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Glutamic acid-112 of the A subunit of heat-labile enterotoxin from enterotoxigenic *Escherichia coli* is important for ADP-ribosyltransferase activity

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A mutant strain of enterotoxigenic *Escherichia coli* (*E coli* pTUH 6A) produced an abnormal heat-labile enterotoxin (LT), the A subunit of which has a single amino acid substitution at position 112 (Glu-112 to Lys-112) As already reported, this mutant LT had no ileal loop and vascular permeability activities [(1990) J Biol Chem 265, 22520–22525] In this paper we report that the mutant LT showed no CHO cell elongation activity and did not activate adenylate cyclase of target cells Moreover, no ADP-ribosyltransferase activity was detected in the mutant LT It is concluded that the amino acid substitution at position 112 abolished the ADP-ribosyltransferase activity of the A subunit and this leads to the loss of toxic activities of LT

Heat-labile enterotoxin (LT), Mutant LT, Adenylate cyclase, ADP-ribosyltransferase

1 INTRODUCTION

Enterotoxigenic *Escherichia coli* produces a heat-labile enterotoxin (LT), which is similar to cholera toxin (CT) from *Vibrio cholerae* and causes severe diarrhea in humans Both LT and CT are composed of A subunit carrying biological activities and B subunit that binds the toxin to G_{M1} ganglioside receptors on the target cells [1] In both LT and CT, the A subunit consists of A_1 and A_2 fragments, which are linked to each other by a disulfide bond. In CT, a proteolytic nick is introduced between the A_1 and A_2 fragments after secretion of the toxin *V cholerae*, whereas the A subunit of LT is almost unnicked and the toxin accumulates in *E coli* cells In any case, the A_1 fragment of both LT and CT activates adenylate cyclase of the target cells by catalyzing the ADP-ribosylation of the GTP-binding protein (Gs) of the cyclase system [2]. In fact, the A_1 fragment has been reported to catalyze the transfer of the ADP-ribose moiety of NAD to arginine or other simple guanidium compounds such as agmatine (ADP-ribosyltransferase activity) [3]. In a previous paper [4], we reported that a single amino acid substitution in the LT-A subunit at position 112 (Glu-112–Lys-112) leads to the loss of LT toxic activities measured in the rabbit ileal loop test and rabbit skin permeability test. Here we report that the loss of toxic activities in the mutant LT is due to the loss of ADP-ribosyltransferase activity

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2 MATERIALS AND METHODS

2 1 *Materials and bacteria*

NAD dithiothreitol and agmatine were purchased from Sigma and [adenylate-^{32}P]NAD (250Ci/mol) from ICN Other chemicals used were of reagent grade Wild strain (*E coli* pEWD 299) was kindly provided by Dr W S Dallas [5] and its mutant strain (*E coli* pTUH 6A) was prepared as described previously [4]

2 2 *Purification of normal and mutant LTs*

Normal and mutant LTs were purified as described previously by Clements and Finkelstein [6] Detailed procedures of cell culture, isolation of crude cell extracts and LT purification by successive chromatography were as described previously [4]

2 3 *Determination of c-AMP response of CHO cell to LTs*

CHO cells 10^4, were cultured in MEM containing 10% fetal calf serum for 2 days, washed 3 times and replaced into fresh medium The cells were treated with the normal and mutant LTs at various concentrations After a 10 min incubation, the concentration of the c-AMP was determined with a c-AMP determination kit (Yamasa Co) as described previously [7]

2 4. *ADP-ribosylagmatine assay*

The formation of ADP-ribosylagmatine was determined as described previously [8] Each sample contained 50 mM potassium phosphate (pH 7 5), 100 μM GTP, 5 mM $MgCl_2$, 100 μM [adenine-U-^{14}C]-NAD, 10 mM agmatine, 0 1 mg/ml of ovalbumin and other materials as described (total volume 300 μl) Toxins were added to the reaction toxins. After 60 min at 30°C, three 50 μl samples were transferred to AG1-X2 column which were washed four times with 1 25 ml water Eluates containing [adenine-U-^{14}C]ADP-ribosyl-agmatine were collected for radioassays

2 5 *ADP-ribosylation*

Assay samples (total volume 300 μl) contained 1 μM [^{32}P]NAD (2 μCi), 10 mM thymidine, 1 mM EDTA, 5 mM $MgCl_2$, 2 mM DTT, 100 mM Tris-HCl (pH 7 5) and other additions as indicated Rabbit leukocyte membranes (100 μg protein/assay) were incubated with mutant LT (10 0 μg), normal LT (10 0 μg) or A subunit of CT (2 5 μg) After incubation at 37°C for 1 h, 2 ml of cold 7 5% trichloroacetic acid and 10 μg of bovine serum albumin were added, and samples kept on ice for 30 min Precipirated proteins were pelleted by centrifugation and dissolved in 1% SDS/5% mercaptoethanol (60°C, 10 min) Samples were subjected to electrophoresis in 12% polyacrylamide gels as described previously [9]

