in Fig. 1. Most other PRPP-utilizing enzymes involved in purine and pyrimidine reutilization and de novo synthesis have higher K_M values for PRPP than does APRT (*5*, pp. 105, 128, 178). This suggests that glutamine limitation may result in a decrease in availability of PRPP for these reactions.

In studies with an HGPRT-deficient line similar to those with WI-L2 described in Fig. 1, 50 μM inosine stimulated adenine conversion to nucleotide in cultures supplemented with 0.1 m*M* glutamine to rates equivalent to those in cultures with 2 m*M* glutamine. The specificity of stimulation of adenine conversion is indicated in Fig. 2. Only substrates of purine nucleoside phosphorylase, namely, inosine and guanosine, stimulated adenine conversion to nucleotide. Guanosine could not overcome the glutamine growth limitation in WI-L2, probably because of toxicity specifically related to guanosine or guanine (*17*). As with other mammalian cells (*5*, pp. 87–88), an active pathway for conversion of these purine nucleosides to PRPP via the intermediates ribose-1-phosphate and ribose-5-phosphate can be observed in supernatants prepared by 100,000*g* centrifugation of extracts from cultured human lymphoblasts. Since (i) purine nucleosides alone are sufficient for maximal stimulation of adenine conversion to nucleotide in glutamine-limited cultures and (ii) in other studies purine synthesis de novo in an HGPRT-deficient cell line was nearly maximal at concentrations of 0.1 to 0.2 m*M* (*18*) if an inosine supplement greater than 10 μM was also present, glutamine limitations may give rise to a lack of availability of a precursor of PRPP, such as ribose-5-phosphate.

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8 March 1976

Cholera Toxin–Peroxidase: Changes in Surface Labeling of Glioblastoma Cells with Increased Time in Tissue Culture

Abstract. *Cholera toxin coupled to peroxidase yielded a highly specific ultrastructural marker of plasma membrane monosialogangliosides. Studies with cultures of brain and brain tumors suggested that long-term culture of tissue in monolayers results in eventual loss of surface monosialogangliosides.*

Fractionation of cells both from brain and from other organs suggests that gangliosides are a constituent of membranes and possibly serve as receptor molecules on the cell surface. Gangliosides are abundant in the central nervous system (CNS). The ultrastructural localization of gangliosides to the plasma membranes of intact CNS cells has not been investigated. In our study we have coupled peroxidase directly to cholera toxin, which specifically binds to the monosialoganglioside G_{M1} (*1*), in order to investigate the distribution of G_{M1} at the surfaces of normal cells and of tumor cells derived from the CNS. We now describe a human glioblastoma tumor line that showed alterations in cholera binding with increasing time in tissue culture.

Highly purified cholera toxin (*2*) was coupled to peroxidase (type VI, Sigma) with glutaraldehyde as the cross-linking agent (*3*). The conjugate (cholera-peroxidase) displayed a single peak on G-22 Sephadex chromatography, and neither free peroxidase nor free cholera toxin was detectable.

Glioblastoma TC 593 cell line was initiated from a human surgical specimen and was maintained in monolayer culture for 14 months (*4*), the cells were frozen and stored in liquid nitrogen for 7½ years and started again in monolayer culture for our experiments. Cholera binding was first tested 2 months after cells were taken from storage, again tested after 7 months and 8 months. Conjugated cholera-peroxidase was added to monolayers of cells in the log phase of growth at a density of about 80 percent after they had been washed several times in phosphate-buffered saline (PBS). Intact cells were exposed to the conjugate for 15 minutes at 22°C, and unbound material was washed off with excess PBS (three washings for 5 minutes each) prior to fixation of the intact monolayer with glutaraldehyde (*5*). In order to demonstrate specificity of the conjugated cholera toxin, the following experiments were done. (i) Excess human ganglioside or purified fractions of human ganglioside (G_{M1} and G_{D1a}) were added to the incubation medium containing cholera-peroxidase in order to prevent binding of cholera-peroxidase to the cell surface. The G_{D1a} was used because it is a disialoganglioside, and thus it should not be as effective as G_{M1} in binding cholera-peroxidase. (ii) Unlabeled toxin was added to the incubation medium to test whether all cholera-peroxidase could be prevented from binding to the cell surface. (iii) The TC 593 cells were first treated with ganglioside fractions and then washed with PBS in order to enhance the binding of cholera-peroxidase when cells were subsequently exposed to the conjugate. This experiment was based on studies showing that fat cells can take up into their plasma membranes exogenously applied ganglioside (*6*). (iv) ^{125}I-Labeled cholera toxin (specific activity 15 μc/μg) was also used to see whether autoradiographic findings

