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Increase of Spacer Sequence Yields Higher Dimer $(Fab\text{-}Spacer\text{-}Toxin)_2$ Formation

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Abstract The divalent antibody-toxins are expected to have increased binding avidities to target cells because of the two cell-binding domains. However, previous studies showed that the refolding yield of divalent antibody-toxin is very low, and it is assumed that homodimer formation of antibody-toxin is strongly interfered by the repulsion between the two large toxin domains that come close to each other during dimer formation. In this study, B3 antibody was used as a model antibody, and its Fab domain was used to construct three different kinds of Fab divalent molecules, $[B3(Fab)\text{-toxin}]_2$. The monomer Fab-toxin molecules were made by fusing the Fab domain of monoclonal antibody B3 to PE38, a truncated mutant form of *Pseudomonas* exotoxin (PE), and a connecting sequence that contained spacer amino acid sequence (G4S)n (n=1, 2, 3) was inserted between Fab and PE38. The prepared divalent molecules were $[\text{Fab-S1, 2, 3-PE38}]_2$ (=$[\text{Fab-SKPCIST-KAS}(G_4S)\text{nGGPE-PE38}]_2$ (n=1, 2, 3)), and they are derivatives of previously studied $[\text{Fab-H2cys-PE38}]_2$ (=$[\text{Fab-SKPCIST-KASGGPE-PE38}]_2$). In $[\text{Fab-S1, 2, 3-PE38}]_2$, two Fab-S1, 2, 3-PE38 monomers were covalently linked by the disulfide bond bridge made from cysteine in the -SKPCIST- sequence. The insertion of spacer amino acids after the disulfide bridge resulted in a 12–18 fold higher yield of dimer formation than previously constructed $[\text{Fab-H1cys-PZ38}]_2$ [7], 3–4-fold higher than $[\text{Fab-ext-PZ38}]_2$ [25]. These two molecules have less amino acid spacer sequence between the disulfide bridge and PE38 domain. The design of $[\text{Fab-PE38}]_2$ in this study gave molecules with a higher refolding yield. The results of cytotoxicity assay showed a higher cytotoxic effect of these divalent molecules than that of the monovalent scFv-PE38 molecule.

Key words: Recombinant antibody refolding, B3 antibody, divalent $(\text{Fab-toxin})_2$, cytotoxicity, divalent immunotoxin, *Pseudomonas* exotoxin A

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Recombinant antibody-toxins for the treatment of cancer have been prepared by connecting "carcinoma-specific" antibodies to toxins that selectively bind and kill cancer cells without harming normal cells [24]. Recombinant DNA technologies made it possible to produce several forms of recombinant antibody-toxins [1, 8, 9, 13, 15, 28]. Fv fragments of antibodies are heterodimers of antibody V_H and V_L domains and are the smallest antibody fragments that contain all the structures necessary for specific binding to antigen [5]. At first, the Fv fragments for the recombinant antibody-toxins were designed in a single-chain form (scFv-toxin), in which the heavy and light chain variable regions (V_H and V_L) were connected by a peptide linker [10, 11, 14, 23, 31, 32]. Subsequently, a method was developed to stabilize the Fv fragments by an interchain disulfide bond that connects structurally conserved framework regions of the V_H and V_L domains [16, 19, 22, 23]. Such disulfide-stabilized Fv(dsFv)-toxins are much more stable than scFv-toxins; however, its cytotoxicities and antitumor activities remain the same [3, 26, 27].

The Fab domains of antibody that are made of Fd (V_H-CH1) and light chain (V_L-CL) have also been used to prepare recombinant antibody-toxins. Fab-toxins showed a higher refolding yield and higher stability in animal blood circulation than scFv-toxin and dsFv-toxin [2, 6].

In our previous studies, divalent molecules in the form of Fab-toxin dimer were constructed to study the effect of divalency on the cytotoxic activities. These molecules were made of the Fab domain of B3 antibody that binds directly to a carbohydrate antigen of the Le^Y family that is found on the surface of many mucous carcinomas of the colon, stomach, ovary, breast, and lung as well as some epidermal carcinomas. The toxin protein was PE38, which is one of the truncated derivatives of *Pseudomonas* exotoxin A (PE). The divalent Fab-toxins previously prepared are $[\text{Fab-H1cys-PE38}]_2$ (=$[\text{Fab-CKPSIST-KASGGPE-PE38}]_2$ made of the Fd-PE38 fusion chain produced from pCE1 and light

Table 1. Plasmids and peptide chains used in this study.

