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# Construction and Expression of Novel Immunotoxin cpIL-4(13D)–PE38KDEL with Increased Activity

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**Abstract**—Interleukin-4 receptors (IL-4Rs) are expressed on a wide variety of human cancer cells, and therefore it may be a good option to treat IL-4R-bearing tumors with IL-4-fusing immunotoxins. In this study, the gene encoding human interleukin-4 mutein cpIL-4(13D) was obtained through overlapping polymerase chain reaction. A chimeric immunotoxin was constructed by genetically fusing the mutein cpIL-4(13D) to a modified version of *Pseudomonas* exotoxin A (PE38KDEL) and was expressed in *Escherichia coli* AD494 (DE3). The expression level of the fusion protein was about 30% of the total bacterial protein assessed by SDS-PAGE analysis. After purification by affinity chromatography and anion exchange chromatography, the chimeric protein was tested for its cytotoxicity. Our data show that cpIL-4(13D)–PE38KDEL has improved cytotoxicity on IL-4R-bearing tumor cells in comparison with other IL-4-fusing immunotoxins and might be useful in treating tumors with a large number of IL-4Rs.

*Key words*: immunotoxin, interleukin-4, *Pseudomonas* exotoxin A

Interleukin-4 receptors (IL-4Rs) are present on a wide variety of human cancer cells derived from carcinomas of the colon, stomach, liver, breast, adrenals, prostate, as well as glioblastoma, epidermoid carcinoma, and melanoma, while there are few interleukin-4 receptors expressed on the surface of normal human cells [1, 2]. Thus, the development of immunotoxins directed at IL-4R may be a good choice for tumor therapy.

However, it is not desirable to construct an immunotoxin by simply fusing native human IL-4 to *Pseudomonas* exotoxin A because the resulting fusion protein binds to the IL-4R with only ~1% of the affinity of native IL-4 [3]. The most probable reason is that the amino terminal and carboxyl terminal of native IL-4 have important binding sites and the binding may be greatly affected if attaching a toxin to either the N-terminal or the C-terminal of IL-4.

To improve the binding affinity of IL-4 toxin to IL-4R, Kreitman et al. [1] constructed a circularly permuted interleukin-4 termed IL-4(38-37), in which codons 1 and 129 were connected with a sequence encoding a peptide linker, and new N-terminal and C-terminal were created between codons 38 and 37. The corresponding circularly permuted IL-4-toxin was constructed by fusing IL-4(38-37) at its new carboxyl terminus to a truncated form of *Pseudomonas* exotoxin(PE38Q), which bound to IL-4R with 10-fold higher affinity than a native IL-4-toxin [4-7].

Although circularly permuted IL-4-toxin has better binding affinity, it is still necessary to improve its affinity to IL-4R to increase its cytotoxicity and reduce its side effects.

Previous studies showed that human IL-4 muteins containing the mutation Thr13 to Asp had higher affinity for IL-4$R_{\alpha}$ than native IL-4 [8]. Based on this fact, circularly permuted IL-4 was further modified in our study by introducing a mutation at amino acid 13 from Thr to Asp, and the resulting IL-4 mutein was termed cpIL-4(13D). By fusing cpIL-4(13D) at the carboxyl terminus to a truncated form of *Pseudomonas* exotoxin (PE38KDEL), immunotoxin cpIL-4(13D)–PE38KDEL was constructed, which had improved affinity to IL-4R compared to the circularly permuted IL-4-toxin and bound IL-4R with 40% of the affinity of native IL-4.

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## MATERIALS AND METHODS

**Plasmid, strain, and cell line.** Expression vector pET32a(+) and *E. coli* strain AD494(DE3) (Novagen, USA) were used in this study. Plasmid pBluescript encoding native human IL-4 was a kind gift from Professor Li-Weber (Germany). Plasmid pMDPEIII encoding a truncated form of *Pseudomonas* exotoxin (PE38KDEL) was constructed in our laboratory. Glioblastoma cell line U251 was purchased from the China Center for Type Culture Collection. The murine fibroblast cell line L929 was kindly provided by the Chinese Medical University.

**Primer.** Sangon Biotechnology Company (USA) synthesized the primers using an oligonucleotide synthesizer:

primer (a) (sense), 5′-TGTTCGTCCGGAGGTAACG-GCGGTCACAAGTGCGATATCAC-3′,

primer (b) (antisense), 5′-CTCAGTTGAGCTCTTG-GAGGCAGCAAAGATGTCTGT-3′,

primer (c) (sense), 5′-TGCTGCCGCCATGGACA-CAACTGAGAAGGAAACC-3′,

primer (d) (antisense), 5′-CTTGTGACCGCCGTTAC-CTCCGGACGAACACTTTGAATATTTCT-3′,

primer (e) (sense), 5′-TCCCGGCCGCCATGGACA-CA-3′,

primer (f) (antisense), 5′-GTTCAAATCTTTGAT-GATCTCCTG-3′,

primer (g) (sense), 5′-CATCAAAGATTTGAACAGC-CTCACA-3′,

primer (h) (antisense), 5′-GCGTTGGGAGCTCTTG-GAGGCA-3′.

