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Direct Evidence That Endogenous G_{M1} Ganglioside Can Mediate Thymocyte Proliferation

Abstract. *The B subunit of cholera toxin, which is multivalent and binds exclusively to a specific ganglioside, G_{M1}, was mitogenic for rat thymocytes. When exposed to the B subunit, the cells proliferated, as measured by ^{3}H-labeled thymidine incorporation. Mitogenesis depended on the direct interaction of the B subunit with G_{M1} on the surface of the cells. This demonstrates that endogenous plasma membrane gangliosides can mediate proliferation in lymphocytes.*

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Various cellular recognition phenomena and some important biological functions have been ascribed to glycolipids, particularly the sialoglycosphingolipids or gangliosides. Although gangliosides are receptors for bacterial toxins (*1*) and viruses (*2*) and can function as tumor antigens (*3*), their normal functioning in the plasma membrane remains unknown. Gangliosides can suppress the immune response in vitro (*4*) and can inhibit the growth of fibroblasts (*5*). In the latter case, the added gangliosides inhibited the interaction of growth factors with their receptors as well as the receptor-associated tyrosine kinase activity. On the other hand, gangliosides seem to stimulate cell growth and differentiation. The ganglioside G_{Q1b}, when added to certain neuroblastoma cell lines, increases both cell number and the length and number of neurites (*6*). Gangliosides, especially G_{M1}, promote neuritogenesis in both primary neurons and neuroblastoma cells (*7*). When hapten-modified gangliosides are inserted into thymocytes, the cells can proliferate in response to multivalent hapten-binding proteins (*8*). The researchers conducting these studies, however, added exogenous gangliosides to elicit the observed effects and did not address the possible functions of endogenous gangliosides. We have found that thymocytes can be induced to proliferate in response to the B subunit of cholera toxin (CT), which binds specifically to the endogenous ganglioside G_{M1} (*1*).

Exposure of rat thymocytes to the B subunit increased incorporation of [^{3}H]-thymidine relative to that of media-treated controls (Table 1). A concentration of B subunit as low as 25 ng/ml (~0.5 n*M*) induced proliferation, which reached a plateau at higher concentrations. Although the stimulation induced varied among experiments, so did that induced by concanavalin A (Con A) (Table 1). The relative responses to the two effectors, however, were of the same magnitude and were comparable between different experiments. Optimal incorporation of [^{3}H]thymidine was observed when cultures were pulsed from 48 to 64 hours after stimulation with the B subunit. The best results were obtained with thymocytes from Sprague-Dawley rats raised under germ-free conditions (Zivic-Miller Labs, Allison, Pennsylvania). In contrast, thymocytes from conventional Sprague-Dawley or Wistar rats responded moderately (fourfold) and thymocytes from C3H/HEN mice did not respond.

To confirm that the observed stimulatory activity was due to the binding of the B subunit to the cells, we incubated the subunit with antibodies to CT. The increase in thymidine incorporation observed was inhibited by the antibodies in a dose-dependent manner; 1:100 and 1:10 dilutions inhibited 40 and 100 percent, respectively (*9*). To further confirm the interaction of the B subunit with the thymocytes, we measured iodinated CT binding to the cells and its inhibition by the B subunit and the antibodies to CT (*10*). Under saturating conditions, 21 fmol of ^{125}I-CT were specifically bound per 10^6 cells. Binding of ^{125}I-labeled CT was completely inhibited (>97 percent) by the addition of unlabeled CT or subunit B or the antibodies. These data make it seem likely that the mitogenic effects of subunit B are due to its specific binding to the cell surface receptor for CT (that is G_{M1}) and that the inhibition of these effects by the antibodies is due to

Table 1. Stimulation of [^{3}H]thymidine incorporation into rat thymocytes by the B subunit. Rat thymocytes were prepared (*8*), washed, and maintained in RPMI 1640 medium supplemented with 5 percent fetal bovine serum, 1 m*M* sodium pyruvate, nonessential amino acids, 2 m*M* L-glutamine, penicillin (100 unit/ml), and streptomycin (100 μg/ml). The thymocytes (2×10^6 per well) were cultured in flat-bottomed microtiter trays in 0.2 ml of medium with the indicated concentrations of Con A or B subunit (Calbiochem-Behring or List Biological). After 48 hours, the cells were pulsed with 1 μCi of [^{3}H]thymidine for 16 hours, collected, and assayed for [^{3}H]thymidine incorporation. Each value is the mean of triplicate wells and each experiment used a different preparation of thymocytes.

