Assay Method for *Vibrio cholerae* and *Escherichia coli* Enterotoxins by Automated Counting of Floating Chinese Hamster Ovary Cells in Culture Medium

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As Chinese hamster ovary (CHO) cells on plastic proliferate, many cells float off into the medium instead of piling up after they form a monolayer. Fewer cells were floating in the medium when CHO cells were incubated with cholera toxin at a concentration as low as 10 pg/ml. The toxin increased the adhesiveness of the cells forming confluent monolayers so that the floating cells accumulated on the adherent monolayers. On the basis of this finding, a simple, quantitative assay method for cholera and *Escherichia coli* enterotoxins was devised by cultivating CHO cells in a Linbro multidish and counting the cells in the medium with a Coulter Counter. The method was sensitive enough to detect toxins in 100- to 200-fold-diluted culture media of toxigenic *E. coli* strains. Little or no activity was detected by this method in the culture medium of nontoxigenic *E. coli*.

It is known that certain bacteria, such as *Vibrio cholerae* (3, 19, 20) and *Escherichia coli* (2, 6-10, 13), that cause diarrhea in human patients produce enterotoxins which activate adenylate cyclase, resulting in the increase of the cyclic AMP level of animal cells. It is important to devise a simple assay method for the toxins to detect toxin-producing bacteria and to prepare toxin, toxoid, and antitoxin.

There are three practical assay methods for cholera-like enterotoxins making use of the toxins' effects in cultured animal cells: morphological alteration of Chinese hamster ovary (CHO) cells (7, 8) or mouse Y1 adrenal cells (1); increase of CHO cell adhesiveness to substratum (14); and growth inhibition of mouse S49 lymphosarcoma cells (F. E. Ruch, J. R. Murphy, L. Graf, and M. Field, J. Infect. Dis., in press). These methods, however, are more or less defective. The first method requires counting cells of slightly altered morphology by microscopic observation. The second is not adequate to handle a large number of samples. The third is simple but takes 2 or 3 days for the assay. Furthermore, there are some difficulties in maintaining toxin sensitivity in cells, especially in S49 cells (J. R. Murphy, personal communication).

Previously, we noticed the characteristic of CHO cells that the newly divided cells rarely pile up after they form monolayers on plastic, but float into the medium (15, 16). We report here the ability of enterotoxins to reduce the floating cells because of increased adhesiveness of the toxin-treated cells and an application of

this cell response for a simple, quantitative assay of cholera and *E. coli* toxins.

MATERIALS AND METHODS

Cell culture. The CHO clone K₁ was used. The cells were routinely grown in modified F12 medium (3 nM H₂SeO₃ was newly added and L-cysteine was reduced to 0.1 mM according to McKeehan et al. [12]) supplemented with 5% heat-treated (56°C, 30 min) fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.) and 50 µg of kanamycin sulfate (Meiji Seika, Tokyo) per ml in plastic culture dishes (Lux, Thousand Oaks, Calif.) under standard tissue culture conditions as described (14).

Measurement of floating cells in the medium. Cells were plated at 0.6×10^5 to 1.0×10^5 cells per cm² in 35-mm plastic culture dishes and incubated for 24 h. Culture medium was aspirated, confluent monolayers were rinsed once with serum-free medium or Puck saline G, and 2 ml of fresh medium, with or without cholera toxin, was added. After 18 h of incubation, culture medium was gently shaken and carefully removed by a Pasteur pipette, and monolayers were rinsed with 3 ml of saline G. Cells in the combined culture and rinse media were counted by Coulter Counter (Coulter Electronics, Hialeah, Fla.). Cells still attached to dishes were also counted after treatment with trypsin (0.05% trypsin [Difco, Detroit, Mich.] in Ca²⁺, Mg²⁺-free phosphate-buffered saline). In later experiments, cells were plated in 16-mm Linbro multidishes (Linbro, Hamden, Conn.) and incubated with 1 ml of medium for 24 h.

Monolayer attachment of CHO cells. Confluent CHO monolayers treated in 35-mm culture dishes with or without 5 ng of purified cholera toxin (choleragen) per ml for 24 h served as adhering monolayers. Culture medium was carefully aspirated, and monolayers were

rinsed twice with 2 ml of serum-free culture medium. CHO cells were cultured in 60-mm dishes until confluency, and floating cells were collected by centrifuging the culture medium at $150 \times g$ for 1 min. Sedimented cells were resuspended in fresh culture medium (2×10^6 /ml), and 2 ml of cell suspension was added to the adhering monolayers. Dishes were incubated at 37°C in a humidified incubator in a CO_2 atmosphere. At various time intervals during 60-min periods, duplicate dishes were removed from the incubator, medium was pipetted out, and monolayers were gently rinsed once with 3 ml of serum-free medium (37°C). Cells in the combined media were counted.

