***Escherichia coli* LT enterotoxin subunit A demonstrates partial toxicity independent of the nicking around Arg192**

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A study was conducted into whether or not nicking of the A subunit of *Escherichia coli* LT enterotoxin at position Arg192 or its neighbouring amino acids Arg192 to The195 is required for its toxicity. The toxic activity of mutants created by substitution or deletion at this position, which lacked ADP-ribosyltransferase activity *in vitro*, was not completely obliterated and cyclic AMP was partially induced in the target cells, showing that they still displayed enzymic activity *in vivo*. Moreover, although the A subunit possesses three potential sites for cleavage by furin, furin was not involved in the partial toxicity and cyclic AMP induction observed. These data suggest that target cells have a nick mechanism that operates at sites other than those around Arg192 or those recognized by furin, which generates an active fragment by processing the A subunit after toxin binding to the cell membrane.

Keywords: heat-labile enterotoxin, enterotoxigenic *Escherichia coli*, nicking, furin

INTRODUCTION

Enterotoxigenic *Escherichia coli* produces a heat-labile enterotoxin (LT), which is structurally and biologically similar to cholera toxin (CT) from *Vibrio cholerae* and which causes severe diarrhoea in humans. CT and LT are composed of an A subunit with toxic biological activities and a B subunit that binds the toxin to G_{M1} ganglioside receptor on the target cells (Finkelstein, 1973; Clements & Finkelstein, 1979; Holmgren, 1981; Spangler, 1992). The A subunit consists of A₁ and A₂ fragments, which are linked by a disulfide bond. A proteolytic nick of CT or LT A subunit at position Arg192 or nearby residues is introduced between the A₁ and A₂ fragments after secretion from *V. cholerae*. The A subunits of both toxins are totally unnicked and accumulate in the periplasmic space of *E. coli* (Clements & Finkelstein, 1979; Pearson & Mekalanos, 1982; Mekalanos *et al.*, 1983; Neill *et al.*, 1983; Finkelstein *et al.*, 1983; Booth *et al.*, 1984; Hirst *et al.*, 1984).

Free A₁ fragments activate adenylate cyclase of the target cells *in vitro* by catalysing ADP-ribosylation of

GTP-binding protein (G_s), whereas the unnicked or nicked A subunits without reduction do not (Gill & King, 1975; Mekalanos *et al.*, 1979). In fact, the free A₁ fragment reportedly catalyses the transfer of the ADP-ribose moiety of NAD to arginine or other simple guanidium compounds such as agmatine (ADP-ribosyltransferase activity) or the ADP-ribosylation of the 42 or 43 kDa protein of the cell membrane (Moss *et al.*, 1979; Freissmuth & Gilman, 1989; Moss & Vaughan, 1990). Therefore, the ADP-ribosyltransferase activity and activation of the adenylate cyclase with ADP-ribosylation of G_s *in vivo* are known to be caused by the free A₁ fragment (Moss & Vaughan, 1990).

As the unnicked LT shows the same toxicity as the nicked LT in the ileal loop test (Rappaport *et al.*, 1976; Clements & Finkelstein, 1979) and CHO cell assay (Kunkel & Robertson, 1979; Tsuji *et al.*, 1984), a trypsin-like protease might completely nick the amino acid sequence around Arg192 in these assays. Moreover, though the biological activity of unnicked LT is lower than that of nicked LT in the rabbit skin permeability (PF) test (Clements & Finkelstein, 1979; Takeda *et al.*, 1981), unnicked LT is considered to be partially nicked at Arg192 or nearby residues by an unknown protease from target cells.

However, in this paper, we demonstrate that LT has partial toxicity and induces cyclic AMP independent of

Abbreviations: CT, cholera toxin; FCS, foetal calf serum; LT, heat-labile enterotoxin; LT-A, A subunit of heat-labile enterotoxin; rLT-B, recombinant B subunit of heat-labile enterotoxin; LT(CE), normal heat-labile enterotoxin from cell extract; PF, rabbit skin permeability.

