Characterization and receptor specific toxicity of two diphtheria toxin-related interleukin-3 fusion proteins DAB_{389}–mIL-3 and DAB_{389}–$(Gly_4Ser)_2$-mIL-3

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Abstract We have constructed two fusion proteins, DAB_{389}–mIL-3 and DAB_{389}-$(Gly_4Ser)_2$–mIL-3, in which the receptor-binding domain of diphtheria toxin is replaced by mouse interleukin-3 (IL-3). Cytotoxic activity of the fusion toxins was observed on three out of six cell lines assayed. This toxicity was mediated through binding to the IL-3 receptor as it was inhibited in a dose-dependent manner with murine IL-3 or anti-IL-3 neutralizing antibodies. DAB_{389}–$(Gly_4Ser)_2$-mIL-3 was up to 5 times more toxic than DAB_{389}–mIL-3, depending on the cell line (0.8×10^{-10} M $< IC_{50} < 3\times10^{-10}$ M). These proteins can be used for the detection of IL-3 receptors on mouse cells and should allow for the selective elimination of IL-3 receptor-positive pluripotent hematopoietic stem cells prior to bone marrow transplantation.

Key words: Diphtheria toxin; Fusion toxin; Interleukin-3; Receptor targeting

1. Introduction

Interleukin-3 (IL-3) can stimulate the proliferation and differentiation of pluripotent hematopoietic stem cells as well as various lineage-committed progenitors [1]. The use of a cytotoxic agent highly specific for cells expressing the IL-3 receptor may have therapeutic potential in the removal of these cells prior to bone marrow grafting. Such treatment could prevent the side effects of conventional bone marrow depletion therapies, including toxicity for more mature hematopoietic cells and for fast dividing cells from other tissues.

In the past 15 years, many efforts have been made to target plant or bacterial toxins to specific cell populations [2]. Genetic fusion of the diphtheria toxin catalytic and translocation domains to various polypeptide hormones led to highly efficient and specific targeted toxins [3] safe enough to be used in humans as anti-tumor [4,5] or immunosupressive therapeutics [6–8].

We describe the construction of the fusion toxins DAB_{389}–mIL-3 and DAB_{389}–$(Gly_4Ser)_2$-mIL-3 aimed at killing cells expressing murine IL-3 (mIL-3) receptors. We demonstrate that the cytotoxic activity of these proteins is dependent on binding to the mIL-3 receptor, and that insertion of the $(Gly_4Ser)_2$ spacer between the diphtheria toxin translocation domain and mIL-3 enhances toxicity up to 5-fold.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli strain JM101 was used for plasmid propagation and cloning and strain BL21(DE3) (Novagen, Madison, WI) was used as the host for fusion toxins production.

2.2. Construction of vectors pCGmIL-3 and pCCmIL-3

The pET11d derived parental plasmid pETJV127 [9] encoding the diphtheria toxin-IL-2 fusion DAB_{389}–IL-2 was digested with *Nco*I to introduce a DNA fragment made by hybridization of the following oligonucleotides: 5′-CATGGGTCATCACCATCACCATCACGATGACGATGACAAAGG-3′ and 5′-CATGCCTTTGTCATCGTCATCGTGATGGTGATGGTGATGACC-3′. As a result, the N-terminal methionine of the encoded DAB_{389}–IL-2 protein is preceded by the following sequence: Met–Gly–His–His–His–His–His–His–Asp–Asp–Asp–Asp–Lys–Gly. The resulting plasmid pCG1 was digested with *Sph*I and *Hin*dIII to remove the IL-2 coding region at the 3′-end of the structural gene for DAB_{389}–IL-2. The coding sequence for mIL-3 was PCR-amplified from the *mIL-3* cDNA (R&D Systems, Abingdon, UK) using primers designed to introduce a *Sph*I and a *Hin*dIII restriction site at the 5′- and 3′-ends of the fragment, respectively: 5′-GGAGGCGGGCGCATGCTGATACCCACCGTTTAACC-3′ and 5′-GGCCGCGAGCAAGCTTTCATTAACATTCCACGGTTCC-3′. After digestion with *Sph*I and *Hin*dIII, the mIL-3-amplified sequence was ligated in plasmid pCG1 to give plasmid pCGmIL-3. Plasmid pCCmIL-3 was derived from pCGmIL-3 by insertion at its *Sph*I site of a DNA fragment made by hybridization of the following oligonucleotides: 5′-GTGGCGGTGGATCCGGCGGTGGCGGTTCGCATG-3′ and 5′-CGAACCGCCACCGCCGGATCCACCGCCACCATG-3′. As a result, the sequence $(Gly_4Ser)_2$ separates the diphtheria toxin translocation domain and mIL-3 in the encoded protein. All DNA insertions were checked by DNA sequence analysis.

