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The B Subunit of Cholera Toxin Enhances DNA Synthesis in Rat Hepatocytes Induced by Insulin and Epidermal Growth Factor[1]

Hiroshi Mitsui, Masao Iwamori*, Naoaki Hashimoto, Haruki Yamada, Yusei Ikeda, Gotaro Toda, Kiyoshi Kurokawa, and Yoshitaka Nagai*

First Department of Internal Medicine, *Department of Biochemistry, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113

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The B subunit of cholera toxin, which binds to ganglioside GM1, enhanced DNA synthesis in rat hepatocytes in primary culture induced by insulin and/or epidermal growth factor. The effect was dose-dependent, and whole cholera toxin, activating adenylate cyclase, showed a higher effect than the B subunit alone. The B subunit acted additively with other agents that also increase cyclic AMP levels. A competitive antagonist of cyclic AMP could not suppress the effect of the B subunit completely. These data suggest that the effect is independent of the cyclic AMP signal pathway, and that GM1 plays a role in hepatocyte proliferation.

Gangliosides and other cell surface glycosphingolipids change in association with the proliferation, differentiation and carcinogenesis of many types of cells (1). Recent studies have demonstrated that gangliosides (especially GM1 and GM3) and their metabolites might directly regulate cell growth (2,3,4).

Cholera toxin (CT) binds by its B subunit to ganglioside GM1 on the plasma membrane, and then the penetrating A subunit activates adenylate cyclase (5). CT has a mitogenic effect on many cells *in vitro* as well as *in vivo*. Recently it has been

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Abbreviations: CT, cholera toxin; CT-B, the B subunit of cholera toxin; GM1, Galβ1-3GalNAcβ1-4[NeuAcα2-3]Galβ1-4Glc1-1Cer; GM3, NeuAcα2-3Galβ1-4Glc1-1Cer; GD1b, Galβ1-3GalNAcβ1-4[NeuAcα2-8NeuAcα2-3]Galβ1-4Glc1-1Cer; EGF, epidermal growth factor; AMP, adenosine mono-phosphate; TLC, thin layer chromatography.

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demonstrated that the B subunit (CT-B) is mitogenic for rat thymocytes (6), quiescent 3T3 fibroblasts (7) and rat thyroid FRTL-5 cells (8), independent of cyclic AMP.

It is well known that insulin and epidermal growth factor (EGF) induce DNA synthesis in primary cultures of rat hepatocytes. CT, like glucagon, is thought to potentiate the DNA synthesis through the cyclic AMP signal pathway (9). In this study, we examined whether CT-B was able to enhance hepatocyte DNA synthesis induced by insulin and EGF through a pathway other than the cyclic AMP-mediated signal transduction system.

Materials and Methods

Reagents. Cholera toxin (CT) and glucagon were purchased from Sigma (St.Louis, MO), and the B subunit of CT from Schwarz/Mann Biotech (Cleveland, OH). Rp-cAMPS was obtained from Biolog (F.R.G.), insulin and EGF from Collaborative Research (Bedford, MA), forskolin from Research Biochemicals (Natick, MA), and collagenase from Wako Chemical Industries (Osaka, Japan).

Hepatocyte culture. Hepatocytes were isolated from rats (Wistar strain, male, 200 g body weight) by collagenase perfusion (10). The isolated hepatocytes, with a viability of over 90%, were suspended in Williams' medium E (WE) containing 5% fetal calf serum and seeded into plastic wells (35 mm diameter, Corning) at a density of 5 x 10^5cells/well. After incubation at 37°C for 24 h under 5% CO_2, the medium was changed to serum-free WE containing insulin, EGF and CT-B or other compounds. Attachment efficiency at 24 h after plating was about 50%, as estimated from photos.

Assay of DNA synthesis. Hepatocytes, 24 h after addition of growth factors, were labeled with [^{3}H]thymidine (2 μCi/well) for 2 h and the radioactivity incorporated was measured in the insoluble fraction precipitated with trichloroacetic acid. Protein was determined by the method of Bradford (11). The background value of [^{3}H]thymidine incorporation was obtained in the presence of 10 mM hydroxyurea. DNA synthesis was represented as cpm/ug protein/h.

Preparation and TLC immunostaining of gangliosides. Gangliosides were extracted from rat hepatocytes as described previously (12). For TLC immunostaining, acidic glycolipids were developed on a silica gel-coated plastic plate, and the plate was then incubated successively with CT-B, rabbit anti-CT-B antibody and peroxidase-conjugated anti-rabbit IgG antibody (Cappel, Malvern, PA) as reported previously (13). Immunostaining kit (Konica, Japan) was used for detection of peroxidase remaining on the plate. The content of GM1 was assessed by measuring the density of spots at 580 nm with a dual-wavelength TLC densitometer (Shimadzu, Japan).