Plasmid name	Name of peptide chain and its amino acid sequence	Ref.
pMCH75	H6-L=$(His)_6$-tagged light chain	[20]
pMC74	Fd-PE38=Fd-SKPSIST-KASGGPE-PE38	[6]
pCE1	Fd-H1cys-PE38=Fd-**C**KPSIST-KASGGPE-PE38	[7]
pCE2	Fd-H2cys-PE38=Fd-SKP**C**IST-KASGGPE-PE38	[7]
pCW1	Fd-ext-PE38=Fd-SKPSIST-KAS**$G_4C(G_4S)_2$**GGPE-PE38	[20]
pMH21	Fd-S1-PE38=Fd-SKPCIST-KAS**$(G4S)_1$**GGPE-PE38	This work
pMH22	Fd-S2-PE38=Fd-SKPCIST-KAS**$(G4S)_2$**GGPE-PE38	This work
pMH23	Fd-S3-PE38=Fd-SKPCIST-KAS**$(G4S)_3$**GGPE-PE38	This work
pMHS22	Fd-AS2-PE38=Fd-**AKPCIAT-Q**AS**$(G4S)_2$**GGPE-PE38	This work

CKPCICT, wild-type hinge sequence; SKPSIST-KASGGPE, connecting sequence between Fab and PE38 containing Cys to Ser substitution modified hinge; Gothic lettered sequence, spacer amino acid sequence inserted in connecting sequence -KAS--GGPE-; PE38, truncated PE toxin with 38-kd molecular weight.

chain from pMCH75) [7], and $[Fab-ext-PE38]_2$ (=$[Fab-SKPSIST-KAS(G_4C)(G_4S)_2GGPE-PE38]_2$ made of the Fd-PE38 fusion chain produced from pCW1 and light chain from pMCH75) [25], (Table 1). $[Fab-H1cys-PE38]_2$ (=$[Fab-CKPSIST-KASGGPE-PE38]_2$) was a dimer formed by the disulfide bond between the cysteines on the first cysteine position in the natural -CKPCICT- hinge sequence. The yield of $[Fab-H1cys-PE38]_2$ was very low, because the space allowed for the two PE38 was small and the steric hindrance between the two PE38 domains made the disulfide bond formation difficult. $[Fab-ext-PE38]_2$ was made to allow more space for PE38 and to increase the dimer formation. In the $[Fab-ext-PE38]_2$, the $(G_4C)(G_4S)_2$ spacer amino acid sequence was inserted, and the number of spacer amino acid between disulfide bond bridge cysteine and PE38 was increased by one amino acid (Table 2). The disulfide bond bridge was formed by the cysteine residue in the inserted $(G_4C)(G_4S)_2$ spacer sequence. The yield of $[Fab-ext-PE38]_2$ increased 4-fold, because the increased space between the disulfide bridge cysteine and PE38 reduced steric hindrance between two PE38s during the dimerization reaction [2].

Based on the result of $[Fab-ext-PE38]_2$, three divalent Fab-toxins were prepared in this study, using the Fab domain of B3 antibody with increasing the number of amino acid in spacer sequences to reduce the steric hindrances. $[Fab-S1, 2, 3-PE38]_2$ (=$[Fab-SKPCIST-KAS(G_4S)nGGPE-PE38]_2$ (n=1, 2, 3)) was constructed to contain the spacer amino acid sequence $(G_4S)n$ in series between Fab and PE38 to allow more spaces between bridge cysteine and PE38. The position of the cysteine residue for the disulfide bridge was the 4th amino acid position after the Fab domain that was the position of the second cysteine residue in the natural hinge sequence (-CKPCICT-).