2.3. Expression and extraction of the fusion toxins

Protein expression was under control of the T7 RNA polymerase promoter in *E. coli* BL21(DE3). Cultures were grown at 30°C in TSB medium (17 g/l Tryptone, 3 g/l Soytone, 5 g/l yeast extract (Difco, Detroit, MI), 2.5 g/l K_2HPO_4, 5 g/l NaCl) supplemented with 5 g/l glucose and 0.2 mg/l ampicillin. Two liters of medium were inoculated with an overnight culture of bacterial cells carrying plasmid pCGmIL-3 or pCCmIL-3 at a 1:50 dilution. When the absorbency at 600 nm reached 0.7, expression of the recombinant protein was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. After 2 h, cells were harvested by centrifugation and resuspended in 20 ml of 50 mM Tris-HCl, pH 8, 10 mM EDTA, 8% sucrose (w/v), 5% Triton X-100 (v/v) supplemented with 0.1 ml of 0.1 M PMSF (Sigma, St. Louis, MO) in ethanol and 1 ml of a 10 mg/ml solution of lysozyme (Sigma).

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After 1 h incubation on ice, the cells were lysed by sonication for 5 min (power setting: 50%; pulse: 1 s; rest: 1 s). The preparation was centrifuged at 14000×*g* for 30 min at 4°C and the supernatant fluid, corresponding to the soluble cytoplasmic fraction, was dialyzed twice at room temperature against 8 M urea, 0.1 M sodium phosphate, 10 mM Tris-HCl, pH 8, supplemented with 10 mM 2-mercaptoethanol.

2.4. Purification and refolding of the fusion toxins

The recombinant proteins were first purified by immobilized metal ion (Ni^{2+}) affinity chromatography (IMAC). Four milliliters of Chelating Sepharose Fast Flow (Pharmacia Biotech, Uppsala, Sweden) was charged with Ni^{2+} in a column according to the manufacturer's instructions and equilibrated with the urea buffer used precedently. The cytoplasmic extract containing the fusion toxin was allowed to pass twice through the column at room temperature. The column was washed with 20 ml of urea buffer, and then with 10 ml of refolding buffer (50 mM Tris-HCl, pH 8, 50 mM NaCl, 5 mM reduced glutathione, 1 mM oxidized glutathione). The flow of refolding buffer was stopped before complete passage, and the column was allowed to rest overnight at 4°C. After washing with 10 ml of 50 mM Tris-HCl, pH 8, 50 mM NaCl, proteins were eluted in 10 ml of 50 mM Tris-HCl, pH 8, 50 mM NaCl, 0.5 M imidazole. The protein sample was concentrated and dialyzed against 20 mM Tris-HCl, pH 7.4, 150 mM NaCl.

Further purification was performed by anti-mIL-3 antibodies (R&D Systems) affinity chromatography. IMAC purified fusion toxin was bound to the column in 27 mM KH_2PO_4, 46 mM NaOH, 10 mM EDTA, 0.75 M NaCl, 0.1% Tween 20, pH 8, and after washing in the same buffer, elution was performed in 0.1 M Na_2CO_3, 0.5 M NaCl, 0.1% Tween 20, at pH 11.3. The eluted sample was neutralized with 1:10 volume of 2 M Tris-HCl, pH 6.8, and concentrated and dialyzed against 20 mM Tris-HCl, pH 7.4, 150 mM NaCl.