Mitogen (μg/ml)	[^{3}H]Thymidine incorporation (counts/min ± S.E.M.)			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
B subunit				
0	3440 ± 273	1690 ± 199	3775 ± 381	483 ± 200
0.025	2770 ± 497	–	–	28400 ± 3140
0.25	10750 ± 1640	–	25200 ± 4625	49300 ± 2450
2.5	1 2400 ± 1410	37900 ± 1805	21460 ± 2160	50050 ± 4630
25.0	16200 ± 2540	40100 ± 667	23700 ± 445	62300 ± 4350
Con A				
2	194000 ± 7650	–	147000 ± 14600	252000 ± 6630

the blocking of the binding of subunit B to the ganglioside.

Direct evidence for the presence of G_{M1} in these cells and its interaction with ^{125}I-labeled CT and the B subunit is shown in Fig. 1A. Gangliosides from rat thymocytes were separated on silica gel–coated aluminum sheets, which were overlain with ^{125}I-labeled CT. The bound CT, detected by autoradiography, was restricted to a component (lane 2) with the same mobility as G_{M1} (lane 1). The binding of CT to thymocyte G_{M1} is through the B subunit, since prior incubation of the chromatogram with the B subunit blocked ^{125}I-labeled CT binding (lane 3). In agreement with studies on other cell types (*1*) including mouse thymocytes (*11*), we were unable to detect any binding of ^{125}I-labeled CT to membrane proteins or glycoproteins by means of a modified Western blot technique (Fig. 1B). All of the toxin binding was restricted to the region at the front of the electrophoretogram where the lipids (and gangliosides) migrate (lane 1). Delipidation of the membranes before electrophoresis eliminated the toxin binding (lane 2), whereas the toxin bound to the lipids extracted from the membranes (lane 3).

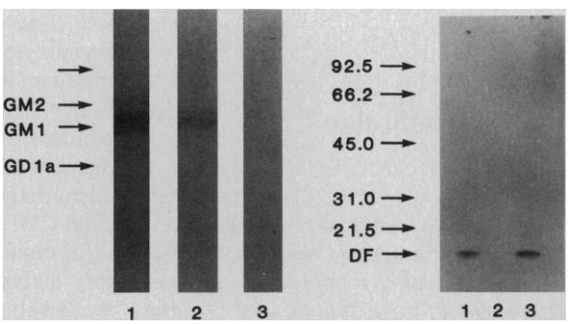

Fig. 1. (A) Specificity of CT and the B subunit for a rat thymocyte ganglioside with the mobility of G_{M1}. Rat thymocytes (10^8 cells), washed in ice-cold phosphate-buffered saline (PBS), were dispersed and sonicated in 1 ml of H_2O. Lipids were extracted with chloroform and methanol (in a ratio of 1:2 by volume) (*21*) and gangliosides were isolated from the crude lipid fraction (*22*). Portions (representing 2.5×10^7 cells) of the purified gangliosides were separated on aluminum-backed silica gel (*15, 23*). The chromatograms were air-dried, dipped in 0.1 percent poly(isobutylmethacrylate) in hexane, and air-dried. Then they were sprayed and soaked with tris-buffered saline (50 m*M* tris-HCl, *p*H 7.4, and 150 m*M* NaCl) containing 0.1 percent bovine serum albumin, overlain with ^{125}I-labeled CT in the same solution for 30 minutes at 4°C and washed (*15, 23*). After the chromatograms dried, bound CT was detected by autoradiography. Lane 1, 0.2 nmol each of standard gangliosides (locations indicated by arrows); lane 2, gangliosides from rat thymocytes; lane 3 is the same as lane 2 except that the incubation with ^{125}I-labeled CT was in the presence of unlabeled B subunit (25 μg/ml). (B) Direct binding of CT to rat thymocyte plasma membrane components. Partially purified membranes were prepared from rat thymocytes and some of the membranes were delipidated. Portions (representing 0.1 mg of protein) of the membranes (lane 1), delipidated membranes (lane 2) and the extracted lipids (lane 3) were dissolved in 1 percent sodium dodecyl sulfate (SDS), separated by SDS–polyacrylamide gel electrophoresis, and electrophoretically transferred to nitrocellulose sheets (*23*). Methanol was omitted from the transfer buffer to improve the retention of the lipids (*23*). The sheets were soaked in tris-buffered saline containing 2 percent bovine serum albumin and then overlain with ^{125}I-labeled CT and washed. The bound toxin was detected by autoradiography. The mobilities and molecular weights (in kilodaltons) of standard proteins are indicated on the left. Abbreviation: DF, dye front.