Preparation of *E. coli* culture filtrates. Toxigenic *E. coli* 339t5 (4), B16-4 (8), and 334 (6, 7) and nontoxigenic *E. coli* 20S0 were cultivated in L broth (containing, per liter, 10 g of tryptone [Difco], 5 g of yeast extract, and 5 g of NaCl; pH 7.2) for 48 h. Cell suspension was centrifuged at $12,000 \times g$ for 10 min, and the supernatant was filtered through a membrane filter ($0.45 \mu\text{m}$, Nihon Millipore, Tokyo).

Materials. Cholera toxin purified essentially according to the method of Finkelstein and LoSpalluto (5) and horse anticholera toxin were obtained from N. Ohtomo.

RESULTS

Serum-dependent increase of floating cells in the medium. When CHO cells grow up confluent on plastic surfaces, the newly divided cells float into the medium instead of piling up on the confluent monolayers (15, 16). Many cells float in the medium as single cells; few if any cells are loosely clumped. Clumped cells are dissociable to single cells by gentle pipetting.

FIG. 1. Dose response curve of serum effect on floating CHO cells. Confluent CHO monolayers were incubated for 18 h in culture medium with various concentrations of serum, and floating cells in the medium were determined by Coulter Counter. The bar indicates standard deviation. This and following experiments were done in duplicate.

FIG. 2. Dose response curve of cholera toxin effect on floating CHO cells. Confluent CHO monolayers were incubated with various concentrations of cholera toxin for 18 h in culture medium containing (○) 5% serum or (●) 10% serum.

Confluent monolayers of CHO cells were incubated for 18 h with media containing various concentrations of serum, and cells floating in medium and attached to dishes were determined (Fig. 1). Cells attached to a plastic culture dish were almost constant in either serum concentration over 1% ($1.97 \pm 0.08 \times 10^6$ cells). Floating cells, however, increased, depending on the serum concentration, up to 20%. We used the culture medium with 5% serum for further experiments unless described otherwise.

Reduction by cholera toxin of floating cells in the medium. Confluent monolayers of CHO

cells were incubated with various concentrations of cholera toxin, and the number of cells in the medium was determined (Fig. 2). Floating cells decreased depending on the dose of cholera toxin, and the minimum effective dose of the toxin was 10 pg/ml. Cells treated with or without 1 ng of cholera toxin per ml are shown in Fig. 3. In untreated dishes, many round cells were seen on confluent monolayers; these floated off into the medium when shaken gently. Very few round cells, on the other hand, were observed on the elongated monolayer cells in cholera toxin-treated dishes. A dose of cholera toxin to reduce the num-

FIG. 3. Cells under phase-contrast microscope ($\times 140$). Confluent CHO cells, (A) untreated and (B) treated with 1 ng of cholera toxin per ml for 18 h.

ber of floating cells to 50% of the control, obtained from three separate experiments, was 67.3 ± 6.1 pg/ml. When monolayers were incubated in the culture medium with 10% serum, the toxin's action was less effective. The cholera toxin dose at 10% serum that reduced floating cells to 50% of control was 170 pg/ml.

Attachment of CHO cells to cholera toxin-treated confluent monolayers. There could be two explanations for the decrease of the floating cells by cholera toxin. One is that the toxin inhibits cell growth. Another is that the toxin increases mutual adhesiveness of cells. To test the former possibility, hamster cells in 35-mm dishes were incubated with or without 2 ng of cholera toxin per ml for 3 days, adding 2 ml of fresh medium every day, and the cells in the medium and attached to the dishes were counted (Fig. 4). The growth rate of toxin-treated cells was slowed down by about 15% as compared with that of untreated cells. Nevertheless, the floating cells in the treated dishes were far fewer

FIG. 4. Effect of cholera toxin on CHO cell growth. Confluent CHO cells were treated with 2 ng of cholera toxin per ml, and cells in the medium and attachment to dishes were determined. Fresh medium (2 ml) with the toxin was added every day. (○) Total cells, untreated; (●) total cells treated with cholera toxin; (□) cells in the medium, untreated; (■) cells in the medium, treated with cholera toxin.

than those in the untreated dishes if compared at the time when total cell number was equal in both dishes. Therefore, the first explanation seems to be unlikely.

