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LT(K63/R72), a New Mutant of *Escherichia coli* Heat-labile Enterotoxin, Exhibits Characteristics More Similar to LT(K63) than LT(R72)

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Abstract LT(K63), a non-toxic mutant and LT(R72), a low toxic mutant of *E. coli* heat-labile enterotoxin are frequently used mucosal adjuvants. In many cases, the adjuvanticity of LT(K63) is lower than that of LT (R72), but LT(K63), which induces a mixed Th1/Th2 response, exhibits a higher level of protection than LT (R72) which induces a polarized Th2-type response. To utilize the advantages of both adjuvants, a double-mutation LT(K63/R72) was generated and purified. The characterization results showed that there was no significant difference in production rate and immunogenicity between wild type LT and LT mutants. The results also showed that the toxicity and the trypsin sensitivity of LT(K63/R72) are between that of LT(K63) and LT(R72). Using HPLC, when samples in an OHpak SB-800 column were eluted by denatural buffer (TEAN containing 10 mg/ml SDS), we found the stability of LT(K63/R72) was higher than that of LT(R72) and lower than that of LT(K63). Through further analyzes, we found that LT(K63/R72) exhibits characteristics more closely related to LT(K63) than LT(R72).

Key words *Escherichia coli* heat-labile enterotoxin; mutants; LT(K63); LT(R72); LT(K63/R72)

Heat-labile enterotoxin generated from enterotoxigenic *Escherichia coli* (ETEC) is one of the main causative agents of human and domestic animal diarrhea. It is homologous to the Cholera toxin (CT) from *Vibrio cholerae* in structure, function and immunogenecity. LT consists of one subunit A (LTA) with a relative molecular mass (M_r) of about 27 kD (M_r about 27 kD), and five B subunits (LTB) each with an M_r of about 11.6 kD, forming an AB5 complex wih an M_r of about 86 kD in total [1]. By interacting with monosialoganglioside GM1 on the outside of the membrane of the target cell through the B subunit, LT is then transferred into the plasma of the target cell. In plasma, LTA is cut down between residues 192 and 195 and this generates an active A1 fragment of about 192 residues (M_r about 21.8 kD) and an A2 fragment (M_r about 5.4 kD) of about 45 residues [2]. By ADP-ribosylating a regulatory component of adenylate cyclase activity, the A1 fragment increases the level of cytoplasmic cAMP, which induces ion flux changes in the intestine. LT can act as potent mucosal adjuvant and induce an immune response against coadministered antigens [3]. Because of high toxicity, nontoxic or low toxic mutants of LT were used as mucosal adjuvants [4]. So far, about fifty mutants have been constructed and combined different antigens; some of the mutants were used as mucosal adjuvant in various animals [4]. Among the most often used mutants, LT(K63) ($S^{63}K$) and LT(R72) ($A^{72}R$) are the most promising [5]. LT(K63) has now been successfully tested in human volunteers with a trivalent subunit influenza vaccine [6]. LT(K63) and LT(R72) both have their own advantages and disadvantages. LT(K63) is nontoxic and LT(R72) retains some toxicity [7]. Some experiments showed the adjuvanticity of LT(R72) was higher than that of LT(K63) [3,8–10]; LT(K63) mainly caused a Th1/Th2 balanced immune response, while LT(R72) mainly a Th1 response, which was thought to be disadvantageous in eliminating infectious pathogen [11,12].

To overcome the disadvantages of LT(K63) and LT (R72), we have generated a new double-mutation LT mutant LT(K63/R72) ($S^{63}K$ and $A^{72}R$), simply expressed

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as LT(KR), with significantly less toxicity than LT(R72), and compared its production rate, interaction between LTA and LTB pentamer, immunogenicity and trypsin sensitivity with wild type LT, LT(K63) and LT(R72). The results show that LT(KR) exhibits characteristics more similar to LT(K63) than LT(R72). LT(KR) may be used as mucosal adjuvant in the future. In this work, we also first report a simple method to test the interaction between LTA and LTB pentamer.