were compatible with studies in which cholera-peroxidase was used. In these experiments excess G_{M1}, unfractionated gangliosides, and unlabeled toxin completely abolished binding of both cholera-peroxidase and ^{125}I-labeled cholera toxin, whereas addition of G_{D1a} had no effect on either peroxidase or labeled toxin (Figs. 1 and 2). No background peroxidase was detected on the cell surface in any of these inhibition experiments. Furthermore, prior treatment of cells with G_{M1} (but not G_{D1a}) increased the binding of conjugate at the cell surface. On the basis of these experiments, we concluded that cholera-peroxidase retained the specificity for G_{M1} that is characteristic of native toxin.

At 2 months (Table 1A) more than 50 percent of the cells of TC 593 revealed intense binding of cholera-peroxidase, and another 25 percent showed weak but detectable binding. Only 14 percent of the cells at this time were completely unlabeled when counts were made with the electron microscope, which is more sensitive for detection of peroxidase. Cells binding cholera-peroxidase, in contrast to those inhibited either by excess ganglioside or unlabeled toxin, showed increased uptake of peroxidase in pinocytotic vesicles (Fig. 1). At 7 months (Table 1B) only a few cells displayed intense labeling; intensely labeled cells now accounted for less than 15 percent of the population. Furthermore, there was a clear-cut increase in cells completely devoid of label, and these now accounted for more than 50 percent of the population. At 8 months (Table 1C) the results were similar; there was a slight increase in unlabeled cells. Autoradiographic studies with ^{125}I at 8 months (Table 1D) showed results similar to those obtained with cholera-peroxidase, and many unlabeled cells were seen; only a few intensely labeled cells were observed, and the numbers were in fair agreement with the number observed with cholera-peroxidase during the same culture period.

No obvious differences were observed in binding of cholera-peroxidase by metaphase cells as compared to interphase cells, and both could be heavily labeled (Fig. 1, A and B). In the experiments at 7 and 8 months both with cholera-peroxidase and with ^{125}I-labeled cholera, unlabeled metaphases were more frequent and thus followed the pattern of the interphase cells. When different bottles of cells grown to different cell densities were sampled, comparable labeling of the cell surface characteristic for that culture period was observed. In all cases under the conditions used, the exposed cell surface was entirely labeled and there was no clustering or aggregation of cholera-peroxidase or ^{125}I-labeled toxin, nor were special regions of the cell surface (as those containing numerous microvilli) preferentially labeled.

Although biochemical analyses of brain show gangliosides enriched in cell membrane fractions, our method demonstrates ultrastructural localization of a specific ganglioside to the plasma membrane of cells derived from the CNS.