**Construction of IL-4 mutein cpIL-4.** Overlapping polymerase chain reaction (PCR), which included three PCR reactions, was performed to obtain the mutant gene of cpIL-4 (Fig. 1). The plasmid pBluescript encoding native human IL-4 was used as the template.

IL-4 codons 1-37 were amplified using primers (a) and (b). The 5′ end of the fragment encoded a GGNGG linker and the 3′ end contained a *Sac*I site. IL-4 codons 38-129 were amplified using primers (c) and (d). The 5′ end of the fragment contained an *Nco*I site and the 3′ end encoded the GGNGG linker. Because the 5′ end of the first fragment and the 3′ end of the second fragment had complementary sequence, the two fragments could be used as the overlapping template in a third PCR reaction using primers (b) and (c). The resulting fragment contained the gene of cpIL-4 encoding IL-4 codons 38-129, GGNGG, and IL-4 codons 1-37, with *Nco*I site and *Sac*I site flanking separately at the N- and C-terminals.

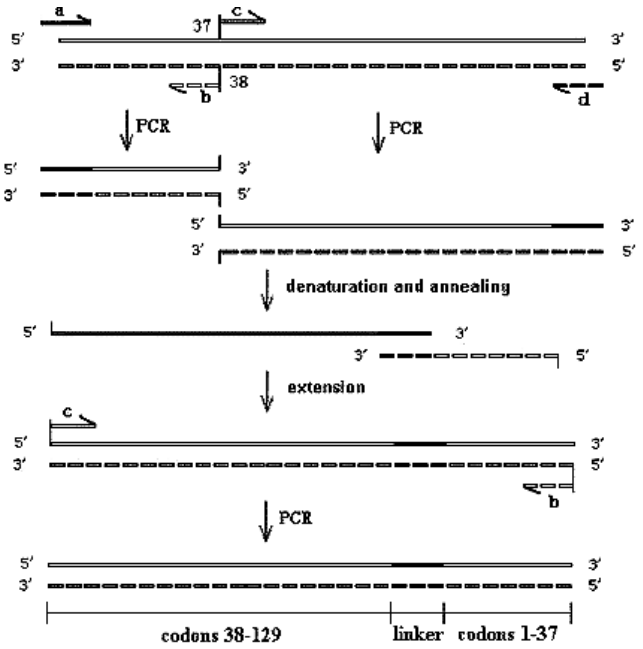


**Fig. 1.** Schematic diagram of the construction of cpIL-4.

**Construction of IL-4 mutein cpIL-4(13D).** Another overlapping PCR reaction was carried out to introduce a point mutation from Thr to Asp into the cpIL-4 gene at amino acid 13. The cpIL-4 gene constructed above was used as a template here.

The cpIL-4 codons 38-129-GGNGG-1-13 were amplified using primers (e) and (f). The sequence of primer (f) had a mutation at the position of codon 13. IL-4 codons 13-37 were amplified using primers (g) and (h). And the sequence of primer (g) had a mutation at the position of codon 13 too. Because the 3′ end of the first fragment and the 5′ end of the second fragment had complementary sequence, the two fragments could be used as the overlapping template in a third PCR reaction using primers (e) and (h). The resulting fragment contained the gene of cpIL-4(13D) encoding IL-4 codons 38-129, GGNGG, and IL-4 codons 1-12, D, and 14-37, with *Nco*I site and *Sac*I site flanking separately at the N- and C-terminals of cpIL-4(13D).

**Construction of the expression vector.** The amplified fragment encoding cpIL-4(13D) was digested with *Nco*I and *Sac*I. Plasmid pMDPEIII encoding PE38KDEL was digested with *Sac*I and *Hind*III. After purification, the two fragments were ligated to the *Nco*I-*Hind*III restriction fragment of plasmid pET32a(+). The resulting expression vector pET32a(+)-cpIL-4(13D)–PE38KDEL encoded a chimeric immunotoxin composed of cpIL-4(13D) and a truncated form of *Pseudomonas* exotoxin (PE38KDEL) (Fig. 2). The expression vector pET32a(+)-cpIL-4–