Table 1. Summary of the mutant toxins prepared in this study

Mutant name	Amino acid deletion(s) or substitution(s)
Group I	
R192G	Substitution from Arg192 to Gly192
Del192	Deletion of Arg192
Group II	
Del192–193	Deletion of Arg192 and Thr193
R192G + Del193–194	R192G plus deletion of Thr193 and Ile194
R192G + Del194–195	R192G plus deletion of Ile194 and Thr195
Del192–194	Deletion from Arg192 to Ile194
Del192–195	Deletion from Arg192 to Thr195
Gly192–194	Deletion of Gly192–Gly193–Gly194
Group III	
R192G + R138G	Double substitutions from Arg to Gly at positions 192 and 138
R192G + R143G	Double substitutions from Arg to Gly at positions 192 and 143
R192G + R146G	Double substitutions from Arg to Gly at positions 192 and 146
R192G + R148G	Double substitutions from Arg to Gly at positions 192 and 148

the nick in the Arg192 region. Moreover, potential cleavage sites of furin are also present in the LT A subunit (LT-A) from positions 138 to 141 (Arg¹³⁸-Leu-His-Arg¹⁴¹), from 141 to 146 (Arg¹⁴¹-Asn-Arg¹⁴³-Glu-Tyr-Arg¹⁴⁶) or from 146 to 151 (Arg¹⁴⁶-Asp-Arg¹⁴⁸-Tyr-Tyr-Arg¹⁵¹). The peptides from 141 to 146 and 146 to 151 are likely to be cleavage sites for furin, but three arginines are essential (Nakayama *et al.*, 1992). Therefore, we determined whether sites of furin cleavage were involved in partial toxicity, as they are in diphtheria (Tsuneoka *et al.*, 1993) and shiga (Garred *et al.*, 1995) toxins.

METHODS

Bacteria. The plasmid EWD299 (Dallas *et al.*, 1979) carrying the LT gene from a porcine enterotoxigenic *E. coli* strain was used. *E. coli* MV 1184 was used as competent cells. The vectors Bluescript II SK(+)-1 and SK(-)-1 were purchased from Toyobo.

Cell lines. BW5147 myeloma cells were donated by the Foundation for Promotion of Cancer Research, Setagaya, Tokyo, Japan. Furin-deficient L₀V₀ cells were kindly given by Professor E. Mekada, University of Kurume, Fukuoka, Japan.

Site-directed mutagenesis. Site-directed mutagenesis was performed by means of the polymerase chain reaction (PCR) as described by Saiki *et al.* (1985). A Bluescript II SK(-)-1 vector carrying the LT gene without the *HindIII*-*EcoRI* or *XbaI*-*HindIII* fragments of the LT-A gene was prepared. Detailed procedures and PCR conditions were as described by Ito *et al.* (1991).

The first PCR generated products with a mutation or a deletion. The second PCR generated *HindIII*-*EcoRI* or *XbaI*-*HindIII* fragments that carried a mutation or a deletion in LT-A. The second PCR product was sequenced using a DNA sequencer (Applied Biosystems) and ligated into the *HindIII*-*EcoRI* or *HindIII*-*XbaI* site of the LT gene.

Purification of mutant and normal toxins. Mutant toxin E112K and recombinant LT B subunit (rLT-B) were prepared as described previously (Tsuji *et al.*, 1990, 1994). Each toxin was purified by Bio-gel A5m or immobilized-D-galactose affinity chromatography as described by Clements & Finkelstein (1979) and Uesaka *et al.* (1994). Detailed procedures for culture of the cells, isolation of cell extracts and purification of LT by successive chromatography in TEAN buffer (50 mM Tris/HCl, 1 mM EDTA, 3 mM Na₂SO₄, 0.2 M NaCl pH 7.4) were as described by Tsuji *et al.* (1990). The LT fraction purified from EWD299 strain contained mainly unnicked LT and a small amount of nicked LT (Clements & Finkelstein, 1979). Therefore, this sample with or without trypsin treatment was referred to as LT(CE) or nicked LT, respectively. Trypsin treatment was performed by mixing 20 µg toxin with 1 µg trypsin at 37 °C for 60 min, as described by Rappaport *et al.* (1976).

NAD:agmatine ADP-ribosyltransferase assay.

The formation of ADP-ribosylagmatine was determined as described by Moss *et al.* (1993). Each sample contained 50 mM potassium phosphate pH 7.5, 100 µM GTP, 5 mM MgCl₂, nicotinamide[U-¹⁴C]adenine dinucleotide [6×10^4 c.p.m. (9.25×10^3 Bq)], 10 mM agmatine, 0.1 mg ovalbumin ml⁻¹ and other materials as described in a total volume of 300 µl. As ADP-ribosyltransferase activity of the toxin is activated by Triton X-100 (Tsuji *et al.*, 1991), 0.1% (v/v) Triton X-100 was added to the reaction mixture. Toxins were then added. After 60 min at 30 °C, each sample was transferred to AG1-X2 columns which were washed four times with 1.25 ml water. Eluate containing [U-¹⁴C]adenine dinucleotide ribosylagmatine was radioassayed.

