Protein Engineering vol.13 no.8 pp.575–581, 2000

Characterization of diphtheria fusion proteins targeted to the human interleukin-3 receptor

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Diphtheria fusion proteins are chimeric proteins consisting of the catalytic and translocation domains of diphtheria toxin (DT_{388}) linked through an amide bond to one of a variety of peptide ligands. The ligand targets the molecule to cells and the toxin enters the cell, inactivates protein synthesis and induces cell death. Diphtheria fusion proteins directed to human myeloid leukemic blasts are a novel class of therapeutics for patients with chemotherapy refractory myeloid leukemia. Because of the presence of interleukin-3 (IL3) receptors on myeloid leukemic progenitors and its absence from mature myeloid cells, we synthesized four bacterial expression vectors encoding DT_{388} fused to human IL3. Different molecules were engineered to assess the effects of modifications on yield, purity and potency of product. The constructs differed in the size of the linker peptide between the DT_{388} and IL3 domains and in the presence or absence of an oligohistidine tag on the N- or C-terminus. *Escherichia coli* were transformed and recombinant protein induced and purified from inclusion bodies. Similar final yields of 3–6 mg of purified protein per liter of bacterial culture were obtained with each of the four molecules. Purity ranged from 70 to 90% after partial purification by anion-exchange, size-exclusion chromatography and/or nickel affinity chromatography. Proteins were soluble and stable at 4°C and –80°C in phosphate-buffered saline at 0.03–0.5 mg/ml. The fusion proteins showed predicted molecular weights by SDS–PAGE, HPLC and tandem mass spectrometry and had full ADP-ribosylating activities. Each was immunoreactive with antibodies to DT_{388} and IL3. Each of the fusion proteins with the exception of the one with an N-terminal oligohistidine tag showed full IL3 receptor binding affinity (K_d = 3 nM) and potent and selective cytotoxicity to IL3 receptor positive human myeloid leukemia cell lines (IC_{50} = 5–10 pM). In contrast, the N-terminal histidine-tagged fusion protein bound IL3 receptor with a 10-fold lower affinity and was 10-fold less cytotoxic to IL3 receptor positive blasts. Thus, we report a series of novel, biologically active DT_{388}IL3 fusion proteins for potential therapy of patients with receptor positive myeloid leukemias.

Keywords: acute myeloid leukemia/diphtheria toxin/fusion protein/interleukin-3

Introduction

Diphtheria toxin (DT), the 535 amino acid residue polypeptide chain secreted from *Corynebacterium diphtheriae* lysogenized by toxin-encoding bacteriophage, is composed of three domains (Choe *et al.*, 1992). These domains include a receptor-binding domain on the C-terminus of the molecule, a transmembrane domain in the middle and a catalytic domain at the N-terminus. After cell binding (Naglich *et al.*, 1992), the toxin internalizes by receptor-mediated endocytosis (Simpson *et al.*, 1998), translocates to the cytosol (Chiron *et al.*, 1994; Oh *et al.*, 1996) and inactivates protein synthesis (Blanke *et al.*, 1994; Falnes *et al.*, 1995). The cell then rapidly undergoes apoptosis (Kochi and Collier, 1993).

Previously, the receptor-binding domain of DT was replaced with a number of cell-selective ligands yielding highly potent and selective fusion proteins (Foss *et al.*, 1998). One such fusion protein, DAB_{389}IL2 (ONTAK) produced a 30% partial and complete response rate in patients with advanced cutaneous T cell lymphoma (Frankel *et al.*, 2000a). Our laboratory previously selected human granulocyte-macrophage colony-stimulating factor (GM-CSF) as a ligand for fusion to a truncated form of DT encoding amino acid residues 1–388 to produce the fusion toxin DTGM (Frankel *et al.*, 1997). After *in vitro* and *in vivo* studies, we initiated a phase I clinical study for patients with relapsed or refractory acute myeloid leukemia (AML) (Frankel *et al.*, 1999). While results to date are encouraging and we are dose escalating, several factors led to our seeking another leukemia-selective ligand for fusion to DT_{388}. A fraction of AML patient blasts and all chronic myeloid leukemic blasts were insensitive to DTGM (Frankel *et al.*, 1998; Hogge *et al.*, 1998; Feuring-Buske *et al.*, in preparation). Further, we have observed a systemic inflammatory response after DTGM patient infusions which we have correlated with DTGM binding to normal mononuclear cells and stimulation of TNFα release (A.E.Frankel *et al.*, in preparation). Thus, we wanted to produce a second-generation diphtheria fusion protein for myeloid leukemias which would target a different group of leukemias and would not stimulate cytokine release.