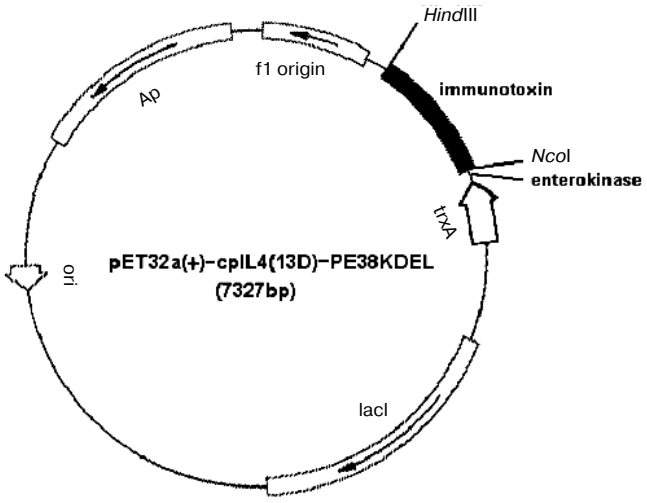


**Fig. 2.** Expression vector pET32a(+)-cpIL-4(13D)–PE38KDEL.

PE38KDEL encoding cpIL-4–PE38KDEL was constructed in the same way as the control.

**Protein expression and purification.** To produce the chimeric immunotoxins, *E. coli* AD494(DE3) cells were transformed with plasmids pET32a(+)-cpIL-4(13D)–PE38KDEL and pET32a(+)-cpIL-4–PE38KDEL, respectively.

After screening by digestion with restriction enzymes and the PCR reaction, the positive clones were incubated in LB media containing ampicillin (50 μg/ml) and kanamycin (30 μg/ml). The cells were incubated with shaking until $A_{600}$ was approximately 0.6. Then isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the incubation was continued for about 4 h. The expression of the target proteins was tested by SDS-PAGE analysis.

The culture was centrifuged at 10,000*g* for 5 min at 4°C, and the pellet was washed with water, phosphate-buffered saline (PBS), and Tris-HCl buffer in turn. Then lysozyme was added to a final concentration of 100 μg/ml and the mixtures were incubated at 37°C for 1 h. After that, the cells were sonicated in Tris-HCl buffer. The sonicated products were centrifuged, and the target proteins in the supernatant were purified by Ni-NTA HisBind® Superflow™ affinity chromatography.

The concentrations of the two chimeric proteins were measured by a modified Folin procedure using bovine serum albumin (BSA) as a standard. Enterokinase was added to the immunotoxin solution in a 1 : 50 mass ratio and the cells were incubated for 3 h at 37°C. After incubation, each immunotoxin was loaded onto 10 ml of DEAE-Sepharose Fast Flow anion exchange resin (Pharmacia Biotech, Germany) and eluted with Tris-HCl buffer containing 0.4 M NaCl. The resulting chimeric toxins had no 109 amino acid Trx·Tag™ thioredoxin protein at the N-terminus.

**Western blotting assay.** After SDS-PAGE, the purified chimeric toxins cpIL-4(13D)–PE38KDEL and cpIL-4–PE38KDEL were electrically transferred from the gel to cellulose nitrate membrane. Biotin-tagged anti-human IL-4 antibody, horseradish peroxidase (HRP)–avidin were added in turn and the film was incubated with the HRP substrate TMB (3,3′,5,5′-tetramethylbenzidine). The reaction was stopped before saturation of the colorimetric reaction by adding 2 M $H_2SO_4$.

**Affinity of immunotoxins to IL-4R bearing cells.** Glioblastoma U251 cells were incubated with 0.3 nM $^{125}$I-labeled IL-4 and various concentrations of unlabeled cpIL-4–PE38KDEL or cpIL-4(13D)–PE38KDEL. After 2 h, the cells were centrifuged and the radioactivity in the pellets was measured in a γ counter.

**Cytotoxicity of the immunotoxin.** Glioblastoma U251 cells ($2 \cdot 10^5$ in 0.2 ml of RPMI 1640 per well) were incubated in 96-well plates with various concentrations of purified cpIL-4(13D)–PE38KDEL, cpIL-4–PE38KDEL, cpIL-4(13D), and BSA for 48 h at 37°C, 5% $CO_2$ in a humidified incubator. The control group was not exposed to drugs. The cells were concentrated and then MTT solution (Sigma, USA) was added. After incubation for 4 h, the cells were concentrated and dimethylsulfoxide was added to the pellets. The absorption values at 570 nm were measured. The cell survival rates were calculated and the killing curves were made according to the following formula:

$$\text{cell survival rate } (\%) = [A_{570} \text{ (of drug-treated group)}/A_{570} \text{ (of control group)}] \cdot 100\%.$$

Data from cpIL-4(13D)–PE38KDEL-treated group were compared with that from cpIL-4–PE38KDEL-treated group using Student's *t*-test. A *p* value of 0.01 was considered significant.

The murine fibroblast cell line L929 without IL-4R was used as control cells.