Rabbit ileal loop test. The rabbit abdomen was opened under anaesthesia and 5 cm loops were ligated to the mid part of the small intestine as described by Lycke *et al.* (1992). Various duplicate doses of purified toxins in 1.0 ml TEAN buffer pH 7.4 were injected into the loops and the abdomen was closed. After 18 h, the rabbits were killed and the contents and length of the ligated loop were measured. Fluid accumulation was expressed as volume per unit length (ml cm⁻¹) and represented means of duplicates with one rabbit. We performed four independent experiments and the results presented are means $\pm$ SD from the four rabbits.

Rabbit skin permeability test. The PF test was performed as described by Craig (1964). Each sample was diluted with borate gelatin buffer and then 0.2 ml was subcutaneously injected into the rabbit skin at four spots for each experiment. After 24 h, 0.01% (w/v) Evans blue [1 ml (kg body wt)⁻¹] was injected intravenously and 1 h later blue spots were scored. Toxin activity was recorded as the dose required to produce a mean blueing score of 25 from the four spots.

CHO cell assay. Morphological changes in CHO cells were examined essentially as described previously (Tsuji *et al.*, 1984). The CHO cells, which were grown in Eagle minimal essential medium (MEM; Eagle, 1959) containing 10% (v/v) foetal calf serum (FCS) under 6% (v/v) CO₂ at 37 °C, were removed from culture vessels by treatment of trypsin. After the cells had been washed with the above medium, they were suspended in MEM containing 1% (v/v) FCS and 3×10^3 cells

were added to each chamber of an eight-chamber slide (Miles Scientific). They were then challenged with toxin for 18 h in a CO₂ incubator at 37 °C. The cells were fixed with absolute ethanol and stained with Giemsa for examination of their morphology.

Determination of the cyclic AMP response of myeloma cells or furin-deficient (L₀V₀) cells. Myeloma BW5147 or furin-deficient L₀V₀ cells (1×10^6) were cultured in MEM or HAM (Ham, 1965) containing 10% FCS for 2 or 4 d, respectively. After three washes with the above medium, the cells were placed in fresh medium and treated with the normal and mutant LTs at the given concentrations. They were incubated for the given periods and harvested in 0.4% (v/v) acetic acid followed by boiling at 100 °C for 5 min. The samples were centrifuged and the concentration of cyclic AMP was determined in the supernatants using a kit (Pharmacia) as described by Lycke *et al.* (1992).

RESULTS

Selection of the mutants

The mutant toxins prepared in this study are described in Table 1 and the mutation sites are shown in Fig. 1. Since it has been reported that R192G has partial toxicity (Grand *et al.*, 1994), we first prepared R192G and Del192 and determined their partial activity in the ileal loop test (Fig. 2).

The nick site from Arg192 to Thr195 is also important for toxin activation (Klapper *et al.*, 1976; Clements & Finkelstein, 1979; Mekalanos *et al.*, 1983; Booth *et al.*, 1984; Ichinose *et al.*, 1992). To determine whether this site might be involved in toxicity, we next prepared deletion mutants as described under group II (Table 1).

As shown in Fig. 1, three kinds of potential furin cleavage sites are also present in LT-A. To determine the role of furin in partial toxicity, we prepared mutant toxins with double mutations, one at position 192 and one at position 138, 143, 146 or 148 (group III of Table 1). Each mutant toxin was then purified and characterized.

Assembly of A and B subunits of the purified toxins

As shown in Fig. 3, R192G and Del192 separated into two bands on SDS-PAGE gels corresponding to the A and B subunits. The mobilities of the subunits were identical to those of LT(CE) and were not affected by trypsin treatment, suggesting that the A subunits of the mutant toxins are not digested by trypsin. The mutant toxins in groups II and III exhibited A and B subunits that were not affected by trypsin treatment.

Moreover, densitograms of these gels were scanned at 660 nm. The ratio (from 35 to 35.5%) of the mutant A to the normal B subunit was identical to that of the normal LT (37.2%). As Bio-gel and immobilized D-galactose have affinity for B subunit but not for A subunit, all of the eluted A subunit from these columns would be expected to bind to the B subunit.

These data suggest that the assembly of mutant A and B

subunits is not affected as a consequence of the mutations that were introduced.