2.5. SDS-polyacrylamide gel electrophoresis and Western blot analysis

Protein containing samples were analyzed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to conventional procedures, using a mini protean II apparatus (Bio-Rad, Ivry sur Seine, France). Proteins on the gels were either stained with Coomassie blue or detected by conventional Western technique using the following primary and secondary antibodies: horse anti-diphtheria toxin (Mérieux, Marcy-l'Etoile, France) or goat anti-mIL-3 (R&D Systems), then rabbit anti-horse IgGs-peroxidase conjugate or rabbit anti-goat IgGs-peroxydase conjugate (Immunotech, Marseille, France).

2.6. Cell cultures

All cell culture media were supplemented with 10% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel) and 2 mM glutamine.

The Ba/F3 cell line transfected with the rabbit mammary prolactin (Prl) receptor cDNA [10] was the kind gift of Dr. Isabelle Dusanter-Fourt, Institut Cochin de Génétique Moléculaire, Hopital Cochin, Paris, France. These cells were cultured in RPMI-1640 medium supplemented with 0.5 nM (10 ng/ml) ovine Prl (NIDDK, Baltimore, MD). The FDC-P1 cell line transfected with the murine erythropoietin (Epo) receptor cDNA [11] was the kind gift of Dr. Patrick Mayeux, Institut Cochin de Génétique Moléculaire. These cells were cultured in α-MEM supplemented with 2 U/ml Epo. The HUT 102/6TG cell line was cultured in RPMI-1640 medium. The cell lines P388D1, A20 and VERO were cultured in D-MEM.

2.7. Cytotoxicity assays

Cells were seeded at a density of 5×10^4 cells per well into 96-well MultiScreen-DV filtration plates (Millipore). The cells were exposed to various concentrations of the fusion toxins with or without recombinant mIL-3 or anti-mIL-3 antibodies in a final volume of 0.2 ml culture medium supplemented with 0.5 g/l gentamycin sulfate (Biological Industries). After incubation for 18 h at 37°C in a 6% CO_2 atmosphere, the medium was removed by aspiration on a Millipore MultiScreen device and replaced with 0.2 ml of leucine-free medium containing 1 μCi/ml [^{14}C]leucine (292 mCi/mmol) (Isotopchim, Ganagobie-Peyruis, France), 2 mM glutamine and 0.5 g/l gentamycine sulfate. After incubation for 3 h at 37°C in a 6% CO_2 atmosphere, the medium was removed by aspiration, the cells were lysed by the addition of 50 μl per well of 0.4 M KOH, and proteins were precipitated by the addition of 200 μl of 10% trichloroacetic acid. Insoluble material was collected on a glass-fiber filter using a TOMTEC Harvester 96 and radioactivity was counted in a 1450 MicroBeta liquid scintillation counter (Wallac, Turku, Finland).

3. Results

3.1. Design, expression and purification of two diphtheria toxin-related mIL-3 fusion proteins

The expression plasmid pCGmIL-3 encoding the diphtheria toxin-related mIL-3 fusion protein DAB_{389}–mIL-3 was derived from plasmid pETJV127 [9] encoding DAB_{389}–IL-2, a diphtheria toxin-related IL-2 fusion protein [12] as described in Section 2. The N-terminus of DAB_{389}–mIL-3 (Met–Gly–His–His–His–His–His–His–Asp–Asp–Asp–Asp–Lys–Gly–Met) which precedes the first glycine of mature diphtheria toxin, displays a six histidine tag for IMAC purification. This tag is followed by an enterokinase proteolytic cleavage site, for removal if needed. The catalytic and translocation domains of diphtheria toxin are then fused to the N-terminus of mIL-3. In mice, two N-terminal variants of native mIL-3, containing either 140 or 134 amino acid residues, have been described. It is believed that the shorter form arises from secondary proteolytic cleavage. Nevertheless, both forms have comparable