Materials and Methods

Plasmids, bacterial strains and cell line

pMD18-LT and pMD18-LTS63K were constructed in our lab [13]. pET11c was purchased from Novagen (Madison, USA). *E. coli* DH5α and *E. coli* BL21(DE3) were stored in our lab. The Chinese hamster ovary (CHO) cell line was purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

Main reagents

A plasmid extraction kit was purchased from Roche (Basel, Switzerland). *Nde*I, *Bam*HI, PCR recover kit, DNA ligation kit, 100 bp DNA marker, protein marker and MutanBEST kit were purchased from TaKaRa (Dalian, China). Cholera toxin (CT) and monosialoganglioside GM1 were purchased from Sigma (Saint Louis, USA). Immobilized-*D*(+) galactose affinity chromatography column was purchased from Pierce (Rockford, USA). Sodium dodecyl sulfate (SDS), galactose and trypsin were purchased from Sangon (Shanghai, China).

Construction of mutants

Mutagenesis of LT(R72) and LT(KR) was performed with MutanBEST kit (TaKaRa, Dalian, China) using pMD18-LT as the template and two synthetic oligonucleotides pairs: P_{721} (AAGTGCTCACTTA*CGT*GGACAGTC, italic nucleotides were mutated) and P_{722} (CTCAAACTAAGAGAAGTGGAAACAT), P_{721} and P_{KR2} (CTCAAACTAAG*TTT*AGTGGAAACAT, italic nucleotides were mutated) from TaKaRa (Dalian, China) as the mutagenic primers respectively. *CGT* in P_{721} made Ala^{72} to be mutagenized to Arg^{72}, and *TTT* in P_{KR2} mutagenized Ser^{63} to Lys^{63}. Mutated genes were confirmed by dideoxy sequencing (TaKaRa, Dalian, China). Destination genes were excised from recombinant plasmids using *Nde*I and *Bam*HI and then inserted into pET11c excised by *Nde*I and *Bam*HI. Recombinant plasmids were transformed into *E. coli* BL21(DE3).

Expression and purification of LT and LT mutants

Experimental procedures used have been described previously [14].

High performance liquid chromatography (HPLC)

HPLC was performed using an OHpak SB-800 column (Shodex, Tokyo, Japan) using Agilent HPLC system. To assay the stability of LT and LT mutants in normal TEAN (50 mM Tris-HCl, 1 mM EDTA, 3 mM NaN_3, 200 mM NaCl, pH 7.4) buffer, a 50 μl (1 mg/ml) sample was applied to the column equilibrated with TEAN (pH 7.4) buffer at a flow rate of 0.25 ml/min and immediately eluted by TEAN (pH 7.4) buffer at the same flow rate, while absorbance at 280 nm (A_{280}) was recorded. To assay the interaction between LTA and LTB pentamer, the same amount of sample was applied to the column but the equilibrium buffer and elution buffer were replaced with TEAN (pH 7.4) containing 10 mg/ml SDS. Areas of elution peaks were measured using the Agilent Technologies ChemStation software [15].

Trypsin digestion

Intact LT and LT mutants assayed by HPLC were diluted to a concentration of 1 mg/ml. 50 μl sample and 5 μl trypsin (100 μg/ml) were added to 1.5 ml Eppendorf tubes. The tubes were incubated at 37 ºC. The digestion reactions were terminated after 15 min, 30 min and 1 h by boiling for 5 min. The same volume of sampling buffer was added to the tube. After another 5 min boiling, samples (15 μg/well) were analyzed by tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE).

GM1 ELISA

The method used has been described previously [14].

Immunogenicity assay

Female C57BL/6 mice, 6 weeks old, were used (Center for Experimental Animal, Sichuan University, China). Groups consisted of five mice each. Mice were immunized intraperitoneal by application of 200 μl sample. Mice were immunized on day 0, 7, 14 and 21 with 20 μg LT, 20 μg LT(K63), 20 μg LT(R72), 20 μg LT(KR) or without toxin respectively. Mice were sacrificed on day 31 and the sera were collected. Individual serum samples were separated by centrifugation. Antibody responses directed

against toxins were determined using an enzyme-linked immunosorbent assay (ELISA) [14]. Briefly, 96-well ELISA plates coated with 100 ng LT per well were incubated with the first antibodies (serum samples serially diluted tenfold in PBS/Tween) and peroxidase-conjugated goat antibodies directed against mouse IgG (1:20,000; Boshide, Wuhan, China). Color developing, absorbance reading and titer determination have been described previously [14].