Interleukin-3 (IL3) is a cytokine which supports the proliferation and survival of multi-potential and committed myeloid and lymphoid progenitors (Suda *et al.*, 1985). The IL3 receptor (IL3R) is composed of α and β subunits (Hara and Miyajima, 1992) and binding of IL3 causes rapid internalization of the ligand–receptor complex (Nicola *et al.*, 1988). Evidence suggests that the receptor is present on human myeloid leukemia cells (Park *et al.*, 1989; Ailles *et al.*, 1997; Frankel *et al.*, 2000b) but is absent from mature mononuclear cells and some primitive stem cells (Ogata *et al.*, 1992). Based on the expression of its receptors on blasts from patients with acute phase CML and AML, the absence of receptors from mature mononuclear cells and some primitive stem cells and rapid receptor-mediated endocytosis on receptor coupling, we chose to fuse IL3 to the catalytic and translocation domains of DT (DT_{388}).

Two other laboratories have synthesized diphtheria fusion proteins directed to the mouse IL3R (Chan *et al.*, 1996; Liger

et al., 1997). Liger *et al.* found that a long linker peptide was required to retain IL3R binding affinity and fusion protein cytotoxicity. In order to determine the optimal DT_{388}IL3 fusion molecule for scale-up and possible eventual clinical development, we engineered a series of bacterial expression vectors with differing linker peptides and peptide extensions on the N- and C-termini. These different constructions were made in order to define the molecule with the greatest potency, stability and ease of preparation and purification prior to production of clinical-grade material. In this work we characterized the yield, purity and bioactivity of these fusion proteins.

Materials and methods

Construction of plasmids

IL3 DNA inserts with *Nde*I/*Hin*dIII restriction sites at the ends were synthesized by PCR using BBG14 DNA (Research Diagnostics, Minneapolis, MN). Primers for the 'M' DT_{388}IL3 containing an $MH(G_4S)_2$ linker were 5′-GCAGTCGAC-CATATGGGCGGAGGCGGAAGTGGAGGAGGAGGCAG-CGCTCCCATGACCCAGACA-3′ and 5′-GCATGCGCAAA-GCTTCTAAAAGATCGCTAGCGACAA-3′. Primers for the 'D' DT_{388}IL3 with an MH linker included 5′-GCAGTCGAC-CATATGGCTCCCATGACCCAGACA-3′ and the second primer above. PCR was carried out as described previously (Frankel *et al.*, 2000b) and products were subcloned in pCRII. pCRII-IL3s and pRKDTGM plasmid (Kreitman and Pastan, 1997) were digested with *Nde*I and *Hin*dIII. The 0.4 kb IL3 DNA fragments and 3.7 kb pRKDT DNA fragment were isolated, ligated and the DT_{388}IL3 encoding M and D plasmids prepared and sequenced. M plasmid encoded DT_{388}IL3 with an $MH(G_4S)_2$ linker between the DT_{388} and IL3 moieties. D plasmid encoded DT_{388}IL3 with only an MH linker. PCR reactions were next carried out to generate DT_{388}IL3 molecules with different restriction sites at the ends for subcloning in expression vectors containing N- or C-terminal oligohistidine tags. The PCR reaction used the 'D' plasmid. Primers for the 'P1' DT_{388}IL3 included 5′-GCAGCTGCACCATGGCTTAT-CCAGAAACCGATTATAAAGACGATGACGATAAGGCT-CCCATGACCCAGACA-3′ and 5′-GCAGCTGCACTCGA-GAAAGATCGCTAGCGACAA-3′. Primers for the 'P5' DT_{388}IL3 included 5′-GCAGCTGCAGCTAGCATGGGCGC-CGACGACGTC-3′ and 5′-GCAGCTGCAAAGCTTCTA-GAATATGGCGAGCGACAAAGTCGTCTGTTG-3′. P1 and P5 DT_{388}IL3 encoding DNAs had *Nco*I/*Xho*I and *Nhe*I/*Hin*dIII restriction sites at the ends, respectively. PCR products were subcloned in pCRII. pCRII-DT_{388}IL3s were then restricted with *Nco*I/*Xho*I or *Nhe*I/*Hin*dIII and the 1.6 kb DT_{388}IL3 encoding DNA fragments ligated to similarly isolated 5.4 kb plasmid fragments of *Nco*I/*Xho*I restricted pET31d and *Nhe*I/*Hin*dIII restricted pET28a, respectively. The P1 pET21d-DT_{388}IL3 plasmid encoded DT_{388}-MH-IL3 followed by LEH_6. The P5 pET28a-DT_{388}IL3 plasmid encoded $MGS_2H_6S_2$ GLVPRGSHMASM followed by DT_{388}-MH-IL3.