We prepared another molecule that has alanine instead of serine on the cysteine position in the natural hinge sequence. We prepared this molecule to find out whether the alanine substitution gives higher yield by avoiding the collision between the two hydrogen atoms of the -OH groups of serine that come close to each other, when the disulfide bridge between -AKPCIAT- forms. The molecule $[Fab-AS2-PE38]_2$, (=$[Fab-AKPCIAT-QAS(G_4S)_2GGPE-PE38]_2$) that has a $(G_4S)_2$ insertion was produced and tested for the yield of divalent antibody-toxin.

The divalent antibody-toxin, $[Fab-S1, 2, 3-PE38]_2$, which has spacer amino acid insertions after the disulfide bridge, showed 12–18-fold higher yield than the $[Fab-H1cys-PE38]_2$ [7], and 3–4-fold higher yield than the [Fab-ext-

Table 2. The yield of divalent Fab-PE38 and the number of spacer amino acids between Cys and PE38 in each Fd-PE38 fusion chain.

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pCE1   ; Fd-------------------CKPSISTKASGGPE----------------PE38
pCW1   ; Fd----SKPSISTKASGGGGCGGGGSGGGGSGGPE--------------PE38
pMH21  ; Fd---------------SKPCISTKASGGGGSGGPE--------------PE38
pMH22  ; Fd---------------SKPCISTKASGGGGSGGGGSGGPE---------PE38
pMH23  ; Fd---------------SKPCISTKASGGGGSGGGGSGGGGSGGPE----PE38
pMHS22 ; Fd---------------AKPCIATQASGGGGSGGGGSGGGGSGGPE----PE38
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Plasmid name	Number of amino acids between Cys and PE38	Yield (%)	Ref.
pCE1	13	0.014%	[7]
pCW1	14	0.06%	[20]
pMH21	15	0.18%	This work
pMH22	20	0.23%	This work
pMH23	25	0.25%	This work
pMHS22	20	0.17%	This work

PE38]$_2$ [25]. These molecules have the structures of the divalent antibody-toxin fusion that give a higher refolding yield than the previous molecules.

These divalent Fab-toxins are expected to have several advantages over monovalent antibody-toxins. The first is the increase of binding avidity, as they have two binding domains. The second is the higher cytotoxicity to cancer cells with higher avidity. The third is higher stability in blood circulation with their highly stable Fab domains. The cytotoxicity assay of these molecules gave higher cytotoxicities than that of the monovalent scFv-PE38 reference molecule, showing that the higher valency of the molecule increased the cytotoxic effects.

MATERIALS AND METHODS

Materials

E. coli BL21 (λDE3) was used in this study to overexpress recombinant proteins. The plasmids used in this study are described in Table 1. Cancer cell lines for cytotoxicity assay were obtained from NCI NIH (Bethesda, MD, U.S.A.).

E. coli culture media used in this study were LB media and its components were purchased from Difco (Becton Dickinson, Sparks, MD, U.S.A.), and other chemicals were from Junsei (Tokyo, Japan) or Sigma (Missouri, U.S.A.). An antibiotic used in this study was ampicillin (150 ug/ml), purchased from Sigma. IPTG (isopropyl-β-thiogalactopyranoside) was purchased from Duchefa (Haarlem, Netherlands), restriction enzymes were from New England Biolab (NEB, MA, U.S.A.), and T4 DNA ligase, Taq polymerase, and dNTP mix were from TaKaRa (Shiga, Japan). Coomassie Plus Protein Assay Reagent was used for protein quantification and purchased from Pierce (Rockford, IL, U.S.A.).

Q-Sepharose column and Source-Q column of Pharmacia (Piscataway, NJ, U.S.A.) were used in the anion-exchange chromatography, and Superdex 200 of Pharmacia was used in the gel filtration chromatography.