Toxicity assay

Cell elongation assay The method used has been described previously [14].

Patent-mouse enterotoxicity assay The method used has been described previously [14].

Statistical analysis

Data are presented as mean ± SD. Comparisons of specific antibody responses were made with the Student's *t* test, where $P<0.05$ was considered to be statistically significant [10].

Results

Sequence analysis for genes coding wild type LT and LT mutants

From the results of sequencing made by TaKaRa, genes coding wild type LT and LT mutants were correctly constructed (**Fig. 1**, whole data not shown). Partial sequences from each gene were aligned in **Fig. 1**.

Expression of LT and LT mutants

To examine the difference in production rate among LT mutants, wet bacteria were weighed and each protein was purified thoroughly from the bacteria (**Table 1**).

As seen in **Table 1**, there are no significant difference in production rate between LT and LT mutants.

Wild type lt	ACT	TCT	CTT	AGT	TTG	AGA	AGT	GCT	CAC	TTA	GCA	GGA
lt(K63)	ACT	AAA	CTT	AGT	TTG	AGA	AGT	GCT	CAC	TTA	GCA	GGA
lt(R72)	ACT	TCT	CTT	AGT	TTG	AGA	AGT	GCT	CAC	TTA	CGT	GGA
lt(KR)	ACT	AAA	CTT	AGT	TTG	AGA	AGT	GCT	CAC	TTA	CGT	GGA

Fig. 1 Partial sequence alignment for genes coding wild type LT and LT mutants

Nucleotides coding wild type or mutated amino acid residues are marked with underlines or boxes respectively.

Stability comparison between LT and LT mutants

To compare the stability of LT and LT mutants, freshly purified LT, LT(K63), LT(R72) and LT(KR) were eluted by TEAN (pH 7.4) in OHpak SB-800 column using Agilent HPLC system. From A_{280} recorded during elution (**Fig. 2**, upper panel), it could be seen that there is a single elution peak in each sample. This indicates that all toxins remain intact. When eluted by TEAN (pH 7.4) containing 10 mg/ml SDS, LT and LT mutants show a greater difference in A_{280} than those eluted by TEAN (pH 7.4) (**Fig. 2**, lower panel). This indicates that LT and LT mutants underwent significant changes in structure: (1) part of the LTA subunit was detached from the holotoxin resulting in two [LT (K63) and LT(KR)] or three elution peaks (LT); (2) alternately part of LTB pentamer collapsed to form an LTB monomer resulting in four elution peaks [LT(R72)]. The area of each peak was calculated by using ChemStation, and the ratios are listed in **Table 2**.

From **Fig. 2** and **Table 2**, it can be concluded that LT (K63) and LT(KR) are more stable than LT, and LT(R72) is the most unstable toxin.

Difference in trypsin sensitivity between LT and LT mutants

To test whether mutation affects the proteolytic degradation of the A subunit, wild type LT, LT(K63), LT(R72) and LT(KR) were digested by trypsin (trypsin:toxin=

Table 1 Production rate of LT and LT mutants

LT and LT mutants	Wet bacteria (g)	Purified LT and LT mutants (mg)	Production rate of LT and LT mutants	
			mg per gram wet bacteria	mg per liter culture medum
LT	485	229	0.473	45.9
LT(K63)	414	270	0.652	54.0
LT(R72)	360	216	0.600	43.2
LT(KR)	427	252	0.591	50.4

