Acta Medica Okayama

Volume 38, *Issue* 5 1984 *Article* 6

OCTOBER 1984

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Abstract

A modified method of passive immune hemolysis (PIH) was applied to the quantitative assay of heat-labile enterotoxin (LT) produced by enterotoxigenic Escherichia coli. The method enabled the measurement of 0.2 to 1.2 ng LT. The production of LT by enterotoxigenic E. coli under various conditions was analyzed using the modified method. LT production was intense during the logarithmic growth phase and decreased during the stationary growth phase. Lincomycin (50 to 100 micrograms/ml) affected cell growth slightly, but enhanced production of LT until the late-stationary growth phase. About 90% of the LT produced was retained in the cell, and the rest was excreted into the culture medium. The initial pH of the culture medium affected LT production. Alkaline pH enhanced LT production, though growth was depressed. Aeration enhanced both growth and LT production.

KEYWORDS: heat-labile enterotoxin, E, coli, passive immune hemolysis

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*PMID: 6393717 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **38,** (5), 461-469 (1984)

QUANTITATIVE ANALYSIS OF THE PRODUCTION OF HEAT-LABILE ENTEROTOXIN BY ENTEROTOXIGENIC *ESCHERICHIA COLI*

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Received May 24, 1984

Abstract. A modified method of passive immune hemolysis (PIH) was applied to the quantitative assay of heat-labile enterotoxin (LT) produced by enterotoxigenic *Escherichia coli.* The method enabled the measurement of 0.2 to 1.2 ng LT. The production of LT by enterotoxigenic *E. coli* under various conditions was analyzed using the modified method. LT production was intense during the logarithmic growth phase and decreased during the stationary growth phase. Lincomycin (50 to 100 μg/ml) affected cell growth slightly, but enhanced production of LT until the late-stationary growth phase. About 90 % of the LT produced was retained in the cell, and the rest was excreted into the culture medium. The initial pH of the culture medium affected LT production. Alkaline pH enhanced LT production, though growth was depressed. Aeration enhanced both growth and LT production.

Key words : heat-labile enterotoxin, *E. coli,* passive immune hemolysis.

Heat-labile enterotoxin (LT) produced by enterotoxigenic *Escherichia coli* is responsible for diarrhea in humans and animals (1, 2). The properties and the mode of action appear to be similar to those of cholera toxin which affects the cell membrane permeability through the stimulation of membrane-bound adenylate cyclase of intestinal epithelial cells (3-5) as well as nonintestinal cells that have G_{M1} ganglioside as a cell-surface receptor (6-9). On the other hand, little information is available regarding the production of LT by enterotoxigenic *E. coli.* In order to investigate the kinetics of LT production by *E. coli,* it was necessary to establish a sensitive and reliable method for the quantitative assay of LT. Various assay methods have been reported for the detection of LT, but they are rather complicated and not very quantitative. Those methods require a large number of animals (10-13), stock of special tissue culture cells (7, 14, 15), radioisotopes (16), or special materials (17). Evans and Evans (18) reported an LT assay method using passive immune hemolysis (PIH), *i.e.,* complement mediated lysis of LT sensitized sheep red blood cells (LT-SRBC) by anti-LT antibody. The PIH method requires only the antitoxin as a special material, and the procedure is rather simple. Above all, the PIH method can be applied to the quantitative

assay of LT with a small amount of LT, assuming the one-hit theory (19) of complement hemolysis through the classical pathway is applicable.

In this paper, the PIH method was modified so as to be more sensitive and more quantitative in the study of the kinetics of LT production.

MATERIALS AND METHODS

Bacterial strain and culture of cells. *E. coli* 240-3, a strain which was isolated from a diarrhea case on Rangoon, Burma, was used. Stock culture was maintained on Dorset egg slants (Nissui Pharmaceutical Co. Ltd., Tokyo). *E. coli* was precultured in CAYE medium (20) at 37℃ for 20 h with shaking at 120 r.p.m. on a New Brunswick rotary shaker model G-25. The cells were cultured at the inoculum size of 1 % (v/v) in CAYE medium supplemented with or without lincomycin hydrochloride (Lincocin Injection, Japan Upjon Ltd., Tokyo) with shaking at 120 r.p.m. The concentration of lincomycin hydrochloride was 90 μg/ml unless otherwise noted. CAYE medium contained (l^{-1}); 20 g Casamino acids (Difco Laboratories, Detroit, Michigan, U.S.A.), 6 g yeast extract (Difco), 2.5 g NaCl, 8.71 g K_2HPO_4, 2.5 g glucose, and 1 ml salt solution [5 % (w/v) $MgSO_4$, 0.5 % (w/v) $MnCl_2$, 0.5 % (w/v) $FeCl_3$, and 0.001 % (v/v) H_2SO_4]. The pH was adjusted to 8.6.

