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Increased Adhesion of Chinese Hamster Ovary Cells to Substratum by Cholera Enterotoxin

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A new, simple assay method using Chinese hamster ovary cells was devised for cholera enterotoxin. The effect of the cholera toxin was measured by an increase in cell adhesion to the substratum. The increase was dependent on the concentration of the toxin used and was effective for a longer period of time than that of adhesion increased by dibutyryl cyclic adenosine monophosphate and theophylline. There was a delay of about 60 min before the increase in adhesion, using the toxin, appeared, whereas the increase caused by dibutyryl cyclic adenosine monophosphate appeared almost at once.

It seems that cholera enterotoxin produces its effect by stimulation of intestinal adenylate cyclase, resulting in exudation of water and electrolytes into the intestinal lumen (5, 7, 13, 18, 21, 22). Stimulation of adenylate cyclase probably explains the various phenomena induced by the toxin in other tissues: morphological change in transformed cells (T. Yokota et al., Proc. 10th U.S.-Japan Coop. Med. Sci. Prog. Cholera Panel. 1974, p. 15; 8), stimulation of lipolysis in fat cells (24) and of steroidogenesis in adrenal cells (4, 25), differentiation in melanoma cells (16), inhibition of the mitogenic transformation in lymphocytes (23), and inhibition in mitogen-induced deoxyribonucleic acid synthesis in fibroblasts (10).

We report here that one of the effects of cholera toxin on Chinese hamster ovary (CHO) cells is to increase cell adhesion to the substratum. Other agents to elevate the intracellular cyclic adenosine monophosphate (cAMP) level such as dibutyryl cAMP (DBcAMP) and theophylline were also effective, as mentioned in a report on mouse fibroblasts (11). The usefulness of this simple, rapid assay method for understanding the biological activities of choleragen and other bacterial toxins that stimulate adenylate cyclase is also discussed.

MATERIALS AND METHODS

Cell culture. The CHO cell clone CHO-K_1, whose characteristics have been described previously (12), was used. The cells were grown in medium F12 (9) supplemented with 5% dialyzed fetal calf serum and antibiotics (50 U of penicillin G and 50 μg of kanamycin sulfate per ml) in plastic culture dishes (Lux, Thousand Oaks, Calif.) under standard tissue culture conditions of 5% CO_2 in air at 37 C in a humidified incubator.

Effectiveness of cell adhesion to the substratum. Cells were plated at 10^5 cells in a 35-mm dish and cultivated overnight. The medium was replaced with fresh medium, and cells were treated with cholera toxin or DBcAMP for the time indicated. After incubation, 500 U of Dispase per ml was added to the medium and cells were incubated at 37 C for a further 25 min. Then, the dish was gently shaken three times and the detached cells were counted by Coulter counter (Coulter Electronics, Hialeah, Fla.). Cells still attached to the dish were scraped off with rubber stopper, suspended in Isoton (Coulter Diagnostic Inc., Miami Springs, Fla.), and also counted. Thus, the percentage of removed and attached cells was calculated.

Chemicals. Fetal calf serum for the culture was purchased from GIBCO (Grand Island, N.Y.). Dispase was obtained from Godo Shusei Co. (Tokyo, Japan), kanamycin sulfate from Sankyo (Tokyo), penicillin G from Meiji Seika (Tokyo), and DBcAMP from Daiichi Pure Chemicals (Tokyo). All chemicals were solubilized in phosphate-buffered saline.

The cholera toxin was partially purified according to the method of Finkelstein and LoSpalluto (6). One microgram of this partially purified toxin was found to be equivalent to 20 ng of choleragen by the bluing test (1).

RESULTS

Increase of cell adhesion to the substratum by using cholera toxin. Dispase is a bacterial neutral protease and effectively works for the dispersion of mammalian cells in the presence of serum (Manual on Dispase, Godo Shusei). Detachment of CHO cells from the plastic

culture dish by various concentrations of Dispase is shown in Fig. 1. With 500 U of Dispase per ml, more than 65% of cells were detached from the dish within 25 min, and this incubation condition was chosen for the following experiments.