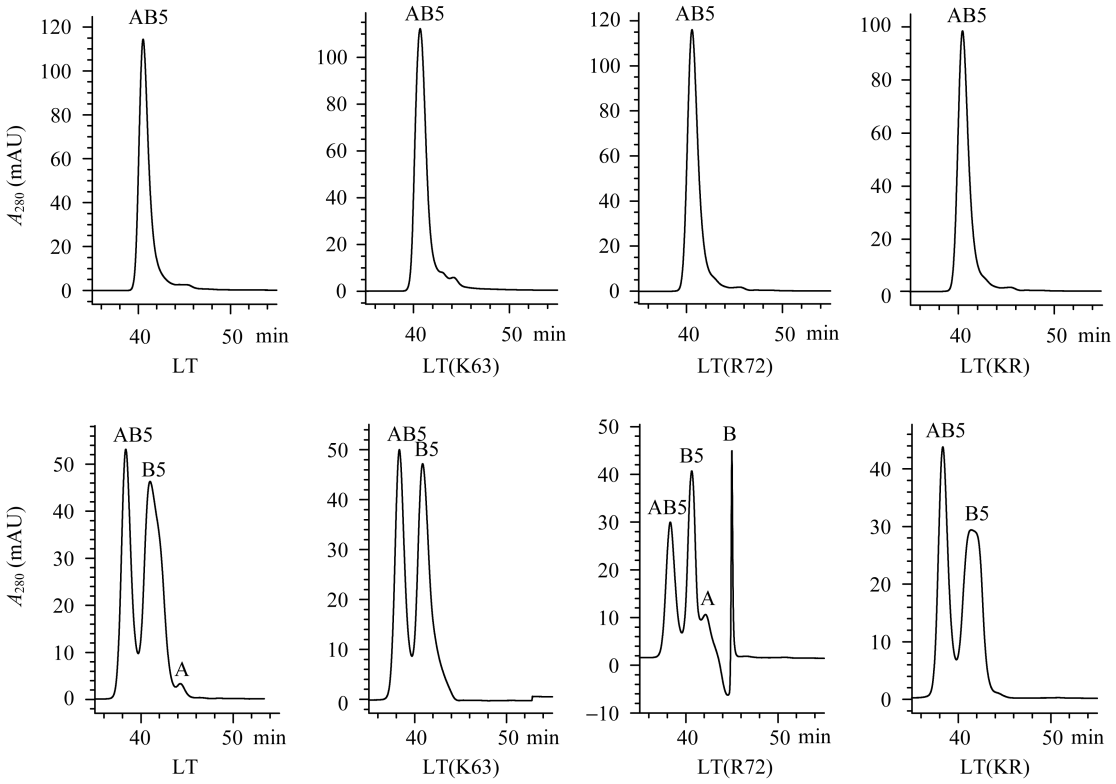

Fig. 2 **A_{280} of LT, LT(K63), LT(R72) and LT(KR) eluted by TEAN with (lower) or without 10 mg/ml SDS (upper)**

AB5, intact toxin; B5, LTB pentamer; A, LTA subunit; B, LTB monomer.

Table 2 **Depolymerization of LT and LT mutants eluted by TEAN with 10 mg/ml SDS**

LT and LT mutants	Ratio of each part (%)			
	AB5	B5	A	B
LT	39	59	2	
LT(K63)	44	56		
LT(R72)	36	53	6	5
LT(KR)	44	56		

1:100, in mass ratio) at 37 °C for 15 min, 30 min and 1 h respectively. The digestion results were analyzed by tricine-SDS-PAGE (**Fig. 3**). The LTB subunit only exists as monomer because the samples were heated at 100 °C. The LTA2 fragment is too small to be seen. The results indicate that LT(R72) and LT are more sensitive to trypsin than LT(K63) and LT(KR). The LTA subunit of LT(K63) is hard to be digested into LTA1 and LTA2 fragments even

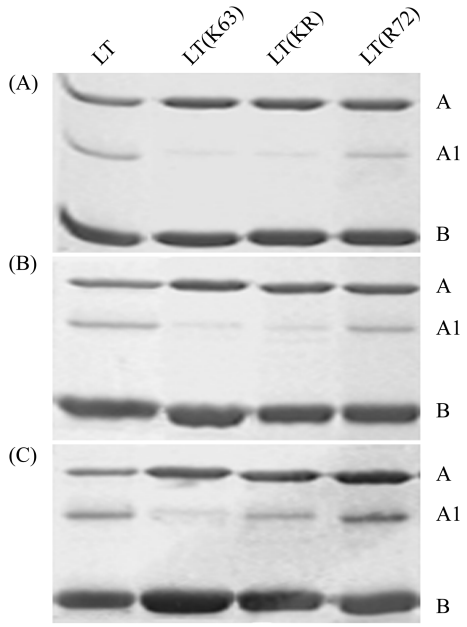

Fig. 3 **Trypsin sensitivity of LT and LT mutants**

(A) Treated with trypsin (mass ratio of trypsin vs. protein, 1:100) for 15 min. (B) Treated with trypsin for 30 min. (C) Treated with trypsin for 1 h. A, LTA subunit; A1, LTA1 fragment; B, LTB monomer.

when digested for 1 h. The trypsin sensitivity of LT(KR) is slightly higher than that of LT(K63).