Preparation of LT and antitoxin. LT was purified as described by Takeda *et al.* (21). The method is essentially the same as that of Clements and Finkelstein (22).

Antitoxin was obtained from rabbit immunized with purified LT. Each rabbit was immunized in the foot pads by injecting 50 μg LT in 1 ml Freund's complete adjuvant, followed by intramuscular injection of 50 μg LT in 1 ml Freund's incomplete adjuvant on the 20th day. The pooled serum was treated at 56℃ for 30 min. The IgG fraction was obtained by ammonium sulfate fractionation, followed by filtration through a DEAE-cellulose column in 0.02 M phosphate buffer, pH 7.2.

Protein was assayed by the procedure of Lowry *et al.* (23), using bovine serum albumin as a standard.

Preparation of LT extracts. LT was released from *E. coli* cells by the polymyxin-release technique (18, 24, 25). Polymyxin B (Taitoh Pfizer Co., Tokyo) was dissolved at the concentration of 50000 IU/ml in N-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer, pH 6.7, containing 0.9 % (w/v) NaCl. For the release of LT, the polymyxin B solution was added to the culture of *E. coli* at the final concentration of 10000 IU/ml, and then incubated at 37℃ for 30 min with shaking at 100 r.p.m. The cells were removed from the supernatant by centrifugation at 40000 × g for 30 min. The resulting supernatant was used as the total LT preparation. The supernatant of centrifugation at 19000 × g for 20 min of the culture without adding polymyxin B was used as the extracellular LT preparation.

Procedure for passive immune hemolysis. Sheep red blood cells (SRBC) were suspended in Tris/HCl-buffered saline-EDTA, which contained 0.018 M tris(hydroxymethyl)aminomethane, 0.12 M NaCl and 0.01 M ethylenediaminetetraacetate, pH 7.4, and heated at 37℃ for 10 min. The cells were washed at least three times with TBS^{++}, pH 7.4, which contained 0.02 M tris (hydroxymethyl)aminomethane, 0.13 M NaCl, and 0.15 mM $CaCl_2$ and 1.0 mM $MgCl_2$. A mixture of 0.1 ml SRBC (2.5 × 10^8 cells/ml TBS^{++}) and 0.5 ml LT sample was incubated at 37℃ for 40 min. The cell suspension was centrifuged, and the pelleted cells (LT-sensitized SRBC ; LT-SRBC) were resuspended in 0.2 ml TBS^{++}. The LT-SRBC suspension was mixed with 0.5 ml antitoxin (20 μg/ml TBS^{++}) and incubated at 37℃ for 60 min, then 1.8 ml TBS^{++} and 0.5 ml complement solution (guinea pig serum diluted to 1 : 100 with TBS^{++}) were added,

followed by further incubation at 37℃ for 60 min. The mixture was cooled in an ice-cold water bath, and then centrifuged at 4℃. The hemoglobin released into the supernatant was determined by spectrophotometry at 412 nm. The average number of effective lytic sites per cell, designated as Z, was calculated by the formula : $Z = -\ln(1-y)$, where y is the fraction of cells lyzed (26, 27). One hundred per cent lysis was determined by complete hemolysis of 0.1 ml SRBC in 2.9 ml H_2O, and 0 % lysis was determined by 0.1 ml SRBC in 2.9 ml TBS^{++}.