Comparison of NAD:agmatine ADP-ribosyltransferase activity of each mutant

As shown in Table 2, ADP-ribosyltransferase activity was determined. R192G and Del192 had no enzymic activity at a dose of 320 µg with or without Triton X-100, though 5 or 40 µg of the nicked LT or LT(CE), respectively, showed some activity. The high dose of LT(CE) also had low enzymic activity as described by Moss *et al.* (1993). Some LT was nicked during the purification procedure, as described by Clements & Finkelstein (1979). Moreover, the mutant toxins in group II and group III also had no enzymic activity at a dose of 320 µg with trypsin treatment.

These data suggest that these mutant toxins do not release an active fragment when treated with trypsin. We then determined whether each mutant toxin had biological activity *in vivo*.

Comparison of biological activity of each mutant toxin by the PF test

As shown in Fig. 4, the dose response curves of R192G and Del192 were determined by the PF test. The dose required to produce a blueing score of 25 was 0.7 ± 0.3 , 6.3 ± 3.0 , 4.5 ± 2.0 , 11.5 ± 5.0 , $> 5 \times 10^3$ or $> 5 \times 10^3$ ng for nicked LT, LT(CE), R192G, Del192, E112K or rLT-B, respectively. Thus, the activity of LT(CE) was less than that of the nicked LT and similar to that of R192G and Del192. Moreover, the doses required to produce a blueing score of 25 for the mutants in group II and III were 5.0 ± 4.0 to 17.0 ± 7.2 , which were indistinguishable for those of LT(CE), R192G and Del192.

These data suggest that nicking around Arg192 and furin sites is not involved in the partial PF activity of the toxin. We then confirmed this finding in the CHO cell assay.

Biological activities of each mutant toxin in the CHO cell assay

In the CHO cell assay, the dose response curves of R192G and Del192 were determined as described in Fig. 5. The doses required to cause 50% elongation were 1.1 ± 1.9 , 1.4 ± 1.0 , 17.7 ± 12.0 , 14.1 ± 7.7 , $> 5 \times 10^3$ and $> 5 \times 10^3$ ng for the nicked LT, LT(CE), R192G, Del192, E112K and rLT-B. Thus, the toxicity of both mutants was about 10-fold less than that of LT. Moreover, the doses required for 50% elongation for the mutant toxins in group II and III were from 14.3 ± 4.2 to 22.5 ± 4.2 , suggesting the toxicity of these mutants is identical to that of R192G and Del192 in the CHO cell assay.

These data suggest that all of the mutant toxins in Table 1 have partial toxicity. We then determined whether they induced cyclic AMP in target cells.

Fig. 1. Amino acid sequence of the A subunit of LT. The arrows (↓) show the potential sites for furin cleavage at Asn142, Asp147 or Asn152. The three underlines between Arg138 and Arg151 show the peptides recognized by furin. 'Mutant sites' show the positions of arginine residues substituted by glycines in group III mutants. The black triangles (▼) show the known nick site (Klapper *et al.*, 1976; Finkelstein *et al.*, 1983; Mekalanos *et al.*, 1983; Booth *et al.*, 1984; Ichinose *et al.*, 1992; Grant *et al.*, 1994).

Fig. 2. Diarrhoeal activity of R192G, Del192, LT, E112K and rLT-B in the ileal loop test. The test was performed as described in Methods. Values are means $\pm$ SD of four independent experiments. Mean values, which gave 0.5 ml cm⁻¹ ileal loop activity, were 44.6 ± 25 , 56.2 ± 30 , 316 ± 157 , 363 ± 120 , $> 10^4$ and 10^4 ng of nicked LT, LT(CE), R192G, Del192, E112K and rLT-B, respectively. ○, Nicked LT; ●, LT(CE); □, R192G with trypsin treatment; ■, Del192 with trypsin treatment; △, E112K with trypsin treatment; ▲, rLT-B.

Comparison of the induction of cyclic AMP by the mutant toxins in myeloma cells

As shown in Fig. 6, cyclic AMP induction by R192G or Del192 was determined. LT(CE), R192G and Del192 induced 366 ± 44 , 239 ± 56 and 127 ± 56 or 201 ± 11 , 211 ± 39 and 150 ± 44 fmol cyclic AMP per 10^6 cells for 5 or 7 h inoculation, respectively. R192G or Del192 induced lower cyclic AMP levels than LT(CE), though

Fig. 3. SDS-polyacrylamide gel electrophoresis of purified normal and mutant LTs. Toxins R192G, Del192 and E112K were purified and digested with trypsin as described in Methods. Each sample was heated with 10 mM DTT at 100 °C for 2 min. SDS-PAGE was performed on a 15% acrylamide gel as described by Laemmli (1970). Lanes: 1, R192G without trypsin treatment; 2, Del192 without trypsin treatment; 3, normal LT without trypsin treatment; 4, R192G with trypsin treatment; 5, Del192 with trypsin treatment; 6, normal LT with trypsin treatment; 7, E112K with trypsin treatment; 8, E112K without trypsin treatment; 9, rLT-B.

the latent time before inducing the maximal response was similar. Moreover, the mutants in group II and III also induced cyclic AMP in myeloma cells.