Expression of fusion proteins

Competent BLR (λDE3) were transformed with 4 μg of M, D, P1 or P5 DT_{388}IL3 expression plasmids. Transformants were expanded into 3 l of Superbroth with 100 μg/ml ampicillin (M, D and P1) or 50 μg/ml kanamycin (P5); recombinant protein expression was induced with 1 mM IPTG at OD_{650} = 0.6; incubation was continued for 2 h.

Purification of fusion proteins

Cells were then harvested and extracted and inclusion bodies isolated as previously described for DTGM (Frankel *et al.*, 1999). Recombinant protein (60–90 mg) was centrifuged; the supernatant was added dropwise to1200 ml of a stirred 0.5 M L-arginine–1 mM EDTA–0.1 M Tris, pH 8–664 mg oxidized glutathione solution at 4°C and and refolding was continued at 4°C for 48 h. Refolded protein was then dialyzed at 4°C against 20 mM Tris, pH 8 and filter-sterilized and the material loaded on a 5 ml HiTrap Q column in buffer A (20 mM Tris, pH 8). Protein was washed with buffer A and eluted with a 25:75 mixture of buffer B (buffer A + 1 M NaCl) and buffer A. The eluate was diluted 6-fold with buffer A and loaded and washed on a 1 ml HiTrap Q column. After elution with a 50:50 mixture of buffers A and B, the eluate was loaded on an S-200 column and eluted with a 75:25 mixture of buffers A and B. The monomer fractions were pooled, filter sterilized and stored at –80°C.

Portions of the P1 and P5 refolded proteins were dialyzed into binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9) and incubated with a charged Ni^{2+} affinity matrix (Novagen). The matrix was washed with binding buffer and eluted with Eelute buffer (binding buffer + 1 M imidazole). The eluates were then redialyzed against 100 volumes of binding buffer.

Biochemical characterization of recombinant DT_{388}IL3 proteins

Protein was quantitated by the Pierce Coomassie Plus assay according to the recommendations of the supplier (Pierce Chemical, Rockford, IL). Aliquots of DT_{388}IL3 proteins, prestained molecular weight protein standards, human IL3 and DTGM were subjected to reducing 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and either stained with Coomassie Brilliant Blue R-250 or transferred to nitrocellulose and immunoblots made with anti-human IL3 and anti-DTA as performed previously (Frankel *et al.*, 2000b). To measure the enzymatic activity of the DT_{388}IL3s, we employed an ADP ribosylation assay (Collier and Kandel, 1971; Hwang *et al.*, 1987) with DAB_{389}EGF (Shaw *et al.*, 1991) as a positive control as described previously (Frankel *et al.*, 2000b). Free thiols were determined by the use of Ellman's reagent and spectrophotometry using a Genesys5 spectrophotometer (Spectronic, Rochester, NY) as described (Ellman, 1959). Two-dimensional PAGE with isoelectric focusing was carried out using pH 3.5–10, 4–6 and 5–7 ampholines by Wallin's modification of the O'Farrell method (Wallin *et al.*, 1984). HPLC of DT_{388}IL3s on a TSK3000 column with PBS at 1 ml/min was performed along with protein standards BSA and ovalbumin. Optical density at 280 nm was monitored. Tandem mass spectrometry was performed on a Quattro II triple-quadrupole mass spectrometer running MassLynx software after reversed-phase HPLC on a C4 Vydak column with 0–70% acetonitrile in 0.05% trifluoroacetic acid as mobile phase as described previously (Frankel *et al.*, 1999).

