Urea-Induced Release of Heat-Labile Enterotoxin from *Escherichia coli*

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Urea induces the release of heat-labile enterotoxin (LT) from cells of LT-producing *Escherichia coli* strains. Optimal conditions were defined by using the checkerboard immunoblotting system. LT release was highest when *E. coli* cells were incubated in 8 M urea, pH 8.0, at 37°C in a water bath for 30 min. Urea was more effective than polymyxin B in inducing the release of LT antigen from *E. coli*; the activity of LT from urea-treated cells was seven times that of LT from polymyxin B-treated cells. Urea also increased the antigenic and biological reactivities of purified LT. This procedure is potentially applicable for the detection of LT-producing *E. coli* strains in the clinical laboratory.

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of acute diarrheal disease in developing countries as well as among travelers in such areas (1-3, 26). ETEC organisms elaborate either a heat-labile enterotoxin (LT), a heat-stable enterotoxin, or both toxins (21). Various bio- and immunoassays which detect LT production in cultures of ETEC have been described. In most of the procedures reported so far, polymyxin B has been used to induce the release of LT from ETEC (8, 11, 13, 15, 25), although Clements and Finkelstein reported in 1979 (6) that lysates were a better source of LT than culture supernatants.

It has previously been demonstrated that urea, a chaotropic agent, caused cell lysis of *E. coli* and of *Vibrio cholerae* (16, 22). In the present study, we report that urea causes the release of LT from ETEC. Using a newly developed immunoassay, checkerboard immunoblotting (CBIB) (18, 19), we investigated the conditions of LT release by urea, compared the effects of urea and polymyxin B, and assayed 60 *E. coli* isolates from humans and from pigs.

MATERIALS AND METHODS

Strains and growth conditions. The reference strains used in this study were *E. coli* H-74-114, isolated in New York City and originally obtained from Ruth Rappaport (24), Wyeth Laboratories, Philadelphia, Pa.; H-10407, isolated in Bangladesh and obtained from D. J. Evans (7), the University of Texas, Houston; H-240-3, isolated from a traveler at Osaka International Airport Quarantine Station and obtained from T. Tsuji, Osaka University, Osaka, Japan; and P-60, obtained from H. W. Moon, National Animal Disease Center, Ames, Iowa. These strains (9, 10) produced H-LT-1 (12, 20), H-LT-2 (29), H-LT-3 (28), and P-LT (17), respectively. These four strains were used, in parallel, in studies to evaluate the optimum conditions for LT release by urea. Mean and standard error were calculated (Sigma-Plot; Jandel Scientific, Corte Madera, Calif.). In addition, 30 *E. coli* strains of human origin, which were provided by T. Honda and Y. Takeda of the Research Institute for Microbial Diseases (Biken) of Osaka University, Osaka, Japan, for the

World Health Organization study of the Biken test for LT (27), and 30 porcine *E. coli* strains, which were provided by Harley Moon of the National Animal Disease Center, were examined. All strains were stored in 20% glycerol in tryptic soy broth (Difco Laboratories) at -70°C until used. Cells were prepared by growing them overnight in nutrient broth at 37°C and then adding a 20- μ l inoculum to 10 ml of Casamino Acids (Difco)-yeast extract-salts medium (23) in 125-ml Erlenmeyer flasks for LT production. The cultures were agitated aerobically in an incubator shaker at 200 rpm at 37°C for 12 h. The cells were harvested by centrifugation at 12,000 $\times g$ for 30 min at 4°C, and the pellet was then washed once by resuspension and centrifugation, as described above, in 10 ml of 0.15 M Tris hydrochloride buffer, pH 8.0.

Urea-induced release of LT. The toxin release buffer was composed of 8 M urea (molecular biology reagent grade; Sigma Chemical Co., St. Louis, Mo.) and 0.15 M Tris hydrochloride, pH 8.0, containing 0.9% NaCl. The cells were prepared as described above, incubated in 0.4 ml of toxin release buffer at 37°C in a water bath for 30 min, and centrifuged for 10 min at 4°C in a tabletop Brinkmann Eppendorf 3200 centrifuge. The supernatant was harvested for the detection of LT. Other samples of urea (American Chemical Society-certified grade [Fisher Scientific, Fair Lawn, N.J.], ultrapure grade [Schwarz Mann Biotech, division of ICN Biomedicals, Inc., Cleveland, Ohio], and enzyme grade [United States Biochemicals Corp., Cleveland, Ohio] [USB]) were also evaluated.