RESULTS

PIH assay for LT. The degree of hemolysis (per cent) was plotted as a function of the amount of total LT preparation (Fig. 1). The percentage of hemolysis showed a good correlation with the amount of LT preparation added. Hemolysis was not observed if either the antitoxin or complement was omitted from the assay medium, indicating that the hemolysis was real PIH. The amount of LT preparation needed for 50 % lysis was about 0.05 μl, and for 100 % lysis about 0.2 μl. The method is sensitive enough to detect LT in culture medium. If the endpoint is properly chosen within the range of the linear proportion of the sigmoidal response curve, it is possible to determine the amount of LT in the sample. The number of lytic sites per cell (Z) were plotted as a function of the amount of purified LT as shown in Fig. 2. The linear correlation between Z and the amount of LT was clearly demonstrated. Using this figure as a calibration curve, LT could be measured precisely in the range of 0.2 to 1.2 ng.

Relation between cell growth and LT production of E. coli. The amount of LT produced at each growth phase of the cells was measured and plotted as shown

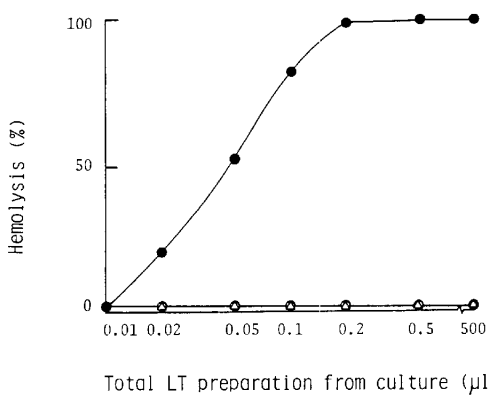

Fig. 1. Assay of heat-labile enterotoxin of *E. coli* by passive immune hemolysis. Percentage of hemolysis of LT-sensitized sheep red blood cells was plotted as a function of the volume of the total LT preparation from the culture. *E. coli* 240-3 (1 % inoculum size) was cultured in CAYE medium supplemented with 90 μg lincomycin/ml at 37℃ for 20 h with shaking at 120 r.p.m. Polymyxin B was added to the culture to make the total LT preparation as described in Materials and Methods. Complete assay medium (●), assay medium without antitoxin (○) and without complement (△).

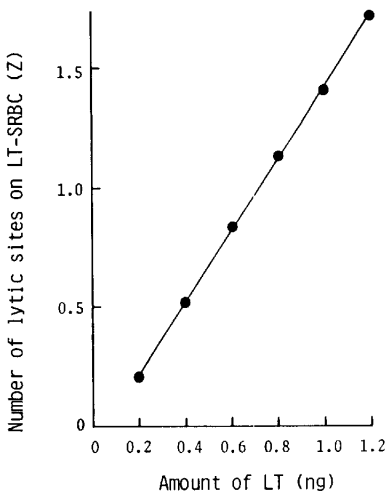

Fig. 2. Assay of heat-labile enterotoxin of *E. coli* by passive immune hemolysis. The average number of lytic sites per cell (Z) was plotted as a function of the dose of purified LT. Z was calculated according to the equation described in Materials and Methods. The assay method for LT is described in Materials and Methods.

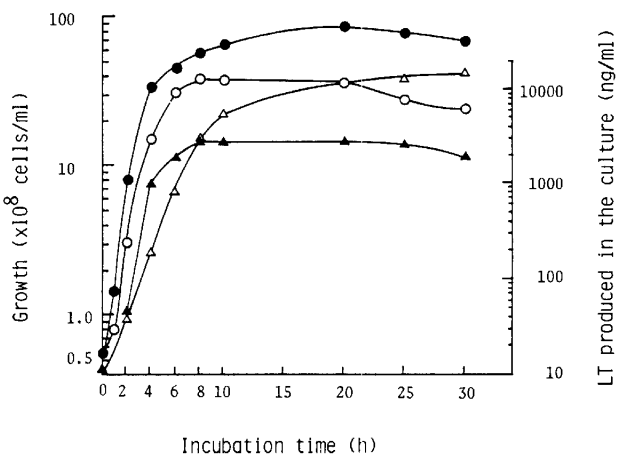

Fig. 3. Time-course of the growth and production of heat-labile enterotoxin of *E. coli*. Growth of *E. coli* 240-3 (○, ●) and amount of LT produced (△, ▲) were determined in the absence (closed symbols) or in the presence (open symbols) of lincomycin (90 μg/ml). *E. coli* (1% inoculum size) was cultured at 37℃ with shaking at 120 r.p.m. Polymyxin B was added to the culture to make the total LT preparation as described in Materials and Methods.

in Fig. 3. The amount of LT increased along with cell growth and reached a maximal amount at 8 h, which is in the end of the logarithmic growth phase.