Results

By TLC immunostaining it was ascertained that CT-B was bound to ganglioside GM1 from rat hepatocytes (Fig. 1). Rat hepatocytes contained about 6 pmol of GM1/10^5cells, this value being compatible with that obtained by Scatchard analysis (14). In rat hepatocytes, another ganglioside GD1b was also detected for the potent receptor for CT-B.

CT-B enhanced the DNA synthesis in hepatocytes induced by insulin (10^{-7}M), EGF (10 ng/ml) or both (Table 1). No effect was observed on DNA synthesis in the cells cultured in the medium with CT-B alone. When the cells were cultured in the medium along with growth factors, [^{3}H]thymidine incorporation was detected 16 h after growth stimulation (data not shown), and sufficient incorporation was obtained at 24 h.

Fig. 2 shows the dose-response curve for incorporation of [^{3}H]thymidine into the cells triggered by insulin and CT or CT-B. The effect of CT-B reached a plateau at 10^3 ng/ml and the rate of [^{3}H]thymidine incorporation was double that with insulin alone. Combination with insulin and whole toxin enhanced the rate of incorporation 7-fold compared with the control.

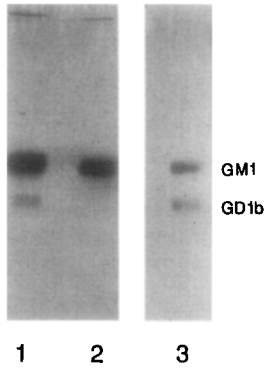

Fig. 1. TLC immunostaining with cholera toxin B subunit. Lane 1, acidic glycolipids from rat hepatocytes (0.8 mg dry weight); lane 2, standard GM1 (containing 4 ng sialic acid); Lane 3, standard GM1 and GD1b. They were developed with chloroform-methanol-0.5%$CaCl_2.2H_2O$ (45:45:10, by vol.) and the spots were detected with CT-B for lanes 1 and 2 and with orcinol reagent for lane 3.

Table 1. Effect of CT-B on DNA synthesis in rat hepatocytes induced by insulin and EGF

Additions	[^{3}H]thymidine incorporation (cpm/μg protein/h)	
	None	B subunit
Control	1.6 ± 0.3	1.4 ± 0.2
I	3.2 ± 0.7	6.0 ± 0.8
E	5.8 ± 0.8	8.9 ± 0.7
I+E	41.6 ± 1.3	79.7 ± 4.1

Rat hepatocytes were incubated with the medium alone (Control), insulin (I, 10^{-7}M), EGF (E, 10 ng/ml), or both in the absence or presence of CT-B (1 μg/ml). [^{3}H]thymidine incorporation was measured as described in Materials and Methods. Each result is expressed as the mean ± SD for triplicate determinations.

To rule out the possibility that the effect of CT-B was due to contamination by the A subunit, a membrane-permeable competitive antagonist of cyclic AMP, Rp-cAMPS, was used as shown in Fig. 3. Similar degrees of enhancement of DNA synthesis were

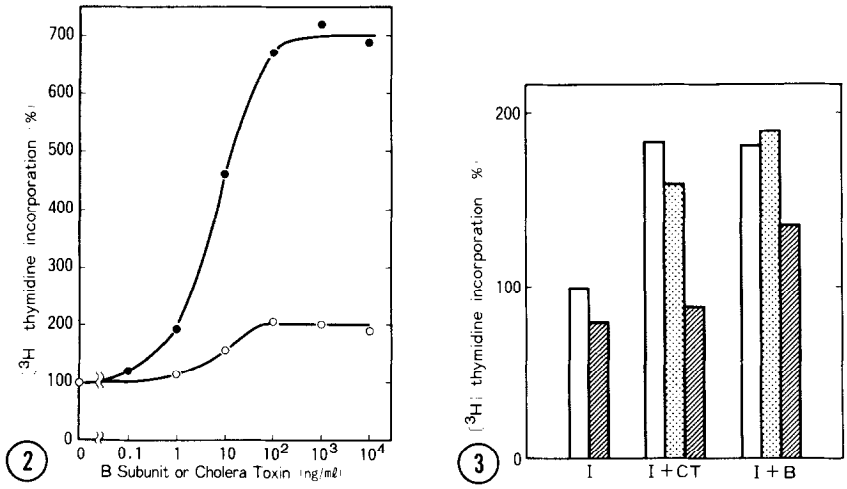

Fig. 2. Dose-dependent effect of cholera toxin and CT-B on DNA synthesis in rat hepatocytes. The cells were incubated with insulin(10^{-7}M) and CT-B (o) or cholera toxin (●). DNA synthesis was determined as described in the text and is expressed as a percentage of the basal value with insulin alone. Each point represents the mean for duplicate determinations.