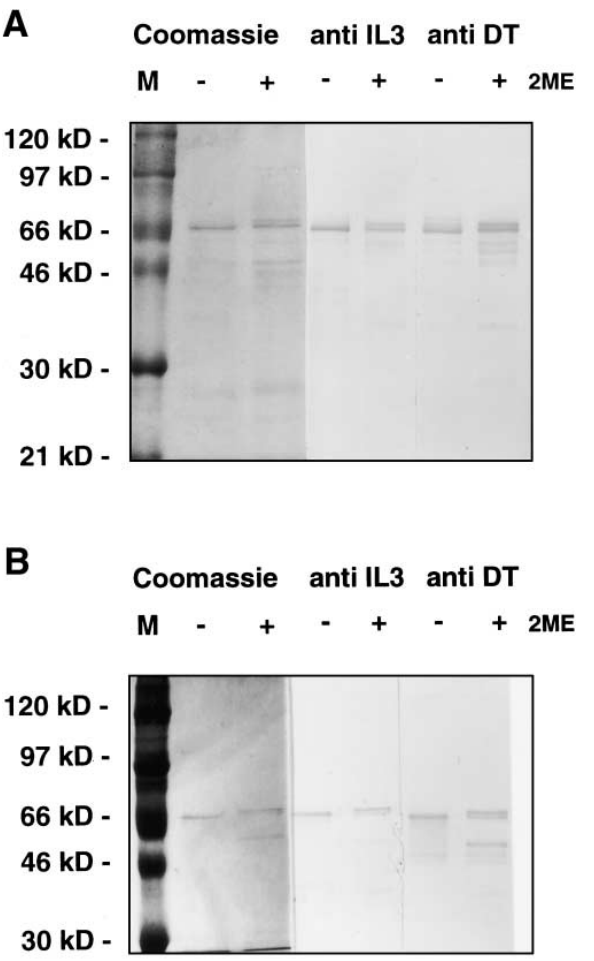

Fig. 1. SDS-PAGE analysis of purified DAB_{389}–mIL-3 (top) and DAB_{389}–$(Gly_4Ser)_2$-mIL-3 (bottom) under reducing or non-reducing conditions (2ME: β-mercaptoethanol) by Coomassie blue staining or Western detection using anti-mIL-3 antibodies or anti-diphtheria toxin antibodies.

activities in vitro [13]. In order to avoid the risk of proteolytic cleavage in vivo, we chose to link the shortest of these variants to the C-terminus of the diphtheria toxin translocation domain. As a result, the sequence at the junction is –His–Lys–Thr–**His–Ala**–Asp–Thr–His–. The first threonine corresponds to Thr^{387} of diphtheria toxin; the amino acid residues in bold characters correspond to the *Sph*I cloning site, and the aspartic acid is the first amino acid residue of the shorter form of mIL-3. (Note: amino acid numbers correspond to that of the DAB_{389}–hormone fusion family and are therefore +1 with respect to mature diphtheria toxin.)

Kiyokawa et al. [14] described that insertion of a peptidic spacer of at least 10 amino acid residues between the diphtheria toxin part and the IL-2 part of the fusion toxin DAB_{389}–IL-2 enhanced its toxicity to IL-2-receptor-bearing cells 5-fold, due to increased accessibility of the hormone for its receptor. We thus derived plasmid pCCmIL-3 from pCGmIL-3 in order to insert the flexible and hydrophilic sequence $(Gly_4Ser)_2$ at the junction of the diphtheria toxin and the mIL-3 sequence of DAB_{389}–mIL-3, leading to the molecule DAB_{389}–$(Gly_4Ser)_2$-mIL-3. As a result, the sequence at the junction is –His–Lys–Thr–**His Ala**–Gly–Gly–Gly–Gly–Ser–Gly–Gly–Gly–Gly–Ser–**His–Ala**–Asp–Thr–His–, both **His–Ala** sequences corresponding to the doubling of the *Sph*I restriction sequence due to the oligonucleotide insertion, although a single nucleotide substitution alters the left-most site.