## RESULTS

**Construction of cpIL-4 and cpIL-4(13D).** Human IL-4 gene was used as a template to amplify codons 1-37 using primers (a) and (b). The resulting fragment was 147-bp long, which was confirmed by agarose gel electrophoresis. IL-4 codons 38-129 were amplified using primers (c) and (d) and the resulting fragment was 310-bp long. Primers (b) and (c) were used to amplify cpIL-4, the full length of which was 427 bp (Fig. 3a).

IL-4 codon 13 was mutated from ACT to GAT by overlapping PCR with cpIL-4 as a template. The resulting cpIL-4(13D) contained a mutation of Thr to Asp at amino acid 13 (Fig. 3b).

**Sequencing of cpIL-4(13D).** cpIL-4(13D) was sequenced as follows:

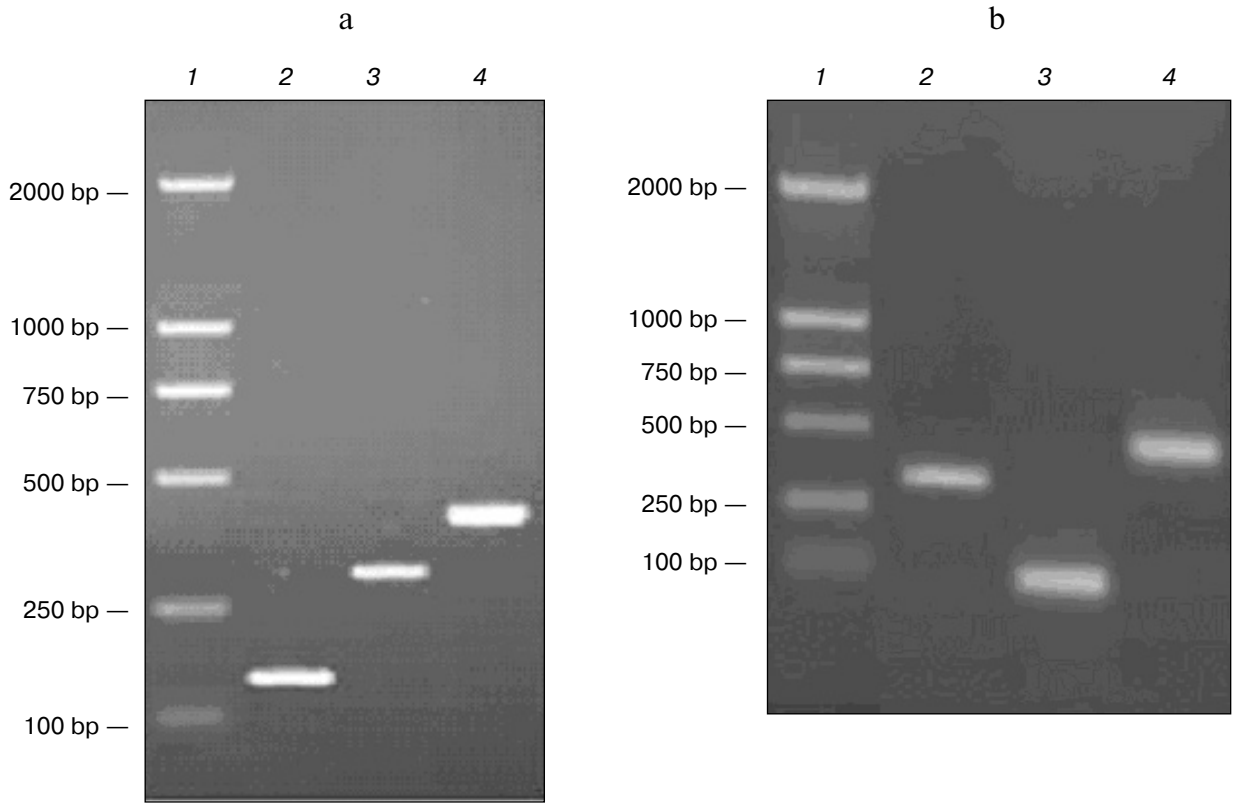


**Fig. 3.** Overlapping PCR amplification of cpIL-4 and cpIL-4(13D). a) Amplification of cpIL-4: *1*) DL2000 DNA marker; *2*) PCR product of the first fragment (147 bp); *3*) PCR product of the second fragment (310 bp); *4*) PCR product of cpIL-4 (427 bp). b) Amplification of cpIL-4(13D): *1*) DL2000 DNA marker; *2*) PCR product of the first fragment (350 bp); *3*) PCR product of the second fragment (94 bp); *4*) PCR product of cpIL-4(13D) (428 bp).