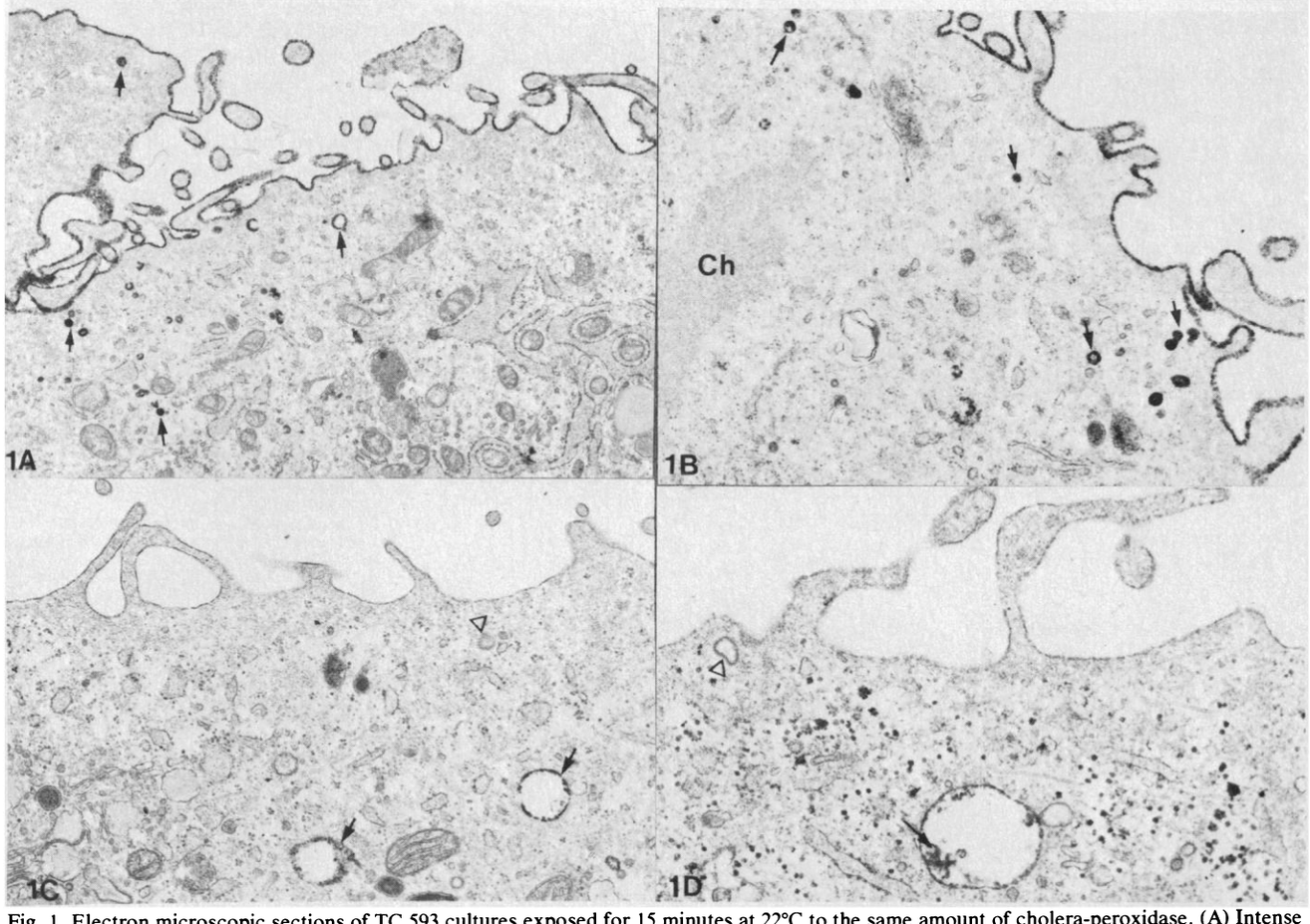

Fig. 1. Electron microscopic sections of TC 593 cultures exposed for 15 minutes at 22°C to the same amount of cholera-peroxidase. (A) Intense peroxidase reaction seen on surface of interphase cell at 2 months. Note many pinocytotic vesicles contain peroxidase (arrows) (× 10,500). (B) Metaphase cell at 2 months shows equally intense surface reaction as interphase cell. *Ch* is chromosome, arrows show uptake into pinocytotic vesicles (× 17,250). (C) Cell exposed to cholera-peroxidase plus a fivefold concentration of unlabeled cholera toxin. There is no surface label; peroxidase, however, is visible in two large vacuoles (arrows) but not in all pinocytotic vesicles (triangle) (× 17,250). (D) Cell exposed to cholera-peroxidase plus 130 μg of whole human ganglioside. No reaction production is visible on the surface; peroxidase is in large vacuole (arrow) but not in coated vesicle near surface of cell (triangle) (× 23,500).

Thus this reagent might be useful as a marker of plasma membrane gangliosides in physiological and biochemical studies. Peroxidase-labeled antibodies to cholera toxin have been used for ultrastructural labeling of intestinal surfaces (*7*); our direct coupling of toxin avoids the production of antibodies and the subsequent purification of antibody moieties specific to cholera. More intriguing than this technological aspect, however, is the apparent loss of G_{M1} on the surface of these glioblastoma cells with more prolonged tissue culture. We do not know whether any of the changes in this line were caused by initial freezing for storage or whether such changes represent an overgrowth by particular cells as compared to eventual loss of ganglioside by all cells in the culture. However, another long-term human glioblastoma line (TC 526) that we have examined with cholera-peroxidase after propagating it for 10 years in continuous subculture showed virtually no labeling of its plasma membranes at any time or cell density. Extensive searches for mycoplasma or viruses, sometimes associated with glycolipid changes in other cells, in TC 526 as in TC 593, were reproducibly negative (*8*). Furthermore, biochemical studies on TC 526 and other long-term glioblastomas have shown reduced ganglioside levels and reduction in labeling by cholera toxin–peroxidase (*9*). Conversely, differentiating short-term organotypic embryonic CNS cultures displayed very intense labeling of all exposed cells (*10*). Possibly, some glioblastomas in long-term tissue culture where no detectable binding of cholera-peroxidase was seen did not initially have a large complement of surface gangliosides. However, a second view, made more likely and attractive by the evolution of TC 593 in culture, is that the conditions of long-term monolayer culture per se can affect the gangliosides in the plasma membranes. In this context it is worth mentioning that, after serial propagation of various glioblastomas in monolayer culture, a change in morphology of tumor cells was observed (*11*). The change in surface gangliosides, as described here for TC 593, may be related to such altered patterns of growth.