DAB_{389}–mIL-3 and DAB_{389}–$(Gly_4Ser)_2$-mIL-3 were expressed under control of the T7 RNA polymerase promoter in *E. coli* BL21(DE3) and were found mainly in the soluble fraction of the cytoplasm. The proteins were purified under denaturing and reducing conditions on a Ni^{2+} IMAC column and refolded while still immobilized on the solid phase of the column, as described in Section 2. This refolding procedure avoids the handling of large volumes of highly diluted protein needed in case of liquid-phase refolding. DAB_{389}–mIL-3 and DAB_{389}–$(Gly_4Ser)_2$-mIL-3 were further purified by affinity chromatography using polyclonal anti-mIL-3 antibodies.

Fig. 1 shows the detection of the fusion proteins after SDS-PAGE of the purified samples, followed by Western blotting. The proteins reacted with both anti-diphtheria toxin and anti-mIL-3 antibodies and have an electrophoretic mobility consistent with the expected molecular mass of 59 000 Daltons deduced from the amino acid sequence. Under reducing conditions, both fusion toxins appear as two bands of slightly lower mobility than the band detected in non-reducing conditions. We suggest that this pattern corresponds to the reduction of either one or both of the disulfide bonds present in the refolded fusion protein. One bond is expected to link the C-terminal part of the diphtheria toxin catalytic domain to the N-terminus of the translocation domain. Another bond is expected in mIL-3.

Coomassie blue staining of the fusion proteins following SDS-PAGE shows that they are purified to about 80–90% homogeneity (Fig. 1).

3.2. *DAB_{389}–mIL-3 is cytotoxic and cytotoxicity is targeted to the mIL-3 receptor*

The toxicity of DAB_{389}–mIL-3 was assayed on the bone marrow-derived murine pro-B lymphoid Ba/F3 cell line transfected with the rabbit mammary Prl receptor cDNA [10]. Ba/F3 cells express the mIL-3 receptor. They are dependent on either mIL-3 or Epo for propagation. However, the expression of a Prl receptor in these cells enables propagation in the absence of mIL-3, provided Prl is present [10], without the loss of mIL-3 receptor expression on the cell surface. Fig. 2 shows that DAB_{389}–mIL-3 inhibited protein synthesis in Prl receptor-positive Ba/F3 cells in a dose-dependent manner, with an IC_{50} of 1×10^{-10} M. On the contrary, protein synthesis of the IL-2 receptor-positive human cutaneous T-cell lymphoma cell line HUT 102/6TG was not significantly affected after overnight incubation with 10^{-8} M DAB_{389}–mIL-3 (Fig. 2A), although this cell line is highly sensitive to the IL-2 receptor-targeted diphtheria toxin derivative DAB_{389}–IL-2 [12].

DAB_{389}–mIL-3 cytotoxicity assays were repeated on Prl receptor-positive Ba/F3 cells in the presence of varying concentrations of recombinant mIL-3 (Fig. 2B). The cytotoxic effect of DAB_{389}–mIL-3 decreased by about 100-fold in the presence of an mIL-3 concentration of 10^{-9} M and about 10-fold in the presence of an mIL-3 concentration of 10^{-10} M. The cytotoxic effect of DAB_{389}–mIL-3 at a concentration of 10^{-8} M was totally abolished in the presence of the same concentration of mIL-3 (10^{-8} M).

Inhibition of cytotoxicity was also found in a dose-dependent manner in the presence of dilutions of purified anti-mIL-3 polyclonal antibodies (Fig. 2C).