Floating cells in untreated dishes were collected by centrifugation, and attachment of these cells to confluent monolayers pretreated for 24 h with the toxin was examined (Fig. 5). The cells, which readily attached to a plastic surface within 40 min, hardly attached at all to untreated monolayers, and 80% of the added cells remained in the medium during a 60-min incubation period. On the other hand, 70% of the cells added to cholera toxin-treated monolayers disappeared from the medium within 60 min. Disappearance of cells from the medium in toxin-treated dishes was not due to clumping of added cells during the incubation, since the number of cells remaining in suspension did not increase after trypsinization, a treatment to dissociate clumped cells. Thus, it was concluded that the added cells were attached to adherent CHO monolayers by the treatment with cholera toxin.

Titration of cholera toxin and anticholera toxin. Horse antisera for cholera toxin and cholera toxin were titrated by the above described assay method. Diluted serum was incubated with 2 ng of cholera toxin per ml in confluent CHO dishes (35 mm), and cells floating in the medium

FIG. 5. Monolayer attachment of floating CHO cells. Attachment of CHO cells (Δ) to plastic culture dish; (○) to CHO confluent monolayers; and to CHO confluent monolayers pretreated with 5 ng of cholera toxin per ml for 24 h, counted (●) before and (□) after trypsin treatment.

FIG. 6. Reversal of cholera toxin effect by horse anticholera toxin. Confluent CHO monolayers were incubated with 2 ng of cholera toxin per ml for 18 h in the presence of anticholera toxin. Floating cells in control cultures were $0.13 \pm 0.03 \times 10^5$ (without antitoxin) and $4.28 \pm 0.04 \times 10^5$ (without cholera toxin and antitoxin).

were counted (Fig. 6). Serum diluted to 1:10,000 effectively reversed the toxin's action.

Assay of *E. coli* enterotoxin. Toxins produced in media by nontoxicogenic and toxigenic *E. coli* strains were measured. For the assay, CHO cells were plated in a Linbro multidish (16 mm) as described in the text. Cells floating in the medium were counted, and the percentage of untreated control was plotted against the dilution of the culture medium (Fig. 7). An ability to inhibit CHO cells from floating off into the medium was detected in the culture filtrates of toxigenic *E. coli* strains (339t5, B16-4, and 334) up to 100- to 200-fold dilution. On the other hand, no activity was detected in 20-fold-diluted culture medium of *E. coli* 20SO, a nontoxicogenic strain.

The toxin produced in culture medium of *E. coli* 339t5 was characterized (Table 1). The toxin was heat labile (LT) and cross-reacted with anticholera toxin.

Detection of *E. coli* strains producing enterotoxins. As an application of this method for detecting *E. coli* strains producing enterotoxins, culture media of nine strains were blindly assayed (Table 2). The number of floating cells was reduced to less than 25% of the control by the diluted (1:20 or 1:25) culture media of three strains known as LT producers; it was not changed, compared with the control, by those of four nontoxicogenic strains. Two strains known as nontoxicogenic showed intermediary percentages (56 and 72%). CHO cells remaining attached to dishes were fixed with methanol and stained with crystal violet. Cells treated with culture

media from three LT-producing *E. coli* strains were highly elongated, but were not morphologically altered by the media from six nonproducers. Therefore, the two intermediary strains seem to produce no toxins, but probably produce an inhibitor for CHO growth. Thus, this assay is applicable for the detection of LT-producing *E. coli*, and false positive strains could be eliminated by simultaneous observation of CHO cell morphology.

DISCUSSION

It was found that cholera toxin increases surface adhesiveness of the CHO cell monolayer, resulting in fewer floating cells in the medium (Fig. 2 and 5). On the basis of this finding, we devised

FIG. 7. Assay of *E. coli* culture filtrates. CHO confluent monolayers in a Linbro multidish were incubated with culture filtrates of *E. coli* strains (□) 339t5; (●) 334; (○) B16-4; or (Δ) 20SO. After 24 h, cells in medium were counted and percentage of control was determined.