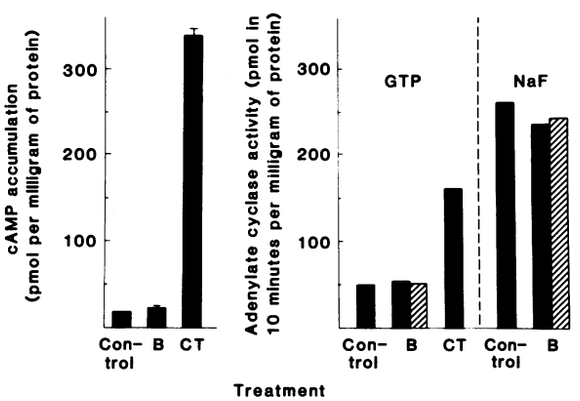

Fig. 2. (A) Effect of CT and the B subunit on cyclic AMP production. Rat thymocytes (3.3×10^6) were incubated in 0.3 ml of Dulbecco's modified Eagle's medium (DMEM) containing 0.1 percent bovine serum albumin, 25 m*M* Hepes, and 1 m*M* 3-isobutyl-1-methylxanthine with no addition (control), CT (1 μg/ml), or the B subunit (25 μg/ml). After 1 hour at 37°C, the samples were boiled for 5 minutes and centrifuged. Portions of the supernatants were analyzed for cAMP by a radioimmune assay (*16*). (B) Activation of adenylate cyclase by CT and the B subunit. Cells suspended in DMEM and Hepes (10^7 cells per milliliter) were incubated at 37°C with no addition, CT (1 μg/ml), or the B subunit (25 μg/ml) 1 hour or with the B subunit (2.5 μg/ml) overnight (hatched bars). After the cells were washed twice with ice-cold PBS and lysed, portions of the lysates were assayed for adenylate cyclase activity with either 50 μ*M* guanosine 5′-triphosphate (GTP) or 10 m*M* NaF (*16*). All values are the means of triplicate determinations from a representative experiment. For each value, the standard error of the mean (S.E.M.) was less than 5 percent of the mean unless otherwise indicated. Similar results were obtained in four additional experiments done on different preparations of thymocytes.

Previous studies leave the role of cyclic AMP in lymphocyte proliferation unclear. A rise and fall in cyclic AMP was observed in Con A–stimulated murine splenocytes, but continual exposure to 8-bromo–adenosine 3′,5′ monophosphate (8-bromo cyclic AMP) inhibited mitogenesis (*12*). Others reported that CT inhibited the stimulation of murine lymphocytes by lectins but enhanced the response of human lymphocytes (*13*). The ability of CT by itself to act as a mitogen, however, was not examined in these studies. CT and cyclic AMP can be mitogenic in other cell types (*14*). To clarify this point, we measured the ability of the B subunit to activate adenylate cyclase and to elevate cyclic AMP in rat thymocytes (Fig. 2). Whereas the intact toxin caused an 18-fold increase in cyclic AMP production, the B subunit had no effect (Fig. 2A). In addition, the B subunit did not activate adenylate cyclase in the cells even after 18 hours, whereas CT activated the enzyme 3.2-fold (Fig. 2B). Furthermore, exposure of the thymocytes to the B subunit did not modulate adenylate cyclase activity since the stimulation by NaF remained unchanged. Thus, the B subunit seemed to be free of any adenylate cyclase–activating A subunit; and the mitogenic effects of the B subunit on rat thymocytes were unrelated to increases in cyclic AMP. We also exposed rat thymocytes to 8-bromo– and dibutyryl–cyclic AMP; by themselves, these derivatives did not stimulate ^{3}H-labeled thymidine incorporation but did inhibit that caused by the B subunit or Con A.