Taken together, these results demonstrate that the cytotoxic

Table 1
Cytotoxic activity of DAB_{389}–mIL-3 and DAB_{389}–$(Gly_4Ser)_2$-mIL-3

Cell line	Toxin $IC_{50}\times10^{-10}$ M					
	$(Gly_4Ser)_2$		$(Gly_4Ser)_2$		$(Gly_4Ser)_2$	
	–	+	–	+	–	+
			+10^{-9} M mIL-3		+1 μg/ml anti-mIL-3	
Ba/F3-rPrl	**1**	**0.8**	>100	**10**	>100	>100
FDC-P1-rEpo	**10**	**2**	>100	**80**	>100	>100
P388D1	**15**	**3**	>100	**100**	>100	>100
A20	>100	>100	>100	>100	>100	>100
HUT102/6TG	>100	>100	>100	>100	>100	>100
VERO	>100	>100	>100	>100	>100	>100

IC_{50} measured from cytotoxicity assays on various cell lines following exposure to DAB_{389}–mIL-3 or DAB_{389}–$(Gly_4Ser)_2$-mIL-3 (– or + $(Gly_4Ser)_2$ linker), in the absence or presence of mIL-3 or anti-mIL-3 antibodies as competitors. Values >100 indicate that at the highest toxin concentration of 10^{-8} M, toxicity if any was lower than 50%. Variation between replicates was lower than 20%. Maximum radioactivity incorporation ranged from 10 000 to 40 000 cpm depending on the cell line.

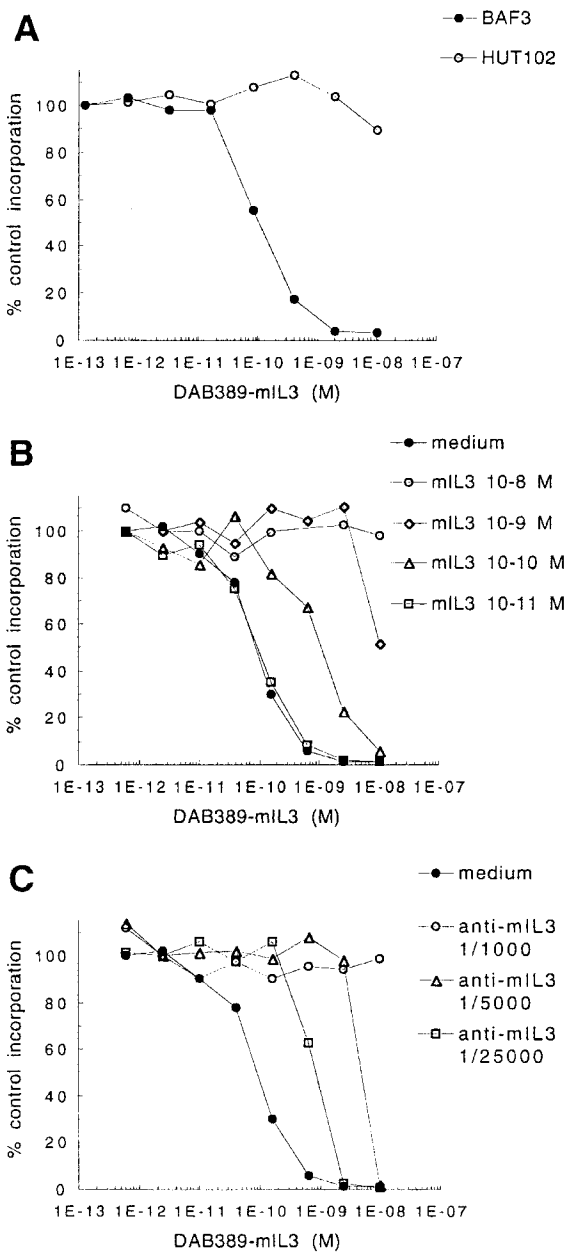

Fig. 2. Cytotoxicity assays following exposure to DAB_{389}–mIL-3. A: Toxicity on Prl receptor-expressing Ba/F3 cells or HUT 102/6TG cells. B: Toxicity on Prl receptor-expressing Ba/F3 cells in the absence or presence of various concentrations of recombinant mIL-3. C: Toxicity on Prl receptor-expressing Ba/F3 cells in the absence or presence of various dilutions of anti-mIL-3 antibodies made from a 1 mg/ml solution. Measures were done in quadruplicates except in the presence of recombinant mIL-3 (B) in which case they were done in duplicate. Variation between replicates was lower than 20%. Maximum radioactivity incorporation reached about 35-40 000 cpm.

effect of the diphtheria toxin-related fusion protein DAB_{389}–mIL-3 is mediated through binding to the mIL-3 receptor and that it is not toxic to a cell line devoid of that receptor.