It was shown that agents which elevated intracellular cAMP concentration such as DBcAMP and theophylline increased the adhesion of mouse fibroblasts to the substratum (11). We studied the effect of cholera enterotoxin on CHO-K_1 cells in the presence of Dispase as compared with DBcAMP and theophylline. CHO cells treated with 10 μg of cholera toxin per ml for 4 h adhered to the disk more strongly and thus remained attached for a far longer period of time compared with the untreated cells (Fig. 2b). Cells treated with the toxin were compared under a microscope with untreated cells 25 min after the addition of Dispase (Fig. 3c and d). All of the untreated cells readily became round, explaining the ready detachment of the cells by mild shaking. On the other hand, the toxin-treated cells produced very few rounded cells, and most of them retained their original shape under the same condition. DBcAMP and theophylline each at 1 mM concentration also increased the adhesion of CHO cells for 30 min (Fig. 2a), suggesting that cholera toxin increased adhesion of CHO cells by the elevation of the intracellular cAMP level. It was clear that cells treated with cholera toxin remained attached to the dish for a longer period than those treated with DBcAMP and theophylline. Less than 40% of the toxin-treated cells were removed from the dish after 70 min of incubation with Dispase.

The cholera toxin induced a morphological change, in the form of a remarkable elongation, of the CHO cells after overnight incubation

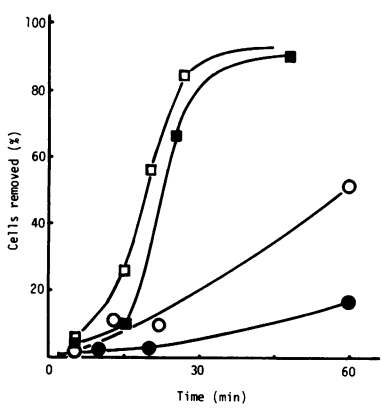

FIG. 1. *Detachment of CHO cells by using various concentrations of Dispase. Symbols:* ●, *100 U/ml;* ○, *200 U/ml;* ■, *500 U/ml;* □, *1,000 U/ml.*

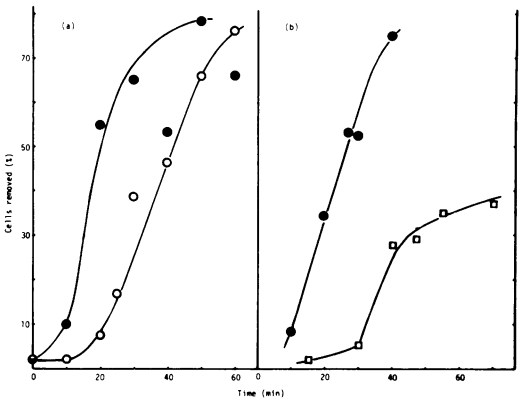

FIG. 2. *Detachment of CHO cells by using 500 U of Dispase per ml after an incubation period with DBcAMP and theophylline, or cholera toxin. Symbols: (a)* ●, *None;* ○, *treated with 1 mM DBcAMP and 1 mM theophylline. (b)* ●, *None;* □, *treated with 10 μg of cholera toxin per ml.*

(Yokota et al., Proc. 10th U.S.-Japan Coop. Med. Sci. Prog. Cholera Panel. 1974, p. 15; 8), which made it possible to assay the toxin easily in vitro. Since the morphology of CHO cells was not so changed after 4 h of incubation with cholera toxin (Fig. 3a and b), it is suggested that the increase of the cell adhesion to the substratum was a phenomenon, possibly due to a change in the properties of the surface membrane, induced earlier than cell elongation, though both phenomena were induced by the elevation of the intracellular cAMP level. Therefore, it became possible to assay cholera toxin rapidly by using this method.