DT_{388}IL3 receptor binding affinities

TF1 cells were maintained in the log phase of cell growth in RPMI 1640 media with L-glutamine and 10% fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate, 1% penicillin–streptomycin and 50 ng/ml GM-CSF in a humidified atmosphere of 5% CO_2–95% air at 37°C. A 5 μg amount of high-affinity IL3 receptor agonist SC65461 (a gift from Dr Barbara Klein, Monsanto, St. Louis, MO (Thomas *et al.*, 1995) was iodinated using Iodogen and $Na^{125}I$ as described previously (Alexander *et al.*, 2000) to a specific activity of 90.5 μCi/μg. Aliquots of washed 1×10^6 cells in RPMI 1640–2.5% BSA–

20 mM HEPES–0.2% sodium azide were mixed with increasing amounts of IL3 (SC50341, a gift from Dr Barbara Klein) or DT_{388}IL3s in a constant amount of ^{125}I-labeled SC65461 in a total volume of 170 µl. Cells were incubated at 37°C for 40 min and then layered over a 200 µl oil phthalate mixture (1 part dioctyl phthalate and 1.5 parts dibutyl phthalate; Aldrich, Milwaukee, WI). After centrifugation at 16 000 *g* for 2 min in a microfuge at room temperature, both pellets and supernatants were separated and radioactivity was counted in a Packard Auto-Gamma 5650 gamma counter gated for ^{125}I with 50% counting efficiency. Background c.p.m. was calculated by linear extrapolation from incubations with excess cold IL3. Non-linear regression analysis was performed with GraphPad Prism software to determine the IC_{50} and K_d values.

Cell cytotoxicity assay

TF1, TF1-Bcl2, TF1-ΔMek-LNL6, TF1-ΔMek-Bcl2 and TF1/ΔRaf were gifts from Dr J.McCubrey, East Carolina University, Greenville, NC. Other cell lines were obtained and maintained as described previously (Frankel *et al.*, 2000b). TF1/ΔRaf cells were cultured identically with TF1 cells. Cells were maintained at (1–5)×10^5 cells/ml in RPMI 1640–10% FBS–penicillin–streptomycin–L-glutamine–L-pyruvate–non-essential amino acids–50 ng/ml GM-CSF; 1.5×10^6 cells were washed with serum-free RPMI 1640 and then resuspended in 10 ml of RPMI 1640–10% FBS–penicillin–streptomycin–L-glutamine–L-pyruvate–non-essential amino acids and 100 µl aliquots containing 1.5×10^4 cells were placed in individual wells of a Costar 96-well flat-bottomed plate. Twelve different concentrations of DTGM or DT_{388}IL3s were added to each well in 50 µl of medium and the cells were maintained at 37°C in 5% CO_2 for 48 h. Then 1 µCi of [^{3}H]thymidine in 50 µl of medium was added to each well and incubation continued for an additional 18 h at 37°C in 5% CO_2. Cells were harvested with a Skatron Cell Harvester (Skatron Instruments, Lier, Norway) on to glass-fiber mats and ^{3}H c.p.m. were counted on a Betaplate reader gated for ^{3}H. The calculated IC_{50}s were the concentrations of toxin which inhibited thymidine incorporation by 50% compared with control wells.

Results

Yields and purity of fusion toxins

Multiple preparations of each DT_{388}IL3 construct were produced. Ten, seven, five and two 3 l fermentations were made with P1, D, M and P5 plasmid transformed *Escherichia coli*. Some differences in protein induction were observed. The P1 inclusion body protein yield was 88 ± 13 (n = 10) mg/l culture, the D inclusion body protein yield was 36 ± 10 (n = 7) mg/l, the P5 inclusion body protein yield was 31 ± 1 (n = 2) mg/l and the M inclusion body protein yield was 25 ± 2 (n = 5) mg/l. The percentage of protein which had the size of DT_{388}IL3 on SDS–PAGE was examined; 59% (n = 1) of the P1 inclusion body was the size of DT_{388}IL3 on Coomassie Brilliant Blue-stained gels, 62% (n = 1) of the D inclusion body, 41 ± 7% (n = 2) of P5 inclusion body and 44 ± 9% (n = 3) of M inclusion body. This results in starting amounts of 52, 22, 13 and 11 mg/l for P1, D, P5 and M recombinant proteins, respectively.