GACACAACTGAGAAGGAAACCTTCT-
GCAGGGCTGCGACTGTGCTCCGGCAGTTC-
TACAGCCACCATGAGAAGGACACTCGCTGC-
CTGGGTGCGACTGCACAGCAGTTCCACAGGCA-
CAAGCAGCTGATCCGATTCCTGAAACGGCTC-
GACAGGAACCTCTGGGGCCTG-
GCGGGCTTGAATTCCTGTCCTGTGAAGGAAGC-
CAACCAGAGTACGTTGGAAAACTTCTTGGAAAG-
GCTAAAGACGATCATGAGAGA-
GAAATATTCAAAGTGTTCGTCCGGAGGTAACG-
GCGGTCACAAGTGCGATATCACCTTACAGGA-
GATCATCAAAGATTTGAACAGCCTCACAGAGCA-
GAAGACTCTGTGCACCGAGTTGACCGTAACA-
GACATCTTTGCTGCCTCCAAG.

In above sequence, GGAGGTAACGGCGGT was the linker encoding GGNGG. IL-4 codons 38-129 were before the linker and IL-4 codons 1-37 were after the linker. Amino acid 13 was mutated from Thr to Asp corresponding to the codon GAT.

**Protein expression and purification.** There was an obvious protein band with the correct size of thioredoxin–cpIL-4(13D)–PE38KDEL on SDS-PAGE analysis of the total cell extract. Since there was a thioredoxin protein of 12 kD at the N-terminal of the target protein when pET32a(+) was used as expression vector, the resulting chimeric protein was about 65 kD, the amount of which was about 30% of that of the total cell protein.

Here, pET32a(+) vector was used to produce a thioredoxin-containing fusion protein to increase the yield of soluble product in the cytoplasm. The use of AD494 host strain allowed the formation of disulfide bonds of expressed proteins in the *E. coli* cytoplasm, which was very important for our chimeric protein since IL-4 has three disulfide bonds. In our study more than 90% of the target proteins were expressed in soluble form, and only a small fraction were in insoluble inclusion bodies.

After purification by $Ni^{2+}$-NTA HisBind® Superflow™ affinity chromatography, analysis showed a single band (about 65 kD) for thioredoxin–cpIL-4(13D)–PE38KDEL on SDS-PAGE gel (Fig. 4, lane *3*). Following further treatment by enterokinase and DEAE-Sepharose Fast Flow anion exchange resin, cpIL-4(13D)–PE38KDEL was obtained in highly purified form and migrated on SDS-PAGE according to its expected molecular mass of 53 kD (Fig. 4, lane *4*). The

purified proteins were shown to be greater than 95% pure, and about 50% of the target proteins expressed in soluble form were recovered.

Immunotoxin cpIL-4–PE38KDEL was expressed and purified in the same way as cpIL-4(13D)–PE38KDEL, with a similar purity of >95%.

**Western blotting of the chimeric protein.** Here western blotting analysis was performed to further confirm the expression of the chimeric toxins. As shown in Fig. 5, an obvious band of cpIL-4(13D)–PE38KDEL (about 65 kD) appeared in the induced total cell protein and the purified product from $Ni^{2+}$ affinity resin. And there was a single band of about 53 kD observed for the final products eluted from anion-exchange chromatography, which corresponded to the molecular mass of cpIL-4(13D)–PE38KDEL without thioredoxin. In contrast, the control gave no band. Western blotting assay also showed that cpIL-4–PE38KDEL could react with anti-human IL-4 antibody (data not shown).

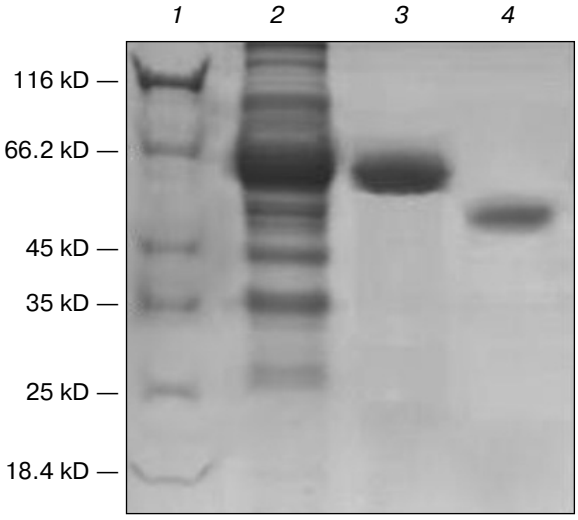


**Fig. 4.** Purification of the recombinant immunotoxin cpIL-4(13D)–PE38KDEL: *1*) protein marker; *2*) induced AD494(DE3)/pET32a(+)–cpIL-4(13D)–PE38KDEL; *3*) cpIL-4(13D)–PE38KDEL purified by $Ni^{2+}$ affinity resin; *4*) cpIL-4(13D)–PE38KDEL purified by anion-exchange chromatography.