The increase in cell adhesion by using various concentrations of the toxin was measured (Fig. 4). The percentage of adhesion of the CHO cells was related to the concentrations of cholera toxin used. The increase was observed with a minimum of 0.2 μg of cholera toxin per ml and it saturated at 2 μg/ml, when the maximum morphological change of CHO cells was observed (Yokota et al., Proc. 10th U.S.-Japan Coop. Med. Sci. Prog. Cholera Panel. 1974, p. 15).

Delayed action of the cholera toxin. Cell adhesion after introduction of the toxin or DBcAMP was periodically measured (Fig. 5). The increase of cell adhesion with 1 mM DBcAMP appeared without any delay, whereas the increase with 100 μg of cholera toxin per ml appeared about 85 min later (60 min after a 25-min incubation period with the toxin and Dispase). This means that it takes about 60 min for cholera toxin to function, presumably to elevate the intracellular cAMP level. This time delay in the appearance of the toxin action has

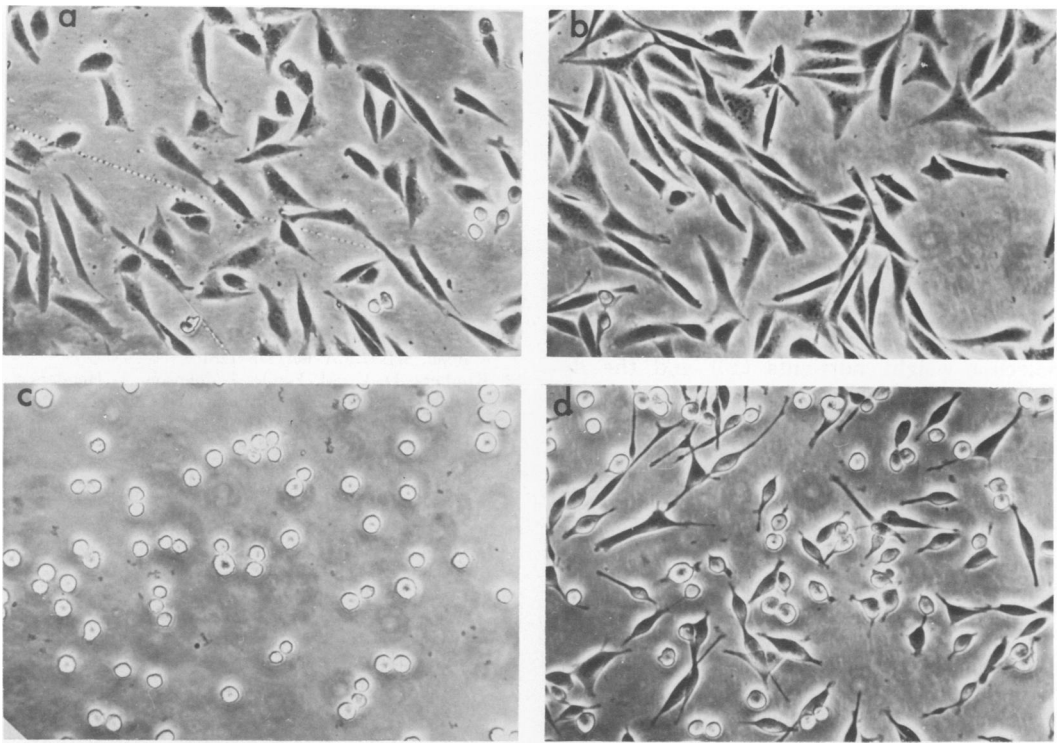

FIG. 3. *Cells under phase-contrast microscope (×100). (a) No treatment; (b) treated with 10 μg of cholera toxin per ml for 4 h; (c) treated with 500 U of Dispase per ml for 25 min; (d) treated with 500 U of Dispase per ml for 25 min after a 4-h incubation period with 10 μg of cholera toxin per ml.*

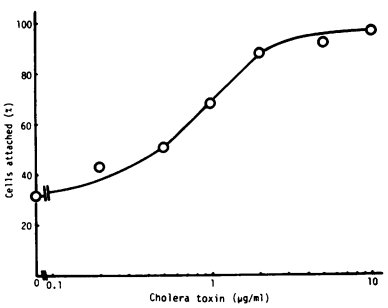

FIG. 4. *Increase of cell adhesion by using various concentrations of cholera toxin. Cells were treated with the toxin for 4 h and then with 500 U of Dispase per ml for 25 min.*