GM1 binding activity of LT and LT mutants

LT attaches to eukaryote cells through the binding of Gly^{33} of the LTB subunit to galactose of GM1, which ubiquitously exists on eukaryote cell membranes. Furthermore, LT enters into the cells and induces water loss. Binding to GM1 also contributes to the immunogenicity of LT. Using the GM1 binding assay *in vitro*, normal biological characteristics of LT could be determined. Judged by ELISA, there was no difference in GM1 binding capacity between LT and LT mutants, all samples gave positive results when the concentration reached to 2 ng/well and the curves of A_{492} showed high consistency (data not shown).

Immunogenicity of LT and LT mutants

To test the immunogenicity of LT and LT mutants, mice were immunized with toxins intraperitoneally as described in "Materials and Methods". The titers of serum antibody IgGs against toxins were assayed (**Fig. 4**). Results in **Fig. 4** indicate that there is no significant difference in immunogenicity between LT and LT mutants.

Toxicity comparison between LT and LT mutants

To detect the toxicity *in vitro*, CHO cells were cultured and treated with toxins as described in "Materials and Methods". When fixed with methanol and stained with 0.4 mg/ml Trypan blue stain (Gibco BRL) and washed and further dried, experimental CHO cells were analyzed for morphological changes by light microscopy and the lowest concentrations of toxins to cause >90% cells elongation were recorded (**Table 3**). As shown in **Table 3**, LT was toxic at the concentration of 20 ng/ml (4 ng/well), LT(R72) caused 90% cell elongation at a concentration of 10 μg/ml (2 μg/well), and LT(K63) and LT(KR) showed no cell elongation at the highest concentration (200 μg/ml) used. When residues 63 and 72 of the LTA subunit were mutated to Lys and Arg synchronously, the toxicity of toxin was significantly decreased.

To detect the toxicity *in vivo*, Patent-mouse enterotoxicity assay was used as described in "Materials and Methods". The gut-to-carcass (G/C) ratios were recorded (**Fig. 5**). **Fig. 5** shows that LT is most toxic; LT(R72) is more toxic than LT(K63) and LT(KR) (P<0.05); LT(KR)

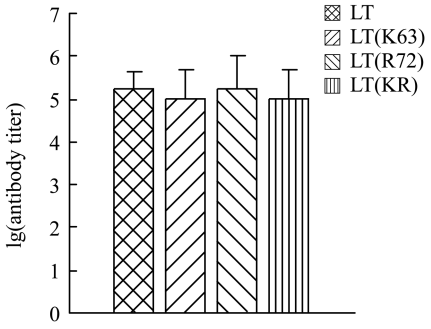

Fig. 4 Serum IgG antibody responses to LT and LT mutants administered intraperitoneally

C57BL/6 mice were immunized i.p. with TEAN (pH 7.4) alone or mixed with 20 μg LT, or 20 μg LT(K63), or 20 μg LT(R72), or 20 μg LT(KR) on day 0, 7, 14 and 21, and mice were sacrificed on day 31. Antibody titers were expressed as the reciprocal serum dilution with the ratio of A_{492} of experimental groups to A_{492} of controlled groups being greater than 2.1. Antibody titers were expressed as geometric mean ± standard deviation, n=5. Comparisons between experimental groups were made by Student's t-test. P<0.05 was considered significant. There is no statistically significant (P>0.05; Student's t-test) difference between LT and LT mutants. Control mice, that received TEAN (pH 7.4) alone, showed no detectable LT-specific antibody titer.

Table 3 ***In vitro*** **toxicity of LT and LT mutants**

LT and LT mutants	Toxicity on CHO cells
LT	Toxic at 20 ng/ml
LT(K63)	Nontoxic at 200 μg/ml *
LT(R72)	Toxic at 10 μg/ml
LT(KR)	Nontoxic at 200 μg/ml *

* is the highest concentration tested.