For 5 or 7 h incubation, the values of cyclic AMP were from 110 ± 62 to 210 ± 55 or from 85 ± 6.0 to $120 \pm$

Table 2. ADP-ribosyltransferase activity of normal and mutant toxins

Toxin concn (μg)	Triton X-100 (0.1%)	[U- ^{14}C]adenine dinucleotide ribosylagmatine concn* (c.p.m.)
LT (nicked)		
2.5	—	0
5.0	—	602
10.0	—	2300
20.0	—	4210
20.0	+	4520
LT(CE)		
20	—	0
40	—	280
80	—	760
160	—	1250
160	+	1320
Mutant (R192G or Del192)		
80	—	0
160	—	0
320	—	0
320	+	0
E112K and rLT-B		
320	—	0
320	+	0

* Determined as radioactivity induced by the toxin minus radioactivity induced by TEAN buffer (pH 7.4, blank). Values are means of two independent experiments.

Fig. 4. Comparison of toxicity of R192G or Del192 with that of LT, E112K and rLT-B by the PF test. The test was performed as described in Methods. Blueing scores were determined as described by Craig (1964) and the values are means $\pm$ SD for three independent experiments. $\circ$, Nicked LT; $\bullet$, LT(CE); $\square$, R192G with trypsin treatment; $\blacksquare$, Del192 with trypsin treatment; $\triangle$, E112K with trypsin treatment; $\blacktriangle$, rLT-B. **Fig. 5.** Comparison of elongation activity of R192G or Del192 with that of LT, E112K and rLT-B in the CHO cell assay. The assay was performed as described in Methods. Values are means $\pm$ SD for three independent experiments. $\circ$, Nicked LT; $\bullet$, LT(CE); $\square$, R192G with trypsin treatment; $\blacksquare$, Del192 with trypsin treatment; $\triangle$, E112K with trypsin treatment; $\blacktriangle$, rLT-B. **Fig. 6.** Comparison of cyclic AMP induction by the mutant toxin with LT, E112K and rLT-B. Myeloma cells (10^6) were cultured in MEM containing 10% FCS and then transferred to fresh medium. The toxin was added at a final concentration of $10 \mu\text{g ml}^{-1}$. After incubation, the samples were prepared as described in Methods and the cyclic AMP concentration determined using a cyclic AMP kit. The induction of cyclic AMP (fmol per 10^6 cells) was determined as concentration induced by the toxin minus concentration induced by TEAN buffer (control). Values are means $\pm$ SD of three experiments. $\circ$, Nicked LT; $\bullet$, LT(CE); $\square$, R192G with trypsin treatment; $\blacksquare$, Del192 with trypsin treatment; $\triangle$, E112K with trypsin treatment; $\blacktriangle$, rLT-B.

65 fmol per 10^6 cells. The cyclic AMP responses slightly differed, but were similar to those of R192G and Del192, indicating that nicking around Arg192 and furin sites is not involved in cyclic AMP induction in target cells.

Table 3. Cyclic AMP induction by normal and mutant toxins in furin-deficient cells

Furin-deficient cells (1×10^6) were incubated with $10 \mu\text{g ml}^{-1}$ of each toxin and lysed after 3, 5, 7 and 9 h. The concentration of cyclic AMP was determined as described in Methods. The induction of cyclic AMP was determined as the concentration induced by the toxin minus the concentration induced by TEAN buffer (control). Values are means $\pm$ SD from three independent experiments.