Polymyxin B-induced release of LT. To examine polymyxin B-induced release of LT (8), the cells, prepared as described above, were incubated in 0.4 ml of polymyxin B (Sigma Chemical Co.) (20,000 U/ml) in 0.15 M Tris hydrochloride (pH 6.0) containing 0.9% NaCl at 37°C in a water bath for 30 min and then centrifuged. The supernatant was tested.

Conditions of urea-induced release of LT. To study the effect of pH on urea-induced release of LT, portions of toxin release buffer were adjusted to various pHs (from pH 4 to 10) with 0.5 N NaOH or 0.5 N HCl. After adjustment, toxin release buffers were added to the bacterial cells. To determine the effects of incubation temperature and time on the urea-induced release of LT, two sets of samples were incubated in toxin release buffer at 37 and 22°C. A sample

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FIG. 1. Effects of concentration of urea and pH on LT release from *E. coli*. (A) Cells were incubated in various concentrations of urea at 37°C for 30 min; (B) cells were incubated in 8 M urea at various pHs at 37°C for 30 min. Circles and bars represent means and standard errors of four LT-producing strains (H-74-114, H-10407, H-240-3, and P-60) in CBIB.

from each set was removed at 5, 10, 20, 30, 60, and 90 min, brought to 4°C, and then assayed.

Enterotoxin assay. The CBIB employed here was recently described in detail (18, 19). Urea-treated supernatants of *E. coli* cultures were initially tested undiluted. To evaluate the effects of different conditions on the release of LT, test and control supernatants diluted in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate [pH 9.6]) containing 8 M urea were immobilized on a nitrocellulose membrane (0.22- μ m pore size) in parallel lanes by using a Miniblotter apparatus (Miniblotter 45; Immunetics, Cambridge, Mass.) and incubated at 37°C for 60 min. Nonspecific binding sites were saturated at 4°C overnight with 5% nonfat dry milk in phosphate-buffered saline containing 0.05% Tween 20. Primary antibodies were then applied in lanes perpendicular to the antigens and incubated at 37°C for 60 min, and the reactions were developed with appropriately labeled secondary antibodies at room temperature for 2 h. After a washing, substrates (200 μ M) in 10 mM Tris hydrochloride buffer (pH 7.4) and H₂O₂ (0.1%, vol/vol) were added. Reactions were stopped by rinsing with water. Positive reactions appear as colored squares at the intersections of antibodies and antigens, giving a checkerboard appearance to the developed blot. The titer of LT was the highest dilution giving a colored square on the nitrocellulose membrane visible to the naked eye. Nonenterotoxigenic *E. coli* was used as a negative control in all tests.

The rabbit skin test for vascular permeability was employed to determine the biological activity of toxins (6, 7). Purified enterotoxins (H-LT-1, H-LT-2, and P-LT), untreated and treated with 8 M urea, were serially diluted with TEAN buffer (4) and then injected in duplicate intracutaneously into the backs of depilated rabbits. The response was determined at 18 h by measuring to the nearest millimeter the diameter of the localized zones of intense blueing 2 h after the intravenous administration of trypan blue. Urea and TEAN were used as negative controls.

Reference LT. H-LT-1, H-LT-2, and P-LT were prepared as described previously (10). H-LT-3 was kindly provided by T. Tsuji (28). The monoclonal antibodies (MAbs) used were directed against the immunodominant B-subunit proteins; of these, one was specific for H-LT-1, one recognized all three H-LTs, one recognized the H-LTs and P-LT, and one was

specific for P-LT, as previously described (10). The preparations of hyperimmune goat anti-H-LT, anti-P-LT, and anti-cholera toxin (CT) sera have been described elsewhere (4, 5, 10, 12).