Construction of Plasmids for Expression of the Fd-S 1, 2, 3-PE38

The plasmids pMH21, 22, 23 encoding Fd-S1, 2, 3-PE38 (=Fd-SKPCIST-KAS(G_4S)nGGPE-PE38(n=1, 2, 3)) fusion protein were constructed by the splicing PCR method [18] using pCE2 encoding Fd-H2cys-PE38 (=Fd-SKPCIST-KASGGPE-PE38) as a template and 6 primers that have codons for amino acid sequences to be inserted, (Gly_4Ser)n (n=1, 2, 3). The primer 1 (T7 promoter primer; 5'-TAA TAC GAC TCA CTA TAG GGA GA-3') was annealed to the 5'-end of the T7 promoter at the upstream of N-terminus of Fd, and the primer 2 (5'-AGA TCC GCC ACC ACC AGA AGC TTT TGT ACT TAT GCT-3') and primer 3 (5'- CCA GAT CCG CCA CCA CCA CTT CCC CCT CCC CCG GAA GCT TTT GTA CTT ATG CTA GGC TTA CT-3') containing parts of the amino acid sequence were annealed to the hinge region. The primer 4 (5'-TGC TGG TGG CGG ATC TGG AGG TCC CGA GGG CGG CAAG C-3') and primer 5 (5'-TGG TGG TGG CGG ATC TGG AGG TGG CGG AAG CGG AGG TCC CGA GGG CGG CAG C-3') containing the other parts of the amino acid sequence were annealed to the -KASGGPE- region in the opposite direction of primer 2, 3. The primer 6 (5'-GCC GCG GGT GCT GAA GCT GAC GTC GCC GCC GTC-3') was annealed to the SacII enzyme site in the PE38 domain of pMC74. Primers 1, 2, and 3 were used to get the Fd fragments, and primers 4, 5, and 6 were used to get the PE fragments. The PCR fragments were purified and splicing PCR was done with primers 1 and 6 with the combination of the Fd and PE fragments as templates. The splicing reaction products were purified and parts of them (-KAS**(G4S)$_n$**GGPE-PE38) were used to replace the corresponding fragment of pCE2 (plasmid encoding (Fd)-SKP**C**IST-KASGGPE-PE38).

The plasmid pMHS22 encoding Fd-AS2-PE38 (=Fd-AKPCIAT-QAS(G4S)$_2$GGPE-PE38) fusion protein that has alanines in place of cysteine residues in the hinge-derived region was constructed using pGN31 (plasmid encoding V_L-Fd-AKPCIAT-QASGGPE-PE38) as a template. The primer 7 (5'-GCG GAT CCG GTG GTG GCG GTT CTG ATG TGA AGC TGG TGG AAT CT-3') was annealed to the 5'-end of the DNA sequence encoding V_L-Fd-AKPCIAT-QASGGPE-PE38, and the primer 8 (5'-GGG AAT TCA TTA AGC TTG TGT AGC TAT GCA AGG CTT AGC ACC ACA-3') containing the -AKPCIATQ- sequence was annealed to the hinge region. The Fd PCR products were purified and used to replace the corresponding fragment of pMH22 (plasmid encoding Fd-S2-PE38).

Expression and Isolation of the Inclusion Bodies

The polypeptide chains Fd-S1, 2, 3-PE38 and H6-L were expressed in *E. coli* BL21 (λDE3) [20, 30]. The bacteria were grown in superbroth at 37°C. The proteins were induced by 1 mM IPTG. After 3 h of incubation, the cells were harvested. Outer membrane and periplasmic proteins were removed. The spheroplast pellets from 1l culture were resuspended in 90 ml buffer containing 50 mM Tris-Cl (pH 8.0), 20 mM EDTA (pH 8.0), and the lysozyme. After incubation at room temperature for 1 h, 10 ml of 5 M NaCl, 10 ml of 25% Triton X-100, and 100 µl of 1 M DTT were added, and the sample was homogenized and incubated for 1 h at room temperature. The inclusion bodies were collected and washed in 90 ml buffer containing 10 ml 25% Triton X-100 and 100 µl of 1 M DTT. The inclusion body pellet was washed again with urea buffer (4 M Urea, 0.1 M Tris-Cl pH 7.4) and 3 times with 50 mM Tris-Cl (pH 7.4), 20 mM EDTA (pH 7.4).