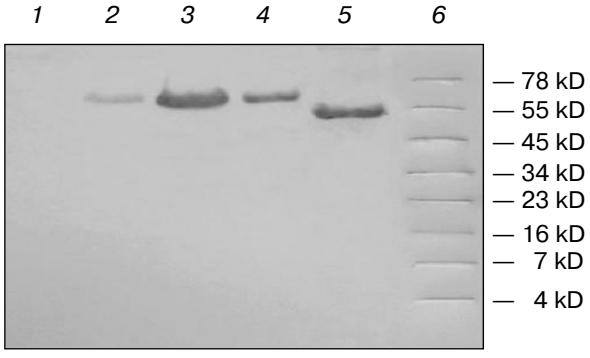


**Fig. 5.** Western blotting of cpIL-4(13D)–PE38KDEL: *1*) induced AD494(DE3)/pET32a(+); *2*) uninduced AD494(DE3)/pET32a(+)–cpIL-4(13D)–PE38KDEL; *3*) induced AD494(DE3)/pET32a(+)–cpIL-4(13D)–PE38KDEL; *4*) cpIL-4(13D)–PE38KDEL purified by $Ni^{2+}$ affinity resin; *5*) cpIL-4(13D)–PE38KDEL purified by anion-exchange chromatography; *6*) SeeBlue® pre-stained protein marker.

Binding of IL-4-toxins to IL-4R-bearing U251 cells

| Molecule | $IC_{50}$, nM | Affinity of IL-4, % |
|---|---|---|
| IL-4 | 0.052 ± 0.001 | 100 |
| cpIL-4-PE38KDEL | 0.430 ± 0.016 | 12.1 |
| cpIL-4(13D)-PE38KDEL | 0.110 ± 0.008 | 47.3 |

Note: $IC_{50}$ is the immunotoxin concentration necessary for 50% displacement of $^{125}I$-labeled IL-4 from U251 cells. The results are presented as the mean ± standard deviation ($n$ = 3).

**Binding affinity of the chimeric protein to IL-4R.** To determine the relative affinities of the immunotoxins to IL-4R, IL-4 displacement assay was carried out. The results showed that cpIL-4(13D)–PE38KDEL bound to IL-4R with 3-fold higher affinity than cpIL-4–PE38KDEL, and the affinity of cpIL-4(13D)–PE38KDEL was about 40% of that of native IL-4 (table).

Because cpIL-4–PE38KDEL and cpIL-4(13D)–PE38KDEL were produced in the same way and had the same purity of greater than 95%, it can be concluded that there was no difference between the two immunotoxins except the Thr13 mutation, and that the superiority in receptor binding properties for cpIL-4(13D)–PE38KDEL was due to the Thr13 mutation.

**Cytotoxicity assay of the immunotoxin.** Cell survival curves showed that IL-4R-bearing glioblastoma U251 was very sensitive to immunotoxin cpIL-4(13D)–PE38KDEL, but had very little response to BSA or cpIL-4(13D) treatment. And the glioblastoma U251 was more sensitive to cpIL-4(13D)–PE38KDEL than to cpIL-4–PE38KDEL. The $IC_{50}$, the toxin concentration necessary for 50% inhibition of protein synthesis, was 12 ng/ml for cpIL-4(13D)–PE38KDEL, compared with 32 ng/ml for cpIL-4–PE38KDEL (Fig. 6a). CpIL-4(13D)–PE38KDEL and cpIL-4–PE38KDEL had no cytotoxic activity against the murine fibroblast cell line L929 that did not express IL-4R (Fig. 6b).

## DISCUSSION

With the development of recombinant DNA technology, fusion proteins such as immunotoxins or bispecif-