3 RESULTS

3 1 *CHO cell elongation caused by wild-type and mutant LTs*

We first compared the CHO cell elongation activities of wild-type and mutant LTs. As shown in Fig 1, wild-type LT caused the elongation of 50% of the cells at a concentration of 5 75 ng/ml, whereas mutant LT did not cause significant elongation even at a concentration as high as 10 μg/ml. Since Gurreant et al [10] have suggested that the elongation of CHO cells by CT and LT is due to an increase in cAMP concentration in the target cells, we next determined the cAMP concentration in CHO cells after exposure wild-type and mutant LTs.

3 2. *cAMP concentrations in CHO cells after exposure to wild-type and mutant LTs*

As shown in Fig 2, the cAMP concentration in CHO cells increased markedly after exposure to wild-type LT The increase was evident even at a LT concentration as low as 0.1 μg/ml Exposure to mutant LT, on the other hand, did not increase the cAMP concentration even at a concentration as high as 161 μg/ml.

These results suggest that mutant LT does not activate adenylate cyclase and thus is unable to elongate CHO cells

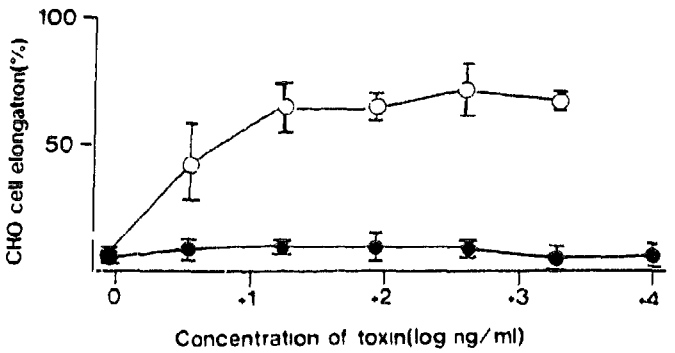

Fig 1 Dose-response curves of normal and mutant LTs in CHO cell assay CHO cell assay was performed as described previously [9] 18 h after each sample was applied to CHO cells, elongation activity was determined Values are means±SE of 3 determinations ( ) normal LT (●) mutant LT

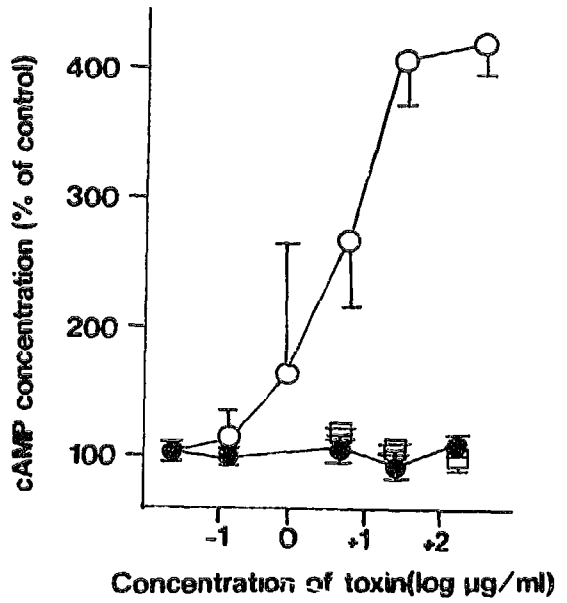

Fig 2 Comparison of the c-AMP response of CHO cells to normal and mutant LTs Cells cultured in MEM containing 10% fetal calf serum were washed and exchanged into fresh medium and the cAMP concentration was determined as described in Materials and Methods The concentration of the toxin is expressed as the final concentration in the medium Values are means ± SE of 3 determinations (○) normal LT, (●) mutant LT, (□) coligenoid

Table I

NAD agmatine ADP-ribosyltransferase activity of normal and mutant LTs with or without Triton X-100

Additions	0 1% Triton X-100	Formation of [adenine-U-^{14}C]-ADP-ribosylagmatine (cpm)
Expt I		
None	–	87±10 7
Cholera toxin A subunit		
(2 5 μg)	–	1648±77 2
(5 0 μg)	–	3349±63 8
Normal LT		
(2 5 μg)	–	181±73 0
(10 0 μg)	–	711±45 3
Mutant LT		
(2 5 μg)	–	85±7 4
(10 0 μg)	–	102±12 1
Expt II		
None	–	97±13 8
	+	95±9 9
Cholera toxin A subunit		
(10 0 μg)	–	1672±52 3
(10 0 μg)	+	2189±104 1
Normal LT		
(10 0 μg)	–	750±43 0
(10 0 μg)	+	972±38 0
Mutant LT		
(9 9 μg)	–	91±36 5
(9 9 μg)	+	91±13 2