Refolding and Purification of the [Fab-S1, 2, 3-PE38]$_2$
Inclusion bodies were solubilized in buffer containing 6 M guanidine-HCl, 0.1 M Tris-HCl, and 2 mM EDTA (pH 8.0). The amounts of Fd-S1, 2, 3-PE38 and H6-L were determined by the Bradford assay method with Coomassie Plus Protein Assay Reagent. These Fd containing long chain and light chain pairs (1:1 molar ratio in 40 mg of total proteins) were put in 5 ml volume, and the mixture was reduced by the addition of 0.06 M DTT. Refolding was done by rapid dilution (1:100) into 495 ml of solutions containing 0.1 M Tris-HCl, 0.5 M L-arginine-HCl, 1.6 mM oxidized glutathione, and 2 mM EDTA. The refolded protein was dialyzed and purified by using Q-Sepharose, Source Q, and Superdex 200 (Amersham, U.K.) chromatographies [6].

Cytotoxicity Assays
The cytotoxicity of the purified [Fab-S1, 2, 3-PE38]$_2$ was evaluated by measuring ID_{50} value on the cancer cell lines, including A431, CRL1739, MCF7, and KB3-1, following the previous procedure [6]. Briefly, each cell line was plated at 2×10^4 cells/well in 96-wells, and serially diluted antibody-toxins were added. Cells were exposed to antibody-toxin for 24 h. For isotope labeling, 1 μCi of tritium-labeled leucine was added into each well and cells were incubated for 14 h. The incorporation of [^{3}H]-leucine into the cell proteins was measured with a Microbeta TriLux Liquid Scintillation Counter (Wallac EG&G co.) [12, 21].

Results

Construction of Plasmids Encoding [Fd-S1, 2, 3-PE38] and Overexpression of Fusion Proteins
The plasmids pMH21, 22, and 23 coding the Fd-S1, 2, 3-PE38 (=Fd-SKPCIST-KAS(G_4S)nGGPE-PE38(n=1, 2, 3)) fusion protein were constructed by modifying pCE2 encoding Fd-H2cys-PE38 (=Fd-SKPCIST-KASGGPE-PE38) as a template with the polymerase chain reaction. The amino acid sequences (G_4S)n were inserted in the middle of the -SKPCIST-KAS--GGPE- sequence in the Fd-H2cys-PE38 molecule. The Fd-AS2-PE38 was constructed to have the hinge-derived sequence with alanines in place of cysteines. In pCE2, the cysteines were replaced by serines. The DNA sequences of pMH21, 22, 23, and pMHS22 were confirmed by sequencing analysis. The polypeptides were expressed as inclusion bodies in *E. coli.* The purities of inclusion bodies of each chain were examined by densitometry (Tina ver.2.0) and calculated to be about 30–40% of the total proteins.

Refolding and Purification of [Fab-S1, 2, 3-PE38]$_2$
The protein chains purified as inclusion bodies were refolded by a redox shuffling method [4]. The refolded

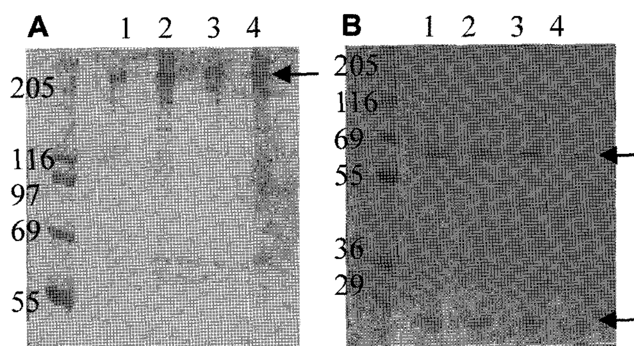

Fig. 1. Purified final antibody-toxins used for cytotoxicity assay.
A. An 8% nonreducing SDS-PAGE gel. The upper arrow indicates the dimers, [Fab-S1, 2, 3-PE38]$_2$ and [Fab-AS2-PE38]$_2$. Lane 1, [Fab-S1-PE38]$_2$; lane 2, [Fab-S2-PE38]$_2$, lane 3, [Fab-S3-PE38]$_2$; lane 4, [Fab-AS2-PE38]$_2$. **B.** A 12% reducing SDS-PAGE gel. The upper arrow indicates Fd-S1, 2, 3-PE38 and Fd-AS2-PE38 chains, and the lower arrow indicates the H6-L chain. Lane 1, reduced [Fab-S1-PE38]$_2$; lane 2, reduced [Fab-S2-PE38]$_2$; lane 3, reduced [Fab-S3-PE38]$_2$; lane 4, reduced [Fab-AS2-PE38]$_2$.

proteins were purified by chromatography. The purified dimers, [Fab-S1, 2, 3-PE38]$_2$, were analyzed by SDS-PAGE under both nonreducing and reducing conditions (Fig. 1).