Fig. 3. Inhibition of DNA synthesis with Rp-cAMPS. Hepatocytes were incubated in medium containing insulin (I, 10^{-7}M), insulin plus cholera toxin (CT, 1 ng/ml) or insulin plus CT-B (B, 10^3 ng/ml) with or without Rp-cAMPS. Open column, medium without Rp-cAMPS; dotted column, with Rp-cAMPS at low dose (5 x 10^{-6}M); hatched column, with Rp-cAMPS at high dose (5 x 10^{-5}M). DNA synthesis is expressed as in Fig. 2. Columns indicate means for duplicate determinations.

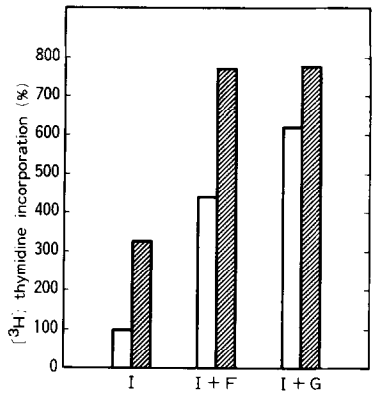

Fig. 4. Additive effect of cyclic AMP and CT-B on DNA synthesis in rat hepatocytes. The cells were incubated with insulin (I, 10^{-7}M), insulin plus forskolin (F, 10^{-5} M), and insulin plus glucagon (G, 5×10^{-8}M). Open and hatched columns represent incubation without and with CT-B (10^{3}ng/ml) respectively. DNA synthesis is expressed as in Fig. 2. Columns indicate means for duplicate determinations.

seen with 1 ng/ml CT and 10^3 ng/ml CT-B. The effect of CT was inhibited slightly by Rp-cAMPS at 5×10^{-6}M and almost completely at 5×10^{-5}M. In contrast, only 25% inhibition of DNA synthesis by insulin and CT-B was observed even at a high concentration of Rp-cAMPS (Fig. 3). Since 5×10^{-5}M Rp-cAMPS also inhibited by 20% the [^{3}H]thymidine uptake induced by insulin, it is unlikely that the effect of CT-B was due to contamination by the A subunit.

Glucagon and forskolin, both of which activate adenylate cyclase, also enhanced hepatocyte DNA synthesis induced by insulin, as reported previously (using insulin and EGF) (9). The effect of CT-B was additive. Nearly 8-fold enhancement was seen using an appropriate combination (Fig. 4), and the degree of enhancement was almost the same as that induced by CT.

Discussion

Recently it was shown that gangliosides might directly regulate cell proliferation by affecting transmembrane signaling. Cholera toxin (CT) binds with its pentameric B subunit (CT-B) to 5 molecules of GM1 on the cell surface. Thus

CT-B is a useful probe for studying the physiological function of GM1. Spiegel and Fishman reported that CT-B stimulated DNA synthesis in quiescent 3T3 cells, but inhibited it in rapidly growing or transformed 3T3 cells (7).

In this study we examined the effect of CT-B on DNA synthesis in rat hepatocytes. If CT-B and cyclic AMP affect cell proliferation in the same manner, it is important to exclude the possibility of contamination with the A subunit in the B subunit preparation, since the A subunit is responsible for production of cyclic AMP (15). Our CT-B preparation failed to induce cyclic AMP production at a concentration of 10 μg/ml (data not shown). In addition, an antagonist of cyclic AMP, Rp-cAMPS, was unable to suppress the effect of CT-B completely, and the rate of [^{3}H]thymidine uptake by CT-B at the plateau level was found to be additive with that of the cyclic AMP-dependent pathway mediated by forskolin or glucagon. All of these data suggest that the effect of CT-B is independent of cyclic AMP. Consequently, whole toxin is thought to affect hepatocyte proliferation through two different pathways. One is through activation of adenylate cyclase with the A subunit, and the other is through CT-B-GM1 interaction. As for the mechanism underlying the latter pathway, reorganization of the cytoskeleton by capping phenomenon involving the CT-B-GM1 complex in thymocytes (16) or involvement of pertussis toxin-sensitive GTP-binding protein in 3T3 cells (17) can be considered. Recently Spiegel suggested cross-talk between the signal transduction induced through gangliosides and protein kinase C in 3T3 cells (18). The present observations might provide new evidence for a glycolipid-mediated signal transduction pathway.

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