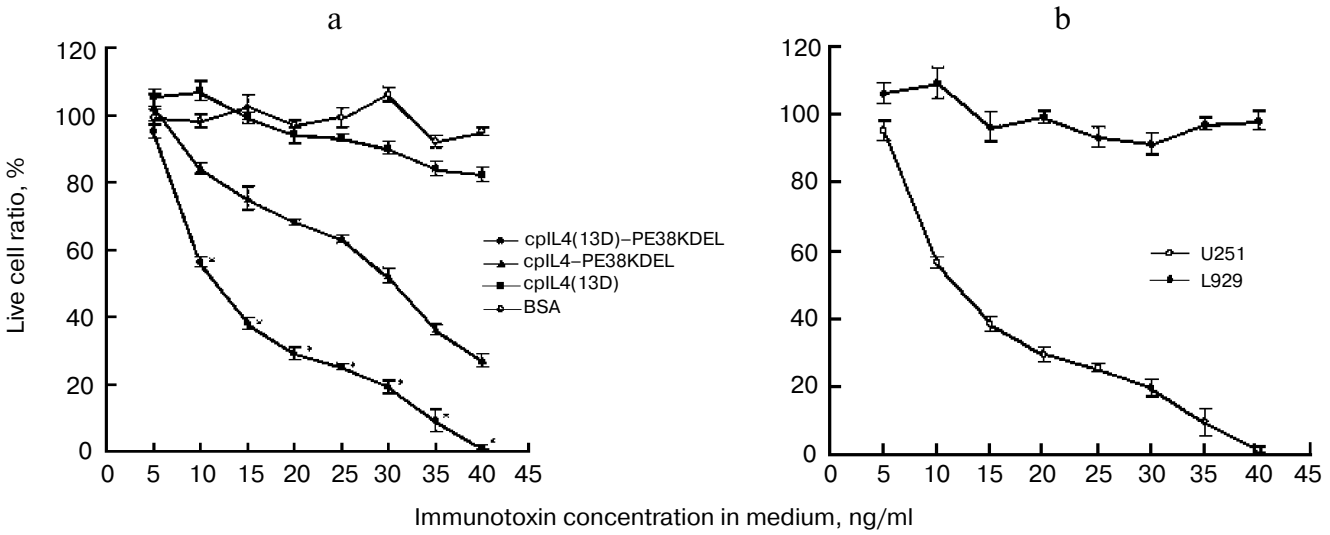


**Fig. 6.** Cytotoxicity of immunotoxins: a) cytotoxicity of immunotoxins to U251; b) cytotoxicity of cpIL-4(13D)–PE38KDEL to U251 and L929. The results are represented as the mean ± standard deviation. Error bars represent standard deviations from means of triplicate samples. * Statistically significant difference between this value and data from the group of cpIL-4–PE38KDEL ($p < 0.01$).

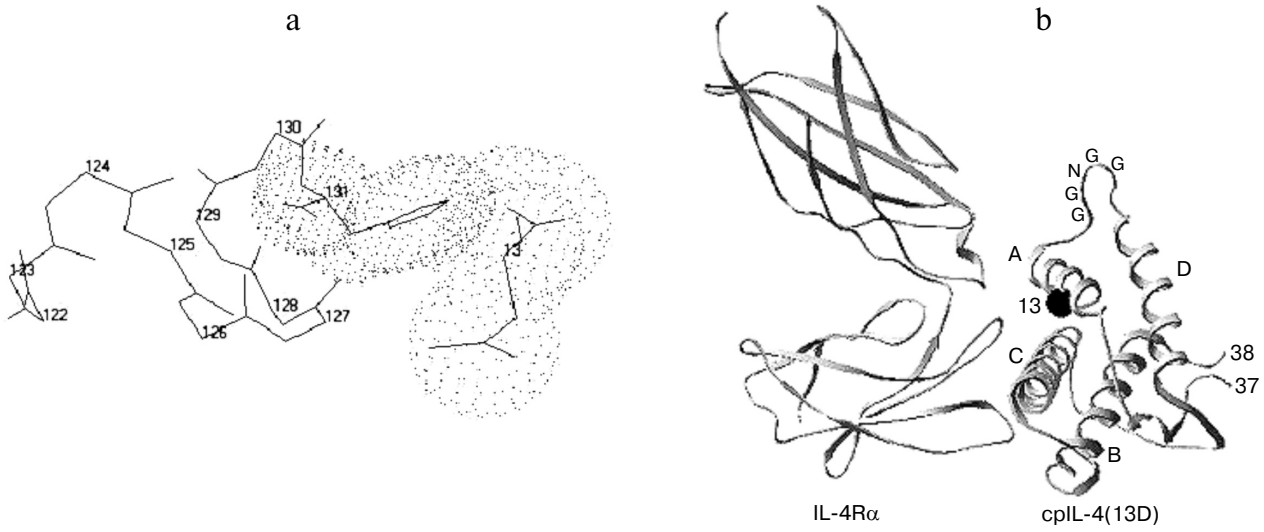


**Fig. 7.** a) Interactions of 13D with adjacent amino acids from IL-4$R_\alpha$. b) Structure of cpIL-4(13D) and IL-4$R_\alpha$.

ic antibodies can be easily obtained. Usually, the fusion protein constructed by directly connecting the two proteins may have impaired biological activity, the reason being that the fusion protein could not correctly fold or react with the receptor due to steric hindrance. That the fusion protein formed by fusing the native IL-4 to the toxin directly cannot effectively interact with the IL-4R is mostly due to steric hindrance.