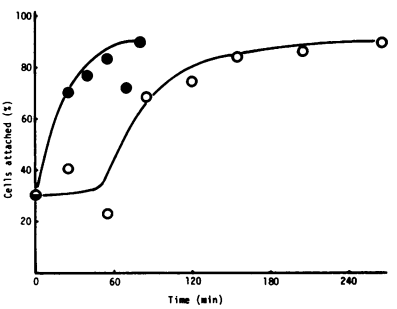

FIG. 5. *Increase of cell adhesion after incubation with DBcAMP and cholera toxin for various times. Cells were treated with 1 mM DBcAMP (●) and 100 μg of cholera toxin per ml (○) for various times after a 25-min incubation period with 500 U of Dispase per ml.*

also been observed in other tissues (2, 21, 24, 25).

DISCUSSION

The cholera toxin induced a remarkable morphological change in CHO cells (Yokoto et al., Proc. 10th U.S.-Japan Coop. Med. Sci. Prog. Cholera Panel. 1974, p. 15; 8) and an increase of cell adhesion to the substratum. Both effects were also observed by the addition of agents to elevate the intracellular cAMP level such as DBcAMP and theophylline (11, 19). It is suggested that the cholera toxin's effect on CHO cells, as in other tissues, is to bind to the membrane ganglioside GM_1 and to activate adenylate cyclase, resulting in the elevation of the intracellular cAMP level (3, 14, 17). It would appear that the effect of cholera toxin is similar to that of hormone action on adenylate cyclase (20), except that with the toxin the

reaction is greatly delayed and maintained for a much longer period (7, 16). These features of cholera toxin were also observed in this assay to measure cell adhesion. Since this assay method is relatively simple, it would be useful for the elucidation of biological activities of the toxin.

The interaction between mammalian cell surface and the substratum is an important characteristic of cells growing in tissue culture (11). cAMP mediated a rapid change in the surface properties of CHO cells (Fig. 5). This change of cell surface caused by cAMP may be related to the rapid suppression of the transport of low-molecular-weight nutrients (15) and the dynamic change in cell surface morphology caused by cAMP (19). These effects were counteracted by colcemid and cyclic guanosine monophosphate (15, 19). Studies of the effect of colcemid and cyclic guanosine monophosphate on cell adhesion are under consideration.

The clinical importance of enterotoxigenic bacteria in diarrheal illness is increasing and requires a simple, rapid assay for detecting enterotoxins produced by such bacteria. It is known that some bacterial enterotoxins such as *Escherichia coli* have a cholera toxin-like ability to activate adenylate cyclase and elongate CHO cells (8). This method may prove useful for the detection and understanding of such bacterial toxins.