RESULTS

Effect of concentration of urea on LT release. Portions of toxin release buffer were adjusted to various concentrations (1 to 10 M) of urea. After adjustment, toxin release buffer was added to the bacterial cells at 37°C for 30 min. Figure 1A shows that 8 M urea caused the maximum release of LT; a further increase in the concentration of urea did not increase the release of LT, and lower concentrations resulted in lower yields.

Effect of pH on LT release. Maximal release of LT was obtained from pH 7 to 10. Below pH 6, less LT was apparently released from *E. coli* under the same incubation conditions (Fig. 1B).

Effects of incubation temperature and time on LT release. LT began to be released during the initial 5-min incubation at 37 and 22°C, but titers were low. Titers of LT reached a maximum at 30 min. Titers of LT were a little higher at 37°C than at 22°C, but this difference was not statistically significant ($P > 0.05$). On the basis of these experimental results, the release of LT from *E. coli* strains was subsequently accomplished with 8 M urea, pH 8, at 37°C for 30 min.

Comparison of the effects of urea and polymyxin B on LT release. Since the report by Evans et al. (8), polymyxin B has been widely used to induce the release of LT from *E. coli*. To compare the effects of urea and polymyxin B on LT release, two sets of the same cells were incubated in 8 M urea or in polymyxin B (20,000 U/ml) at 37°C in a water bath for 30 min and then assayed. Figure 2 shows that the mean titer of LT in the supernatant of urea-treated cells, $1:266 \pm 60$, was approximately seven times that of polymyxin B-treated cells, $1:36 \pm 16$ ($P < 0.01$). Interestingly, adding 8 M urea to the supernatant of polymyxin B-treated cells increased the titer of LT twofold (Fig. 2). This result suggested that urea not only induced the release of LT from *E. coli* strains but also increased the antigenic reactivity of the LT.

Effects of various urea preparations on LT release. All of the data in this report were obtained by using urea from

FIG. 2. Comparison of effects of urea and polymyxin B (PMB) on LT release from *E. coli*. The cells were incubated in 8 M urea (open bar) or polymyxin B (20,000 U/ml) (hatched bar). Urea (8 M) was added to the supernatant of polymyxin B-treated cells (Sup+Urea) (solid bar). Values are means and standard errors of four LT-producing strains in CBIB.

Sigma (lot 99F-0691). Examination of samples of urea from various other sources (Fisher, ICN, and USB) revealed that there were no significant differences in inducing the release of LT from *E. coli* in various urea preparations (results not shown).

Effect of 8 M urea on various purified LTs. Purified LTs, including H-LT-1, H-LT-2, H-LT-3, and P-LT, were serially diluted with 8 M urea and then assayed with several MABs and polyclonal antibodies. Figure 3 shows that reactions between urea-treated LTs and antibodies were much stronger than those between untreated LTs and antibodies. LT was easily detectable at concentrations of 2.5 $\mu\text{g/ml}$ (i.e., ~ 5 ng per square), especially with goat polyclonal anti-H-LT-1. As in previous studies (10, 19), one MAB was specific for H-LT-1, one was reactive with each of the H-LTs but not with P-LT, one recognized all the LTs, and one was specific for P-LT. The MABs were slightly less reactive than the polyclonal goat anti-H-LT-1 under the conditions described in these tests. Polyclonal anti-P-LT reacted with each of the LTs, but it reacted more strongly with the homologous antigen. Goat anti-CT also reacted with all of the LTs, but not strongly.

The results of rabbit skin tests showed that, if anything, urea enhanced the biological activity of LT; the minimal amounts that caused a 7-mm blueing reaction were approximately 0.25 ng for urea-treated LTs and 7 ng for untreated LTs.

Detection of urea-induced LT from 60 *E. coli* strains. To evaluate the possible application of urea-induced release of LT from *E. coli* strains in a clinical setting, 20 LT-producing *E. coli* strains of human origin, 20 of porcine origin, and 20 LT⁻ *E. coli* strains, as determined in the latex particle agglutination test (11) and CHO cell assay (14), were studied. Figure 4 shows that all strains which previously gave positive reactions in the latex particle agglutination test and CHO cell assay were also positive in the CBIB, whereas all nonenterotoxigenic strains gave negative reactions.