The maximum refolding yields of [Fab-S1, 2, 3-PE38]$_2$ were 0.17–0.25% (Table 2). It is about 12–17 fold higher than that of [Fab-H1cys-PE38]$_2$ (0.014%) [7] that has a total of 13 spacer amino acids, -KPSISTKASGGPE-, between the disulfide bridge and PE38. It is about 2.8–4.1 fold higher than that of [Fab-ext-PE38]$_2$ (0.06%) [20] that has a total of 14 spacer amino acids, -GGGGSGGGGSGGPE-. The refolding yields of [Fab-H1cys-PE38]$_2$ and [Fab-H2cys-PE38]$_2$ were found to be very low, similar to the previous study, and the refolding yield of [Fab-H2cys-PE38]$_2$ was not measured [7]. The amino acid spacer sequence between the disulfide bridge and PE38 determines the degree of the freedom and steric hindrances between two PE38 domains. Increase of the spacer length enhances the dimer formation yield as it lessens the repulsion between the two PE38 domains from each monomer. The maximum refolding yield of alanine-substituted [Fab-AS2-PE38]$_2$ was 0.17%, and it was similar to the 0.23% maximum yield of [Fab-S2-PE38]$_2$.

Cytotoxicity of [Fab-S1, 2, 3-PE38]$_2$ Toward B3-Antigen-Expressing Cancer Cells
The cytotoxicity of antibody-toxin was analyzed by the [^{3}H]-leucine-incorporation method. The cytotoxicity assay was tested in triplicate, and the assay was repeated three times. In this assay, scFv-PE38 was used as a reference antibody-toxin, and the KB3-1 cancer cell line was used as the negative control.

The ID_{50} is the concentration that reduces [^{3}H]-leucine incorporation into target cells by 50%. The ID_{50} of antibody-toxins were obtained from the graphs in ng/ml concentration, and they were converted in pM concentration and presented in Table 3. The average ID_{50} values of [Fab-S1-PE38]$_2$,

Table 3. Average ID_{50} values of antibody-toxins from cytotoxicity assays.

Cell line	A431		CRL1739		MCF7		KB3-1
B3 antigen expression	+		++		++		-
Antibody-toxin \ ID50	ng/ml	pM	ng/ml	pM	ng/ml	pM	ng/ml
scFv-PE38	5.8	90.2	7.2	112.5	8.4	131.2	>1,000
[Fab-S1-PE38]$_2$	5.4	30.4	12.0	68.1	11.4	64.7	>1,000
[Fab-S2-PE38]$_2$	6.4	36.3	4.7	26.3	10.3	58.2	>1,000
[Fab-S3-PE38]$_2$	7.1	39.7	4.7	26.4	9.8	54.9	>1,000
[Fab-AS2-PE38]$_2$	16.0	90.3	9.2	51.7	13.7	77.1	>1,000

The ID_{50} values of antibody-toxins were obtained from the graphs in ng/ml concentration. They were then converted to pM concentration.

[Fab-S2-PE38]$_2$, [Fab-S3-PE38]$_2$, and [Fab-AS2-PE38]$_2$ were 30.4 pM, 36.3 pM, 39.7 pM, and 90.3 pM on A431 cell lines, 68.1 pM, 26.3 pM, 26.4 pM, and 51.7 pM on CRL1739 cell lines, and 64.7 pM, 58.2 pM, 54.9 pM, and 77.1 pM on MCF-7 cell lines. These ID_{50} values were lower than the average ID_{50} values of reference molecule, scFv-PE38, that were 90.2 pM on A431, 112.5 pM on CRL1739, and 131.2 pM on MCF-7. These results are shown in Table 3, and Fig. 2 shows the result of one set of the cytotoxicity assays. Cytotoxicities of the divalent antibody-toxins [Fab-S1, 2, 3-PE38]$_2$ in pM concentration are higher than that of positive control molecule scFv-PE38 on all positive cell lines.