From these results, we conclude that the B subunit stimulates the thymocytes by directly binding to and possibly cross-linking several molecules of G_{M1} ganglioside on the cell surface. The B subunit, which is pentavalent, binds only to the oligosaccharide chains of G_{M1} exposed on the cell surface (*1, 11, 15*). In addition, both CT and the B subunit undergo redistribution and capping on lymphocytes (*11, 16, 17*). These observations show that an interaction with endogenous gangliosides results in lymphocyte proliferation (*18*). The advantage of the B subunit as a mitogen is that its receptor is a single defined membrane component, whereas other mitogens can interact with numerous membrane components. Although we do not know the

mechanism by which binding of the B subunit to G_{M1} leads to a mitogenic response, changes in intracellular free Ca^{2+},K^+ channel gating, and Na^+-H^+ exchange have all been implicated in the mitogenic response (*19*). Thus, cross-linking of G_{M1} by the multivalent B subunit may modulate ion channels in the plasma membrane (*20*).

Our findings also raise the possibility that other physiological processes may be triggered by an interaction with gangliosides on the cell surface. Gangliosides have been implicated as inhibitors of both mitogen-stimulated lymphocyte proliferation (*4*) and the growth of fibroblasts in response to serum and growth factors (*5*); they also mediate neuronal growth, survival, and differentiation (*6*, *7*). Thus, gangliosides may play a role as membrane transducers of both positive and negative signals that regulate cell growth and differentiation.

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10. Rat thymocytes (3.9×10^6) were incubated in 0.2 ml of a solution (*21*) containing 0.5 n*M* ^{125}I-labeled CT (1120 cpm/fmol). Some samples contained CT (5 μg), B (2.5 μg), or antibodies to CT (1:10). After one hour at 37°C with constant shaking, the cells were washed three times with 3 ml of the ice-cold solution containing 0.2 percent bovine serum albumin by centrifugation at 1875*g* for 10 minutes. The washed cells were then counted for bound ^{125}I-labeled CT in a gamma counter.
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Colocalization of Band 3 with Ankyrin and Spectrin at the Basal Membrane of Intercalated Cells in the Rat Kidney

Abstract. *An immunoreactive form of the anion channel protein of erythrocytes, band 3, has been identified in the rat kidney. It is found in the intercalated cells of the distal tubule and collecting ducts. Immunostaining specific for band 3 is confined to the basolateral plasma membrane of these cells, where this protein probably mediates the transport of bicarbonate across the tubular wall. Double-immunolabeling studies demonstrate that band 3 is colocalized with immunoreactive forms of ankyrin and spectrin along the basolateral plasma membrane. The polarized distribution of band 3 may be the result of the association of its cytoplasmic domain with ankyrin, which in turn links band 3 to spectrin and the cytoskeleton. These observations help to explain how the collecting ducts of the kidney can direct the transport of bicarbonate ions, thus maintaining the acid-base balance.*

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The plasma membrane of transporting epithelial cells can be divided into apical and basolateral regions, which differ in protein composition and function. The mechanisms responsible for the placement of membrane proteins at specific sites on the cell surface are under active investigation (*1*). One way by which membrane proteins might be confined to a particular region of the cell surface could be by association with cytoskeletal proteins.

The membrane of erythrocytes provides an example in which a membrane-spanning (integral) protein, the anion channel protein (band 3), is linked by ankyrin to spectrin, which forms a fibrous meshwork on the cytoplasmic surface of the membrane (*2*). Proteins closely related to erythrocyte ankyrin and spectrin have been purified and characterized from various nucleated cell types (*3*), and immunoreactive forms of band 3 have also been described in nucleated cells (*4*), including the chicken kidney (*5*). We describe here the localization of an immunoreactive form of band 3 in the intercalated cells of the distal tubules and collecting ducts in the mammalian (rat) kidney. Band 3 is confined to the basolateral plasma membrane of these cells. This protein probably mediates reabsorption of bicarbonate at the basolateral membrane surface and thus may regulate acid-base balance. Immunoreactive forms of ankyrin are colocalized with band 3 along the basolateral surface of the intercalated cells; nonerythroid spectrin is also present at this site. Linkage of the anion channel analog via ankyrin to the spectrin-based membrane cytoskeleton may serve to maintain the polarized localization of this transport protein at the basolateral membrane.

Membrane proteins of the rat kidney were precipitated with antibodies to either rat or human erythrocyte band 3. Western blot analysis of the precipitated proteins (Fig. 1) revealed a polypeptide with an apparent molecular weight (M_r) of 120,000 migrating above rat erythrocyte band 3 (M_r ~100,000). In whole kidney membranes, band 3 migrated at M_r ~140,000. A slightly lower molecular weight (M_r ~115,000) has recently been determined for band 3 in the chicken kidney (*5*). In tissue sections of the adult rat kidney, affinity-purified antibodies to either the cytoplasmic or the membrane-spanning domain of both human and rat