3.3. *Comparison of the cytotoxic effects of DAB_{389}–mIL-3 and DAB_{389}–$(Gly_4Ser)_2$-mIL-3 on various cell lines*

The toxicity of both DAB_{389}–mIL-3 and DAB_{389}–$(Gly_4Ser)_2$-mIL-3 was assayed on six cell lines including the Prl receptor-positive Ba/F3 cells and the HUT102/6TG cells. The four other cell lines were the following: (1) the murine myelomonocytic leukemia cell line FDC-P1 transfected with the murine erythropoietin (Epo) receptor cDNA [11]. FDC-P1 cells express the mIL-3 receptor and are dependent on mIL-3 for propagation. However, the expression of the Epo receptor enables propagation in the absence of mIL-3, provided Epo is present [11]; (2) the murine macrophage-like cell line P388D1 which was shown to respond to mIL-3 by secreting IL-1 [15]; (3) the African green monkey kidney cell line VERO, highly sensitive to native diphtheria toxin, but necessarily deprived of mIL-3 receptors; and (4) the murine B cell lymphoma cell line A20, for which the mIL-3 receptor expression status is unknown to our knowledge.

Each assay was performed with varying concentrations of toxins, in the absence or presence of 10^{-9} M mIL-3 or 1 μg/ml purified anti-mIL-3 antibodies. The results are summarized in Table 1.

Both toxins inhibit protein synthesis in the three cell lines known to express mIL-3 receptors but not in cells devoid of that receptor. Interestingly, DAB_{389}–$(Gly_4Ser)_2$-mIL-3 was found 5 times more toxic on two cell lines and slightly more toxic on a third one. In all cases, cytotoxicity could be inhibited with either mIL-3 or anti-mIL-3 antibodies.

4. Discussion

We have shown that both fusion toxins DAB_{389}–mIL-3 and DAB_{389}–$(Gly_4Ser)_2$-mIL-3 were cytotoxic to cells expressing the mIL-3 receptor, and that cytotoxicity was mediated through binding to that receptor. However, efficiency of toxicity varies depending on the targeted cell line. Several explanations could account for this observation: the number of receptors on the cell surface, their affinity, or their rate of internalization, as it was shown that both diphtheria toxin [16,17] and the diphtheria toxin-related fusion toxins [18–21] need to be internalized into the cell by receptor-mediated endocytosis in order to exert their toxic action.

While preparing this manuscript, we were aware of a work describing a diphtheria toxin–mIL-3 fusion, $DT\text{-}_{390}$–mIL-3 [22], similar to DAB_{389}–mIL-3. Unfortunately, one cannot compare the values of IC_{50} found in both studies, due to important differences in the cytotoxicity assay conditions. However, DAB_{389}–$(Gly_4Ser)_2$-mIL-3 was found 5-fold more toxic than DAB_{389}–mIL-3 on two of the three cell lines assayed, while mIL-3 at a concentration of 10^{-9} M was less efficient to inhibit DAB_{389}–$(Gly_4Ser)_2$-mIL-3 than DAB_{389}–mIL-3 (Table 1). These results suggest that the presence of a peptidic spacer between the diphtheria toxin part of the fusion toxin and its mIL-3 part favors the accessibility of the hormone for its receptor, hence increasing the binding affinity of the fusion toxin. Such increase in affinity correlated with an increase in toxicity was demonstrated in case of DAB_{389}–IL-2 fusion toxin derivatives when the size of the peptidic spacer was comprised between 10 and 20 amino acid residues [14].

We anticipate that our toxins will be cytotoxic to murine pluripotent hematopoietic stem cells in vivo and we shall investigate the treatment conditions to allow the specific removal of those cells prior to bone marrow grafting in mice.

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