DISCUSSION

Antibody is one of the most powerful and widely used tools in biotechnology [17, 29]. In this study, we prepared cancer therapeutic agent divalent Fab-toxins with increased

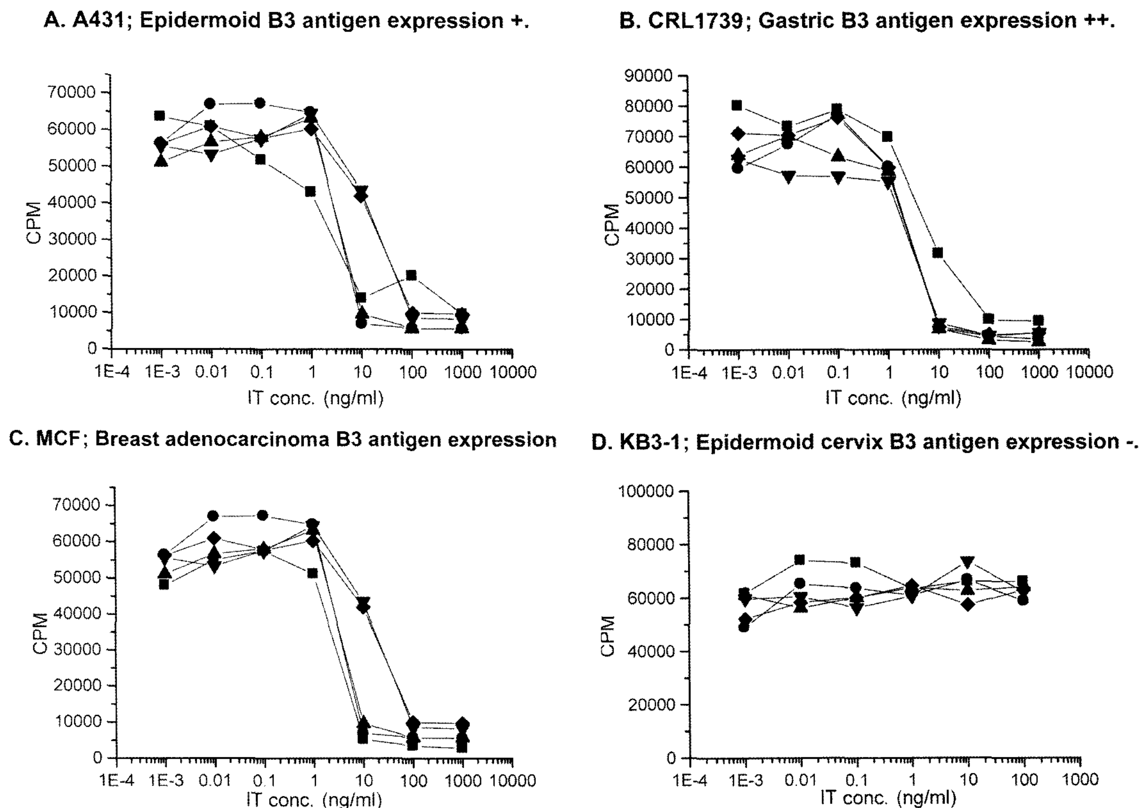

Fig. 2. Cytotoxicity assays of [Fab-S1, 2, 3-PE38]$_2$, [Fab-AS2-PE38]$_2$, and control molecule scFv-PE38 on four cancer cell lines.
(**A**) A431, (**B**) CRL1739, (**C**) MCF7, and (**D**) KB3-1 cell lines. A431, CRL1739, and MCF7 have B3 antigen, but KB3-1 does not. ■, scFv-PE38; ●, [Fab-S1-PE38]$_2$; ▲, [Fab-S2-PE38]$_2$; ▼, [Fab-S3-PE38]$_2$; ◆, [Fab-AS2-PE38]$_2$. Each data point is the average value of triplicate samples, and the assay was repeated three times. The average ID_{50} values from three assays obtained in ng/ml were converted to pM concentration, and they are shown in Table 3.

number of spacer amino acids between the disulfide bridge and PE38 to achieve higher refolding yields. The refolding yields of divalent Fab-toxins increased 12–17-fold higher than that of $[Fab\text{-}H1cys\text{-}PE38]_2$ and 3–4-fold higher than that of $[Fab\text{-}ext\text{-}PE38]_2$.