The anti-H-LT-specific MAB reacted only with strains of human origin, the anti-P-LT-specific MAB reacted only with porcine strains, and the anti-LT MAB reacted with LT from both human and porcine strains under the conditions de-

FIG. 3. Effect of urea on purified LTs. CBIB reactions of various monoclonal antibodies (mAb) (1:10) and polyclonal antibodies (pAb) (1:5,000) with LT proteins in urea-treated (U) and untreated (N) forms at various concentrations are shown. α denotes goat anti-toxin. CT, Cholera toxin.

scribed. Polyclonal antisera reacted with LTs of both human and porcine strains, but the reactions were stronger with the homologous LTs.

DISCUSSION

Since the initial report of Evans et al. (8), polymyxin B has been widely used to liberate periplasmic LT from *E. coli* cells, particularly for the detection of LT in the laboratory. In 1979, Clements and Finkelstein reported (6) that the whole-cell lysate was a better source of *E. coli* LT than the culture supernatants, and they used sonication to lyse fermentor-grown cells for extracting LT and purifying it to homogeneity. However, sonication is not a convenient method for releasing LT in a clinical setting. In 1981, Ingram (16) observed that urea caused the lysis of growing *E. coli* cells. The observations were confirmed in 1984 by Lohia et al. (22), who also reported that urea caused the rapid lysis of stationary-phase cells of *V. cholerae*.

The present study reveals that urea promotes the release of LT from *E. coli*. In order to define the optimum conditions for LT release from *E. coli*, the effects of various concentrations of urea, pH, incubation time, and temperature on LT release, as measured with CBIB, were investigated.

Our results show that 8 M urea caused maximal LT release at 37°C for 30 min. A further increase in the concentration of urea did not affect LT release, and lower concentrations

FIG. 4. Detection of urea-induced LT from 60 *E. coli* isolates in CBIB. Strains H-74114, H-10407, H-240-3, and P-60 produced H-LT-1, H-LT-2, H-LT-3, and P-LT, respectively. Strains 1 through 30 were from humans and 31 through 60 were from pigs. Positive results (yellow-brown squares visible to the naked eye) were given by strains 1 through 20 and 31 through 50. Negative results were given by strains 21 through 30 and 51 through 60. Gα denotes goat antitoxin.

were less effective. LT release began as early as 5 min after contact with urea and was maximal at 30 min. LT release was slightly, but not significantly, better at 37°C than at 22°C.

The pH of the toxin release buffer is potentially important for maintaining LT activity. We found that LT release was maximal from pH 7 to 10; however, below pH 6.0, LT activity was decreased. The differences in LT yield with change of pH suggest that either *E. coli* cells released less LT or a substantial amount of released LT was inactivated when the pH of toxin release buffer was decreased (below pH 5).

In the present study, release of LT by urea was compared with that by polymyxin B; it was found that the LT titer was much higher in the supernatant of urea-treated cells than in the supernatant of polymyxin B-treated cells. It was interesting to observe that the titer of LT increased when urea was added to the supernatant of polymyxin B-treated cells. Further, when urea was added to the purified LTs, the reactions between urea-treated LTs and antibodies were

much stronger than those of untreated LTs and antibodies. Further, permeability factor assays in rabbit skin indicated that the biological activities of the LTs (H-LT-1, H-LT-2, and P-LT) were enhanced by urea treatment. These results suggest that urea not only induces LT release from *E. coli* but also increases the antigenic and biological reactivity of LT, perhaps by changing the conformation of the native holotoxins. This aspect is under further study in our laboratory (19).

Results with urea treatment of 60 *E. coli* isolates from humans and pigs were identical to those obtained by the latex particle agglutination test and CHO cell assay (11, 14). Both MABs and polyclonal antibodies are suitable to assay urea-released LT. The potential application of the urea release technique to the identification of LT-producing *E. coli* in the clinical laboratory is also under investigation in our laboratory.

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