Mutant	Cyclic AMP concn (fmol per 10^6 cells)			
	3 h	5 h	7 h	9 h
LT(CE)	39.2 ± 8.3	203.3 ± 35	192.3 ± 30	186.7 ± 11
R192G	ND	92.1 ± 30	148.2 ± 25	171.1 ± 16
Del192	25.0 ± 6.5	98.7 ± 25	159.4 ± 22	181.7 ± 21
Del192-192	ND	69.4 ± 28	137.4 ± 45	181.7 ± 14
R192G + Del193-194	ND	64.5 ± 32	143.9 ± 28	197.8 ± 12
R192G + Del194-195	ND	64.5 ± 32	143.9 ± 28	197.8 ± 12
Del192-194	15.6 ± 7.9	129.7 ± 45	181.7 ± 31	186.8 ± 14
Del192-195	23.6 ± 8.0	106.8 ± 35	148.2 ± 21	203.3 ± 31
Gly192-194	21.8 ± 10.0	38.4 ± 29	127.5 ± 38	181.7 ± 10
E112K	ND	ND	< 6.0	ND
rLT-B	ND	ND	< 6.0	ND

However, it is possible that the point mutations blocking the furin sites left one or two other sites that might be involved in activation of toxin *in vivo*. Therefore, we examined the importance of furin for toxin activation in furin-deficient cells.

Cyclic AMP induction by mutant toxins and LT in furin-deficient L_0V_0 cells

Cyclic AMP induction by mutant toxins from group I and group II was compared with that of normal LT in furin-deficient cells. As shown in Table 3, cyclic AMP was induced by LT(CE), R192G, Del192 and each deletion mutant from Arg192 to Thr195. Though the latent time to induce the maximal cyclic AMP response was about 5 h in myeloma cells (Fig. 6), the latent time in furin-deficient cells was longer. At this time, we do not understand the reason for this observation.

These data suggest that a deficiency of furin does not extinguish cyclic AMP induction in the target cells and that the three kinds of furin sites are not involved in toxicity.

DISCUSSION

The nick at Arg192 or nearby residues is thought to be essential for activation of biological and enzymic activity of CT or LT (Klapper *et al.*, 1976; Finkelstein *et al.*, 1983; Mekalanos *et al.*, 1983; Booth *et al.*, 1984; Ichinose *et al.*, 1992; Grant *et al.*, 1994). However, although mutant toxins lacking these nick sites have no ADP-ribosyltransferase activity, they showed partial toxicities in the ileal loop test (Fig. 2), PF test (Fig. 4) and CHO cell assay (Fig. 5) and induced less cyclic AMP (Fig. 6 and Table 3) than LT. These nick sites are therefore essential for ADP-ribosyltransferase activity,

but not for biological activity and cyclic AMP induction. This is consistent with the fact that partial toxicity and adjuvant activity is independent of nicking at Arg192 (Grant *et al.*, 1994; Dickinson & Clements, 1995).

Thus, how might these mutant toxins activate the adenylate cyclase/cAMP system? There are two possible mechanisms apart from nicking of the abnormal A subunit. (1) There might be another pathway by which the abnormal A subunit activates adenylate cyclase *in vivo*. (2) The abnormal unnicked A subunit might directly activate adenylate cyclase dependent on or independently of ADP-ribosyltransferase activity.

There are no reports indicating another pathway that activates adenylate cyclase by CT except for the ADP-ribosylation of G_s , which inhibits its GTPase activity (Freissmuth & Gilman, 1989). In this system, free A subunit is essential. Therefore, it is unclear whether or not there is another pathway.

The abnormal A subunits did not show ADP-ribosyltransferase activity (Table 2). Therefore, the mutant A subunit cannot activate adenylate cyclase dependent on ADP-ribosyltransferase. Moreover, there is no information to show that the A subunit can directly activate adenylate cyclase independently of ADP-ribosyltransferase.

Therefore, there might be a nick mechanism that generates an active fragment by processing the A subunit, after toxin binding to the cell membrane. Since unnicked LT stayed on the cell surface longer than nicked LT (Tsuiji *et al.*, 1984), the A subunit might be nicked by a membrane protease.

A subunits might be nicked after they penetrate cells. Crystal analysis of LT (Sixma *et al.*, 1991) has indicated how the A subunit might penetrate the membrane and

reach the target protein. B subunits bind to the G_{M1} ganglioside receptor and the polysaccharide of G_{M1} forms a loop around the B subunits and pushes the A subunit into the cell membrane. The toxin might also enter the cell by endocytosis and a function of the Golgi apparatus is necessary to generate the A₁ fragment (Orlandi *et al.*, 1993). It is possible that after the A subunit penetrates the cell, it is processed by an intracellular endopeptidase normally used for post-translational processing of a protein other than furin.

Therefore, LT has partial toxicity independent of the nick sites at Arg192 or nearby residues. To demonstrate partial toxicity, nicking mechanisms that are independent of furin may function subsequent to toxin-binding to the cell membrane to release an active A fragment.

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