The differences in the insertions, $(G_4S)_n$ from n=1 to n=3, did not make significant differences in the yields of divalent Fab-toxins. They gave a similar yield of dimer formation, and this is most likely due to the fact that 15 amino acid spaces between the disulfide bridge and PE were enough to reduce the steric hindrance between two PE38s.

Twenty-five amino acid space between the disulfide bridge and PE38 in $[Fab\text{-}S3\text{-}PE38]_2$ were thought that it might be too long and allow the Fab domain and PE38 domain on a same chain to come close together during refolding processes, causing intra-chain (Fd to PE38) or intra-monomeric (Fab to PE38) refolding interferences. If this happens during the refolding process, it could interfere the tertiary structure formation of Fab and PE38 domain by scrambling the beta-sheet strands of Fab and PE38. The results showed that the yield of $[Fab\text{-}S3\text{-}PE38]_2$ was similar to the other two constructs, $[Fab\text{-}S1, 2\text{-}PE38]_2$, and the fact that the monomer formation did not decrease indicated that the folding of Fab and PE38 does not interfere with each other even under the large steric freedom given by twenty five amino acid spacer between the disulfide bridge and PE38.

$[Fab\text{-}S1, 2, 3\text{-}PE38]_2$ have disulfide bridge at the 4th amino acid position in the hinge-derived sequence after the Fab domain. These dimers showed increased yields, compared with $[Fab\text{-}ext\text{-}PE38]_2$ (=$[Fab\text{-}SKPSIST\text{-}KAS(G_4C)(G_4S)_2GGPE\text{-}PE38]_2$, which has a disulfide bridge cysteine after the serine-substituted hinge. It is highly likely that the cysteine in the hinge-derived sequence might have a higher chance of meeting with each other to form a disulfide bridge if the two hinge sequences have self-affinity. This could be an alternative explanation of why the $[Fab\text{-}S1, 2, 3\text{-}PE38]_2$ molecules have a higher dimer formation than the $[Fab\text{-}ext\text{-}PE38]_2$ molecule. However, the question of why $[Fab\text{-}H1cys\text{-}PE38]_2$, which has cysteine derived from the hinge, has a lower yield than $[Fab\text{-}ext\text{-}PE38]_2$ remains to be studied.

Substitution of the cysteine residue in the hinge sequence with alanine, $[Fab\text{-}AS2\text{-}PE38]_2$, did not increase the formation of the divalent antibody-toxin, compared with the $[Fab\text{-}S2\text{-}PE38]_2$ that has serine in place of cysteine in the natural hinge. This can be interpreted that the structure of the hinge region formed by two hinge sequences can allow enough spaces for the hydrogen atom of the -OH group of serine.

The cytotoxicities of divalent $[Fab\text{-}S1, 2, 3\text{-}PE38]_2$ were higher than that of the monovalent reference molecule scFv-PE38, when they were compared in pM unit. This was expected from the previous results of $[Fab\text{-}ext\text{-}PE38]_2$ that showed a 12-fold higher cytotoxicity than scFv-PE40 [25]. We assume that these include the cases where the divalent $[Fab\text{-}S1, 2, 3\text{-}PE38]_2$ bind only one antigen.

The results presented in this study demonstrated a new structure that could be used to produce more cytotoxic recombinant antibody-toxin molecules. The activity of antibody-toxin can be dependent on the antigen environment on the cell surface. If the antigens are on a long flexible structure and can come close enough for a divalent antibody-toxin to bind two of them together at the same time, the divalent antibody-toxin will show higher binding avidity than the monovalent antibody-toxin. On the other hand, if the antigens are on a rigid structure and the distances between them are too far for a divalent antibody-toxin to bind two of them at the same time, the divalent antibody-toxin will have the same affinity as the monovalent antibody-toxin. It remains to be seen whether the design of molecules in this study can be used to produce higher cytotoxic antibody-toxin with other kinds of antibodies.

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