IL-4 contains four antiparallel alpha-helical domains (A-D, from N- to C-terminal) and two β sheets. And IL-4R consists of two chains, IL-4$R_\alpha$ and IL-2$R_\gamma$ (or IL-13$R_{\alpha 1}$). The interaction of IL-4 and IL-4R involves a sequential, two-binding event that results in a 1 : 1 : 1 ternary complex. The first binding event involves the binding of helices A and C of IL-4 with IL-4$R_\alpha$, the first chain of IL-4R. The second binding event occurs when the IL-4/IL-4$R_\alpha$ complex recruits a second chain of IL-4R, IL-2$R_\gamma$ (or IL-13$R_{\alpha 1}$), in which the region interactive with IL-2$R_\gamma$ (or IL-13$R_{\alpha 1}$) is located on helix D of IL-4 [9, 10]. Therefore, helices A and C determine the affinity of IL-4 to its receptor, and helix D is responsible for the selective binding of IL-4 to different kinds of IL-4Rs.

Since the regions associated with the binding and signaling are near the N-terminal and C-terminal, ligation of a big toxin such as PE to the C-terminal of the native IL-4 could mostly interfere with the binding affinity of immunotoxin to IL-4R. To resolve this problem, an IL-4 mutein (cpIL-4) has been constructed, which encodes native IL-4 codons 38-129, GGNGG, and 1-37. Immunotoxin constructed by fusing the toxin PE to the C-terminal of cpIL-4 would weaken the steric hindrance resulting from the introduction of PE. The binding activity of the immunotoxin to IL-4R is 10% of that of free IL-4.

It should be noted that such binding efficiency is still very low in comparison with native IL-4; thus we made further modification on cpIL-4 to improve its binding activity. To determine the optimum mutation site, the steric structure of IL-4/IL-4$R_\alpha$ complex was carefully analyzed using Swiss PDB Viewer. It was found that the binding surface of helix A is from about position 5 to 16, and in helix C, it is from about position 77 to 89. Thus, amino acid substitutions occurred in these regions may increase the affinity of the resulting IL-4 mutein for IL-4$R_\alpha$. Of those potential mutation sites, position 13 is the most attractive because if it is changed to a negatively charge amino acid, the electrostatic interaction between this position and the positive charge-rich region of IL-4$R_\alpha$ will be enhanced (Fig. 7). This analysis is in agreement with Srivannaboon's work [8]. When they substituted Thr13 with Asp, an IL-4 mutein with 18-fold affinity increase was produced.

Provided that the steric structure of cpIL-4 is similar to that of the native IL-4, the mutein of cpIL-4 containing the corresponding amino acid mutation in the A helices should also show improved affinity. To test this idea, cpIL-4(13D) was constructed in this study. Cytotoxicity assay shows that the immunotoxin cpIL-4(13D)–PE38KDEL has 3-fold higher affinity than immunotoxin cpIL-4–PE38KDEL. Hence, the mutation of amino acid 13 could obviously increase the affinity between cpIL-4(13D) and IL-4R and improve the cytotoxicity of immunotoxin cpIL-4(13D)–PE38KDEL.

In this study, we construct a novel immunotoxin with increased activity, which is named cpIL-4(13D)–PE38KDEL. Because the ligand cpIL-4(13D) possesses improved affinity to its receptor, the cytotoxicity of the resulting immunotoxin has been greatly increased.

## REFERENCES

1. Kreitman, R. J., Puri, R. K., and Pastan, I. (1995) *Cancer Res.*, **55**, 3357-3363.
2. Debinski, W., Puri, R. K., and Pastan, I. (1994) *Int. J. Cancer*, **58**, 744-748.
3. Debinski, W., Puri, R. K., Kreitman, R. J., and Pastan, I. (1993) *J. Biol. Chem.*, **268**, 14065-14070.
4. Kreitman, R. J., Puri, R. K., and Pastan, I. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 6889-6893.
5. Puri, R. K., Hoon, D. S., Leland, P., Snoy, P., Rand, R. W., Pastan, I., and Kreitman, R. J. (1996) *Cancer Res.*, **56**, 5631-5637.
6. Puri, R. K., Mehrotra, P. T., Leland, P., Kreitman, R. J., Siegel, J. P., and Pastan, I. (1994) *J. Immunol.*, **152**, 3693-3700.
7. Husain, S. R., Behari, N., Kreitman, R. J., Pastan, I., and Puri, R. K. (1998) *Cancer Res.*, **58**, 3649-3653.
8. Srivannaboon, K., Shanafelt, A. B., Todisco, E., Forte, C. P., Behm, F. G., Raimondi, S. C., Pui, C. H., and Campana, D. (2001) *Blood*, **97**, 752-758.
9. Walter, M. R., Kood, W. J., Zhao, B. G., Cameron, R. P., Jr., Ealick, S. E., Walter, R. L., Jr., Reichert, P., Nagabhushan, T. L., Trotta, P. P., and Bugg, C. E. (1992) *J. Biol. Chem.*, **267**, 20371-20376.
10. Hage, T., Sebald, W., and Reinemer, P. (1999) *Cell*, **97**, 271-281.