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LITERATURE CITED

1. Craig, J. P. 1966. Preparation of the vascular permeability factor of *Vibrio cholerae*. J. Bacteriol. **92**:793-795.
2. Cuatrecasas, P. 1973. Cholera toxin-fat cell interaction and the mechanism of activation of the lipolytic response. Biochemistry **12**:3567-3577.
3. Cuatrecasas, P. 1973. Gangliosides and membrane receptors for cholera toxin. Biochemistry **12**:3558-3566.
4. Donta, S. T., M. King, and K. Sloper. 1973. Induction of steroidogenesis in tissue culture by cholera enterotoxin. Nature (London) New Biol. **243**:246-247.
5. Field, M. 1971. Intestinal secretion: effect of cyclic AMP and its role in cholera. N. Engl. J. Med. **283**:1137-1144.
6. Finkelstein, R. A., and J. J. LoSpalluto. 1970. Production of highly purified choleragen and choleragenoid. J. Infect. Dis. **121**:S63-S72.
7. Guerrant, R. L., L. C. Chen, and G. W. G. Sharp. 1972. Intestinal adenyl-cyclase activity in canine cholera: correlation with fluid accumulation. J. Infect. Dis. **125**:377-381.
8. Guerrant, R. L., L. L. Brunton, T. C. Schnaitman, L. I. Rebhun, and A. G. Gilman. 1974. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive in vitro assay for the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. Infect. Immunity **10**:320-327.
9. Ham, R. G. 1965. Clonal growth of mammalian cells in a chemically defined, synthetic medium. Proc. Natl. Acad. Sci. U.S.A. **53**:288-293.
10. Hollenberg, M. D., and P. Cuatrecasas. 1973. Epidermal growth factor: receptors in human fibroblasts and modulation of action by cholera toxin. Proc. Natl. Acad. Sci. U.S.A. **70**:2964-2968.
11. Johnson, G. S., and I. Pastan. 1972. Cyclic AMP increases the adhesion of fibroblasts to substratum. Nature (London) New Biol. **236**:247-249.
12. Kao, F-T., and T. T. Puck. 1968. Genetics of somatic mammalian cells. VII. Induction and isolation of nutritional mutants in Chinese hamster cells. Proc. Natl. Acad. Sci. U.S.A. **60**:1275-1281.
13. Kimberg, D. V., M. Fields, J. Johnson, A. Henderson, and E. Gershon. 1971. Stimulation of intestinal mucosal adenyl cyclase by cholera enterotoxin and prostaglandins. J. Clin. Invest. **50**:1218-1230.
14. King, C. A., and E. van Heyningen. 1973. Deactivation of cholera toxin by a sialidase-resistant monosialosylganglioside. J. Infect. Dis. **127**:639-647.
15. Kram, R., and G. M. Tomkins. 1973. Pleiotypic control by cyclic AMP: interaction with cyclic GMP and possible role of microtubules. Proc. Natl. Acad. Sci. U.S.A. **70**:1659-1663.
16. O'Keefe, E., and P. Cuatrecasas. 1974. Cholera toxin mimics melanocyte stimulating hormone in inducing differentiation in melanoma cells. Proc. Natl. Acad. Sci. U.S.A. **71**:2500-2504.
17. Pierce, N. F. 1973. Differential inhibitory effects of cholera toxoids and ganglioside on the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. J. Exp. Med. **137**:1009-1023.
18. Pierce, N. F., C. C. Carpenter, Jr., H. L. Elliot, and W. B. Greenough. 1971. Effects of prostaglandins, theophylline and cholera exotoxin upon transmucosal water and electrolyte movement in the canine jejunum. Gastroenterology **60**:22-32.
19. Puck, T. T., C. A. Waldren, and A. W. Hsie. 1972. Membrane dynamics in the action of dibutyryl adenosine 3':5'-cyclic monophosphate and testosterone on mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **69**:1943-1947.
20. Robison, G. A., R. W. Butcher, and E. W. Sutherland. 1971. Cyclic AMP and hormone action, p. 17-47. *In* G. A. Robison (ed.), Cyclic AMP. Academic Press Inc., New York and London.
21. Schafer, D. E., W. D. Lust, B. Sircar and N. D. Goldberg. 1970. Elevated concentration of adenosine 3':5'-cyclic monophosphate in intestinal mucosa after treatment with cholera toxin. Proc. Natl. Acad. Sci. U.S.A. **67**:851-856.
22. Sharp, G. W. G., and S. Hynie. 1971. Stimulation of intestinal adenyl cyclase by cholera toxin. Nature (London) **229**:266-269.
23. Sultzer, B. M., and J. P. Craig. 1973. Cholera toxin inhibits macromolecular synthesis in mouse spleen cells. Nature (London) New Biol. **244**:178-180.
24. Vaugham, M., N. F. Pierce, and W. B. Greenough. 1970. Stimulation of glycerol production in fat cells by cholera toxin. Nature (London) **226**:658-659.
25. Wolff, J., R. Temple, and G. H. Cook. 1973. Stimulation of steroid secretion in adrenal tumor cells by choleragen. Proc. Natl. Acad. Sci. U.S.A. **70**:2741-2744.