Values are calculated from 3 independent experiments

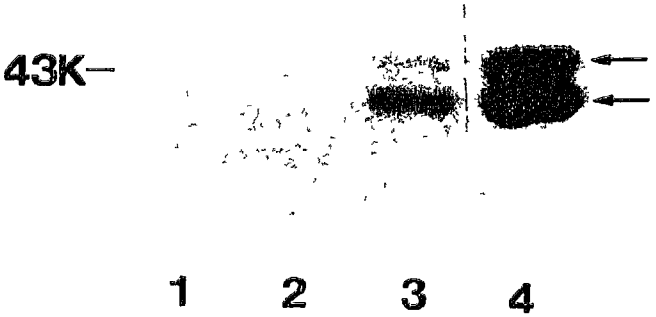

Fig 3 ADP-ribosylation of rabbit leukocyte membrane protein by mutant LT, normal LT, or A subunit of CT A rabbit leukocyte membrane preparation (100 μg protein) and 1 μg [^{32}P]NAD (2 μCi) were incubated without toxin (lane 1) and with 10 0 μg mutant LT (lane 2), 10 0 μg normal LT (lane 3), or 2 5 μg CT A subunit (lane 4) in the presence of 100 μM GTP as described in Materials and Methods The autoradiogram of the SDS-polyacrylamide gel (12%) of the labelled protein is shown 43K is marker and arrows show ADP-ribosylation of membrane proteins

3 3. *Comparison of the ADP-ribosyltransferase activity of the normal LT with mutant LT*

It has been reported that by ADP-ribosylation of the cyclase Gs protein, the A1 fragment activates adenylate cyclase and its ADP-ribosyltransferase catalyzes it [3]. We determined whether or not the A subunit of mutant LT had ADP-ribosyltransferase activity. As shown in Table I, normal LT had ADP-ribosyltransferase activity, which increased with the addition of Triton X-100. However, mutant LT did not show such activity with or without Triton X-100.

These data suggest that as the A subunit of mutant LT does not have ADP-ribosyltransferase activity, adenylate cyclase is not activated and that Glu-112 is important for this activity

3 4 *ADP-ribosylation caused by wild-type and mutant LTs*

We determined whether mutant LT could make ADP-ribosylation of membrane protein As shown in Fig. 3, normal LT and A subunit of CT could make ADP-ribosylation of two proteins, which contained Gs protein of adenylate cyclase, as described previously [11] However, mutant LT could not do this

These data suggest that mutant LT cannot transfer ADP-ribose to Gs protein of adenylate cyclase

4 DISCUSSION

The relationship between structure and function of the A subunit of LT has been performed by chemical modification and mutagenesis. Chemical modification determined that Arg-146 of the A subunit is important for the auto-ADP ribosylation site though lately recent results have indicated otherwise [12,13] By mutagenesis, Harford et al [14] reported that one amino acid substitution (Ser-61 to Phe-61) affected the biological activity by causing a defect in ADP-ribosyltransferase Moreover, we also reported that one amino acid substitution (Glu-112 to Lys-112) resulted in the loss of its toxic activity [4] but affected neither its binding ability to coligenoid nor its nicking site on the A subunit by trypsin [4] We examined which enzymatic activity might be defective in this mutant LT-A subunit

As shown in Figs 1 and 2, mutant LT could not elongate CHO cells due to an alteration in its ability to stimulate adenylate cyclase As the mutant LT A subunit did not have any ADP-ribosyltransferase activity (Table I), it could not activate cyclase without ADP-ribosylation of Gs protein In fact, ADP-ribosylation of the Gs protein by this mutant LT did not occur (Fig 3). Moreover, NAD glycohydrolase activity decreased in the mutant LT and increasing dehydration of NAD did not result in a loss of ADP-ribosylation of Gs protein

Though CT and LT activate adenylate cyclase and increase c-AMP in intestinal epithelial cells, the relationship between diarrheal activity and the cAMP increase in epithelial cells has been examined but has not yet been clarified [15] However, as shown above, mutant LT resulted in a simultaneous loss of activation of adenylate cyclase and diarrheal activity Though it is impossible to say that in this mutant, adenylate cyclase activation is the only mechanism for diarrhea, at least the active sites of adenylate cyclase and diarrhea in the A subunit exist very close to each other and that Glu-112 is involved in both active sites

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