Effect of lincomycin was also examined (Fig. 3). The addition of 90 μg

TABLE 1. EFFECT OF LINCOMYCIN ON THE PRODUCTION OF HEAT-LABILE ENTEROTOXIN BY *E. COLI*.

Lincomycin concentration (μg/ml)	Cell yield ($\times 10^8$/ml)	Total LT (μg/ml)	Total LT (ng/1$\times 10^8$ cells)	Extracellular LT (μg/ml)	Extracellular LT (%)
0	58	1.80	31.0	0.156	8.7
1	56	1.98	35.5	0.148	7.5
10	53	2.33	44.0	0.092	3.9
50	39	5.80	148.7	0.165	2.8
100	28	5.03	179.6	0.315	6.3
300	20	0.89	44.5	0.078	8.8

E. coli 240-3 was cultured in CAYE medium containing various amounts of lincomycin at 37℃ for 20 h by shaking at 120 r.p.m. Assay samples for total LT and extracellular LT were prepared as described in Materials and Methods.

lincomycin/ml to the culture medium did not severly affect cell growth, however, the amount of LT produced increased up to the late-stationary growth phase. The addition of lincomycin results in about a fivefold increase in LT production compared with the nonaddition after 20 h incubation.

The correlation between the concentration of lincomycin and LT production was examined as shown in Table 1. The LT production of *E. coli* was maximally stimulated by the addition of 50 to 100 μg lincomycin/ml, whereas growth was slightly inhibited. At a concentration below 10 μg/ml, neither production of LT nor growth was affected. Lincomycin (300 μg/ml) barely stimulated LT production, and 500 μg lincomycin/ml inhibited the growth of *E. coli* severely.

The amount of extracellular LT was less than 10 % of the total LT produced. Lincomycin did not stimulate the excretion of LT into the medium.

Effects of pH on growth and LT production of E. coli. The effect of pH of the medium on LT production was examined (Fig. 4). LT production was enhanced as the initial pH of the medium was raised from 6.5 to 9.0, while the growth yield of *E. coli* decreased as the pH was raised. The excretion rate of LT into the medium was hardly affected by the initial pH of the medium. However, at the pH 6.5, LT production was very little, and the excretion rate was about 0.2 % of the total LT produced.

Effects of aeration on growth and LT production of E. coli. Fig. 5 shows the effects of aeration on growth and LT production. The aeration effect was examined with or without shaking at 120 r.p.m. Both growth and LT production were markedly enhanced by aeration. The amount of LT produced per cell was increased about ninefold by aeration. Kinetics of LT production was similar whether aerated or not. The excretion of LT into the culture medium was not enhanced by aeration. The ratio of extracellular LT to total LT was somewhat less in aerated culture than in noneaerated culture (data not shown).

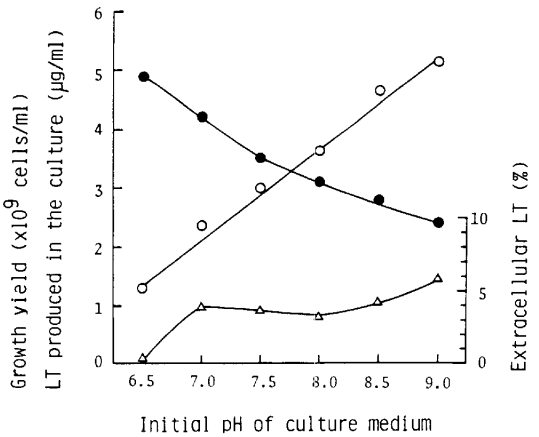

Fig. 4. Effect of pH of the medium on the growth and production of heat-labile enterotoxin of *E. coli*. *E. coli* 240-3 was cultured in CAYE medium supplemented with lincomycin (90 μg/ml) at 37℃ for 20 h. Initial pH of the medium adjusted as indicated. The amount of LT in the culture (○), the growth yield (●) and extracellular LT (△) expressed as a percentage of the total LT produced.