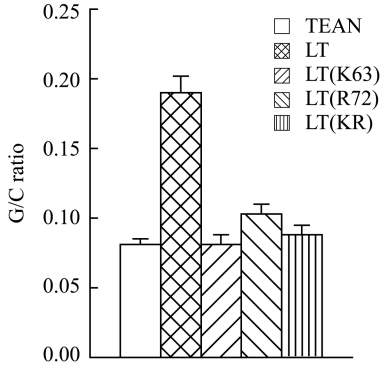

Fig. 5 Results of *in vivo* patent-mouse toxicity assay of LT and LT mutants

The G/C ratio of TEAN group is 0.081 ± 0.004; LT, 0.190 ± 0.012; LT(K63), 0.081 ± 0.007; LT(R72), 0.103 ± 0.007; and LT(KR), 0.088 ± 0.007. Data were represented as mean ± SD, n=5.

retains toxicity to some extent; and LT(K63) is nontoxic.

Discussion

That LT(K63) is a nontoxic mutant and LT(R72) is a low toxic mutant has been confirmed [7,16]. In LT(K63), amino acid residue 63, Gly, situated between β_3 strand and α_3 helix, is replaced with Lys, and this causes a great change in the conformation of the substrate binding domain and results in poor substrate binding [16]. Whether this conformation change could affect the stability was not reported. Magagnoli *et al*. [2] noticed that the storage stability of LT(K63) was higher than that of wild type LT, but they gave no explanation. From the results of HPLC assay, LT(K63) exhibits more resistance to SDS denaturation suggesting that conformation change of LT(K63) may increase interaction between the LTA and LTB pentamer. The trypsin sensitivity of LT(K63) is lower than that of wild type LT, suggesting that conformation change of LT (K63) also affects the A1/A2 proteolytically sensitive site. In contrast, when eluted by TEAN containing 10 mg/ml SDS, LT(R72) is even more unstable than wild type LT and it may be rationally deduced that conformation change of LT(R72) may decrease interaction between the LTA and LTB pentamer. From the results of trpsin digestion, conformation change of LT(R72) seems have little effect on the A1/A2 proteolytically sensitive site. Based on our observations, that the toxicity of LT(R72) is higher than that of LT(K63) may be interpreted as follows. After entering into cell plasma, because of the weak interaction of the LTA and LTB pentamer, the LTA fragment of LT(R72) is prone to be separated from the LTB pentamer; furthermore, because of high trypsin sensitivity, LTA is more easily broken down to LTA1, which determines the low toxicity. LT(KR) exhibits significantly reduced toxicity, significantly lower trpsin sensitivity and significantly increased interaction of the LTA and LTB pentamer than LT(R72). These maybe attribute to the effect of LT(K63) covering up the effect of LT(R72).

LT and LT mutants will be depolymerized after a long time in storage. The stability assay was performed using gel filtration chromatography. The intact hexamer has only one peak, and two or more peaks were presented if there were other oligomers and monomers [2]. To compare the stability differences of intact LT and LT mutants, one can not simply use gel filtration chromatography, and another method should be used. Rodighiero *et al.* [17] tested the stability difference of LT and LT mutants using GM1-ELISA. Namely, 96-well plates were coated with GM1, then LT and LT mutants were added, elution buffer containing 5 mg/ml SDS was added to wash part of LTA, monoclonal antibodies against LTA and HRP-IgG were added successively, and the stability differences were determined by comparing the absorbance differences between LT and LT mutants. This method was sensitive, but more time and costs were needed to purify LTA and prepare monoclonal antibody. Through applying LT and LT mutants to an OHpak SB-800 column in an HPLC device and assaying the differences of A_{280} under elution of TEAN (pH 7.4) containing 10 mg/ml SDS, we directly observed the stability differences between LT and LT mutants. This method has not been reported before.

Newly generated double-mutation LT(KR) is less toxic and more stable than LT(R72), moreover, it retains the same immunogenicity. Furthermore, LT(KR) may be used as a mucosal adjuvant in the future.

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