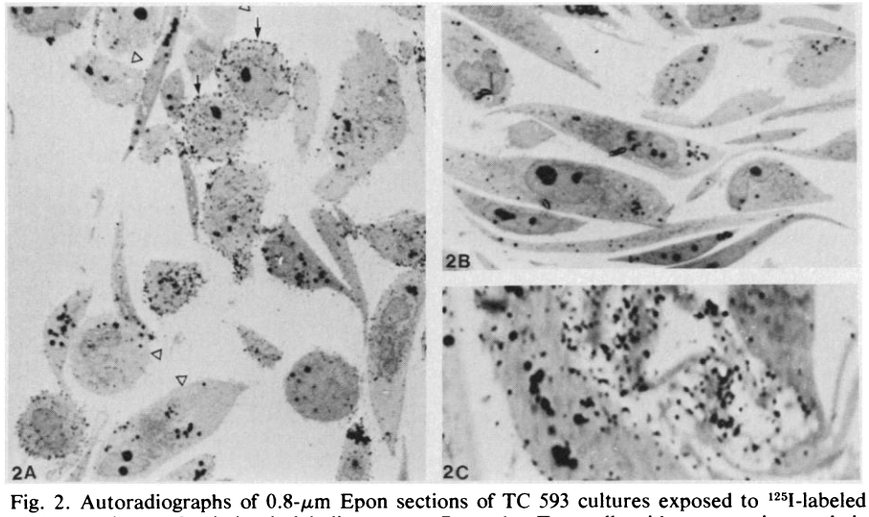

Fig. 2. Autoradiographs of 0.8-μm Epon sections of TC 593 cultures exposed to ^{125}I-labeled cholera toxin. (A) Variation in labeling seen at 7 months. Two cells with many grains on their surfaces (arrows), as well as several cells completely devoid of label (triangles), are seen in this field (× 1,370). (B) Culture exposed to ^{125}I-labeled cholera plus 5.5 μg of human G_{M1}. No grains are visible on cell surface (× 2,000). (C) Culture exposed to ^{125}I-labeled cholera plus 5 μg of human G_{Dia}. Grains are still visible on surface of cells (× 2,560).

Table 1. Cholera toxin–peroxidase or ^{125}I-labeled cholera toxin of TC 593 at different times. The percentage of reaction was judged by the labeling observed by light microscopy (LM) and electron microscopy (EM). More than 100 cells were counted for each determination.

Substance	Reaction (percent)		
	Intense	Weak	None
A. Labeling at 2 months			
Cholera-peroxidase			
LM	51	26	23
EM	61	25	14
Control:			
Cholera-peroxidase plus ganglioside			
LM	0	0	100
EM	0	0	100
B. Labeling at 7 months			
Cholera-peroxidase			
EM	13	36	51
C. Labeling at 8 months			
Cholera-peroxidase			
LM	10	23	67
EM	11	33	56
Control:			
Cholera-peroxidase plus unlabeled toxin			
EM	0	0	100
D. ^{125}I-Labeled cholera toxin at 8 months			
LM	10	20	70
Control:			
^{125}I-labeled Cholera plus G_{M1}.			
LM	0	0	100

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12. Supported by PHS grants CA 15044-03 and NS 06209 and a grant from the Esther A. and Joseph Klingenstein Fund, Inc. L.M. is the recipient of an NIH grant, RCDA 1 K04 00101-01. The technical assistance of R. Nicholes and P. Johnson is appreciated; we thank R. Yu for the gift of G_{M1} and G_{Dia} and G. Van Bennett for ^{125}I-labeled cholera toxin.

2 February 1976