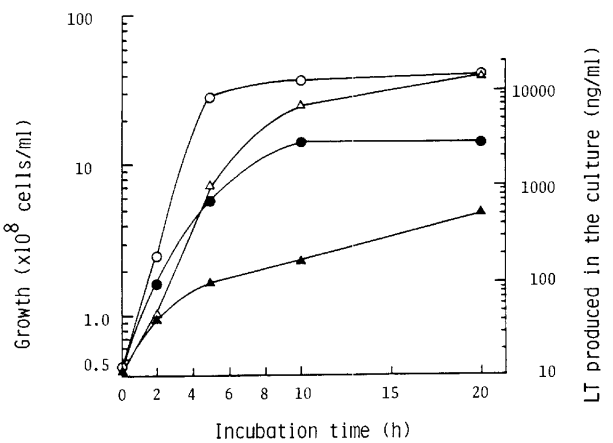

Fig. 5. Effects of aeration on the growth and production of heat-labile enterotoxin of *E. coli*. Growth of *E. coli* 240-3 (○, ●) and amount of LT produced (△, ▲) were determined with (open symbols) or without (closed symbols) shaking at 120 r.p.m. Lincomycin (90 μg/ml) was added, and the inoculum size was 1%. Polymyxin B was added to the culture to make the total LT preparation as described in Materials and Methods.

DISCUSSION

Heat-labile enterotoxin of enterotoxigenic *E. coli* has been detected by various methods. Although the methods appear to be specific for LT, the procedures are rather complicated and not very quantitative. The availability of purified

LT increased by the purification method first described by Clements and Finkelstein (22) and slightly modified by Takeda *et al.* (21), and antibody made it possible to measure the amount of LT by various immune reactions. Evans and Evans reported the PIH method for the assay of LT. A good correlation between the PIH method and Y-1 adrenal cell assay was demonstrated with enterotoxigenic *E. coli* isolated from human feces (18).

In this paper, the procedure for passive immune hemolysis was modified. Tris-buffered saline containing Ca^{2+} and Mg^{2+} was used as a working buffer instead of phosphate buffer. Ca^{2+} and Mg^{2+} are essential for the hemolysis through the classical pathway of complement reaction. The LT preparation was adsorbed to sheep erythrocytes, and the unbound contaminants were removed from the assay medium by washing LT sensitized sheep erythrocytes. Purified anti-LT IgG was used as antitoxin. The degree of hemolysis was measured at the wavelength of 412 nm instead of 420 nm (26). The use of 412 nm enhanced the sensitivity. The degree of hemolysis was expressed as the average number of effective lytic sites per cell (Z) (26, 27). With the modifications described above, the LT assay can be performed very sensitively, quantitatively and consistently.

Studies on the optimal nutritional conditions for synthesis of LT (20, 28), and on factors affecting the release of LT into the culture medium (29) using less quantitative methods have been extensively reported. We examined the correlation between cell growth and production of LT under various conditions using the modified PIH method. Production of LT was intesive from the middle to the late logarithmic growth phase. It was not produced during the stationary growth phase. Levner *et al.* (30) reported that lincomycin enhanced LT production, especially in a lincomycin resistant strain of *E. coli* ; however, they did not examined the effect of lincomycin on the relation between cell growth and LT production. In the presence of lincomycin, the production continued up to the stationary growth phase. Lincomycin-induced production was detected both intracellularly and extracellularly. Lincomycin did not stimulate the excretion of LT from the cells into the culture medium. Thus, lincomycin seems to stimulate the synthesis of LT. The *E. coli* strain used in this study was able to grow in CAYE medium supplemented with 50 and 100 μg lincomycin/ml, though the cell yields decreased by 33 % and 52 % after 20 h incubation, respectively. Nevertheless, the amount of LT produced was markedly increased by addition of 50 to 100 μg lincomycin/ml, indicating that lincomycin at the concentration where the growth of *E. coli* is slightly inhibited stimulates the production of LT.

The initial pH of the culture medium affected cell growth and production of LT. The growth yield was inhibited as the pH was raised to 9.0, whereas LT production was enhanced. These results show that the optimal condition for growth is not consistent with the optimal condition for LT production. Thus, there may be some control mechanism for the production of plasmid-coded protein by host cells.

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