TABLE 1. Characteristics of *E. coli* enterotoxin in culture filtrate^a

Addition	No. of CHO cells in medium $\times 10^5$	(%)
None	1.62 ± 0.17	(100)
<i>E. coli</i> 339t5 toxin (1:10 dilution)	0.07 ± 0.02	(4.3)
Heated (95°C, 20 min) <i>E. coli</i> toxin (1:10)	1.19 ± 0.09	(73.5)
<i>E. coli</i> toxin (1:10) + horse anticholera toxin (1:200)	0.88 ± 0.14	(54.3)
<i>E. coli</i> toxin (1:10) + horse anticholera toxin (1:1,000)	0.90 ± 0.04	(55.6)

^a Culture filtrate of *E. coli* 339t5 was assayed in a Linbro multidish with CHO cells.

a simple, quantitative assay method for cholera and *E. coli* toxins. This method detected cholera toxin at a concentration as low as 10 pg/ml. We calculate that 60 to 70 molecules of cholera toxin per CHO cell is necessary to exert a detectable effect in the culture with 5% serum (medium, 2 ml; cholera toxin, 82,000 daltons [22]; and cells, 1.9×10^6 to 2.6×10^6). To detect a lower concentration of cholera toxin, it would be better to reduce the number of CHO cells in the assay system by employing a smaller culture vessel. In later experiments (Fig. 7, Tables 1 to 3), we employed a Linbro multidish (16 mm) for that reason and also for easier handling of a large number of samples. Lower serum concentration in the culture medium would also be important for raising the sensitivity of the assay, since the serum antagonized the toxin's action (Fig. 2).

Diluted horse anticholera toxin reversed the effect of cholera toxin, confirming that the observed response of CHO cells is due to the toxin's action to raise the intracellular cyclic AMP level by activating adenylate cyclase (7). Dibutyryl cyclic AMP showed the same effect as cholera toxin on floating CHO cells (Table 3). *E. coli* enterotoxin also increases the intracellular cyclic

AMP level (2, 6–10, 13). Culture filtrates of toxigenic *E. coli* strains inhibited CHO cells from floating off into the medium (Fig. 7). This activity of *E. coli* was heat labile and partially reversed by anticholera toxin (Table 1), showing characteristics of *E. coli* LT (6).

Our method was useful not only for the assay of cholera toxin but also for the detection of *E. coli* toxin produced in culture medium. Guerrant and Brunton detected *E. coli* LT in the culture medium of *E. coli* 334 diluted up to 170-fold by their method of counting elongated CHO cells (6). Our method was as sensitive for the detection of *E. coli* 334 toxin as theirs (Fig. 7). Estimation by this method of the dual effects of the toxin on CHO cells, i.e., decrease of floating cells (Fig. 7, Table 2) and morphological elongation (Fig. 3), would be advantageous in screening of LT-producing *E. coli*.

Lowered cell adhesiveness as well as increased cell growth is considered one of the characteristics of transformed cells (18). Cholera toxin affected CHO cell growth little but increased cell adhesiveness greatly (Fig. 4 and 5). CHO cells grown on fibrin films also showed increased cell adhesiveness, but no difference in cell growth as compared with cells grown on plastic (15, 16). Therefore, the control mechanism of CHO cell growth seems separable from that of cell adhesiveness. This coincides with a recent conclusion by Pouyssegur et al. from an experiment on 3T3 BALB/c variant cells (17).

TABLE 2. Detection of LT-producing *E. coli*^a

<i>E. coli</i> strain	Toxin identified	Dilution of culture filtrate	No. of cells in medium $\times 10^5$ (%)
TD218C1 ^b	LT LT-ST ^c (11)	Control	1.00 (100)
		1:25	0.19 $\pm$ 0.01 (19)
		1:25	0.20 $\pm$ 0.02 (20)
		1:25	0.56 $\pm$ 0.04 (56)
		1:25	1.06 $\pm$ 0.12 (106)
		1:25	1.22 $\pm$ 0.08 (122)
B2C	LT-ST (21)	Control	0.86 $\pm$ 0.04 (100)
		1:20	0.21 $\pm$ 0.04 (24)
		1:20	0.62 $\pm$ 0.01 (72)
		1:20	0.82 $\pm$ 0.12 (95)
		1:20	0.94 $\pm$ 0.06 (109)
H10407			
CSH38			
HfrH			
W3110			
E111			
594			
K-12			

^a *E. coli* strains were cultivated for 48 h in L broth. Culture filtrates were assayed in a Linbro multidish with CHO cells.

^b Obtained from R. B. Sack.

^c ST, Heat-stable toxin.

TABLE 3. Effect of dibutyryl cyclic AMP on CHO cells in the medium^a

Concn of dibutyryl cyclic AMP (mM)	No. of cells in medium $\times 10^5$ (%)
None	1.51 $\pm$ 0.02 (100)
0.03	1.39 $\pm$ 0.16 (92)
0.1	1.06 $\pm$ 0.09 (70)
0.3	0.61 $\pm$ 0.05 (40)
1	0.14 (9)

^a Confluent monolayers of CHO cells in a Linbro multidish were incubated with dibutyryl cyclic AMP for 24 h.

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