The recovery of protein after dialysis was good for all the samples we examined. P1 recovery was 62%; D recovery 71%; P5 recovery 100%; and M recovery 87 ± 18% (n = 2). As expected, the percentage of total protein that was the correct molecular weight was not changed after dialysis: P1 was 63%, D was 62%, P5 was 40% and M was 44%. Thus, most of the expressed proteins were loaded on to anion-exchange matrices.

The majority of all four DT_{388}IL3s bound the HiTrap Q anion-exchange matrices. In the absence of EDTA in buffers A and B, essentially no protein was lost in flow-through or wash steps. With 0.25 or 0.5 parts of buffer B, the DT_{388}IL3 proteins eluted rapidly in high yield. The fractional recovery from the 5 ml Q matrix was 0.25, 0.5, 0.62 and 1.0 of the loaded 60 kDa protein. Protein recovered from the 5 ml Q matrix bound and eluted quantitatively from the 1 ml matrix with negligible losses. Thus, the anion-exchange chromatography concentrated the DT_{388}IL3 from a starting volume of 1400 ml to 2.5 ml (560-fold).

The size-exclusion chromatography step removed aggregates and dimers. This constituted a large fraction of P1, P5 and M and 25% of D. The final yields were 3 mg/l for P1, 6 mg/l for D, 3 mg/l for P5 and 4 mg/l for M. The final purities based on Coomassie Brilliant Blue-stained SDS–PAGE were 85% for P1, 81% for D, 90% for P5 and 70% for M.

When P1 and P5 were subjected to Ni^{2+} affinity chromatography, recombinant proteins were recovered with 86 and 88% of the starting material for P1 and P5, respectively. Final protein purities were 99 and 97%, respectively.

Biochemical characterization

Coomassie Brilliant Blue-stained SDS–PAGE showed that most of the protein contaminants were of lower molecular weight than the 60 kDa DT_{388}IL3s (Figure 1A). Immunoblots confirmed the reactivity of all four DT_{388}IL3 molecules with both anti-IL3 and anti-DTA antibodies (Figure 1B and C). A weaker band migrating in the region of the lower molecular weight contaminants (50 kDa) reacted with anti-DTA but not anti-IL3. It was most visible in the partially purified D, P5 and M DT_{388}IL3s. HPLC using a TSK3000 size-exclusion column revealed a predominant protein species of 60 kDa for each of the preparations. The P1 and D preparations showed a small amount (5–10%) of small (25 kDa) polypeptides. Using Ellman's reagent, we detected two free thiols in ricin toxin A chain and no free thiols in DTGM. P1, D and P5 had no measurable free thiols and M1 yielded 0.24 free thiols per molecule. ADP ribosylating activity was present in all the DT_{388}IL3 proteins. The specific activities of D, M, P1 and P5 DT_{388}IL3 were 3-, 10-, 10- and 32-fold less active, respectively, than DAB_{389}EGF (Figure 2). Two-dimensional IEF gels revealed p*I*s of 6.5, 6.5, 7 and 7 for D, M, P1 and P5, respectively (Figure 3). Tandem mass spectrometry showed excellent agreement between the calculated and observed molecular masses. P1 was calculated to be 58 738 Da and was observed to be 58 757 Da, D was calculated to be 57 691 Da and was observed to be 57 689 Da, P5 was calculated to be 60 222 Da and was observed to be 60 269 Da and M was calculated to be 58 321 Da and was observed to be 58 322 Da.

IL3 receptor affinities

IL3 competed for [^{125}I]IL3 binding to TF1 cells with a half-maximum binding inhibition concentration (equivalent to the K_d) of 3 ± 1 nM (n = 7) (Figure 4). The D and M DT_{388}IL3s had nearly intact IL3 receptor binding affinities with K_ds of 4 ± 2 nM (n = 3) and 4 ± 1 nM (n = 3), respectively. In contrast, P1 had a moderately reduced affinity with a K_d of 11 ± 8 nM (n = 4) and P5 had a dramatically reduced affinity with a K_d of 49 ± 28 nM (n = 3).

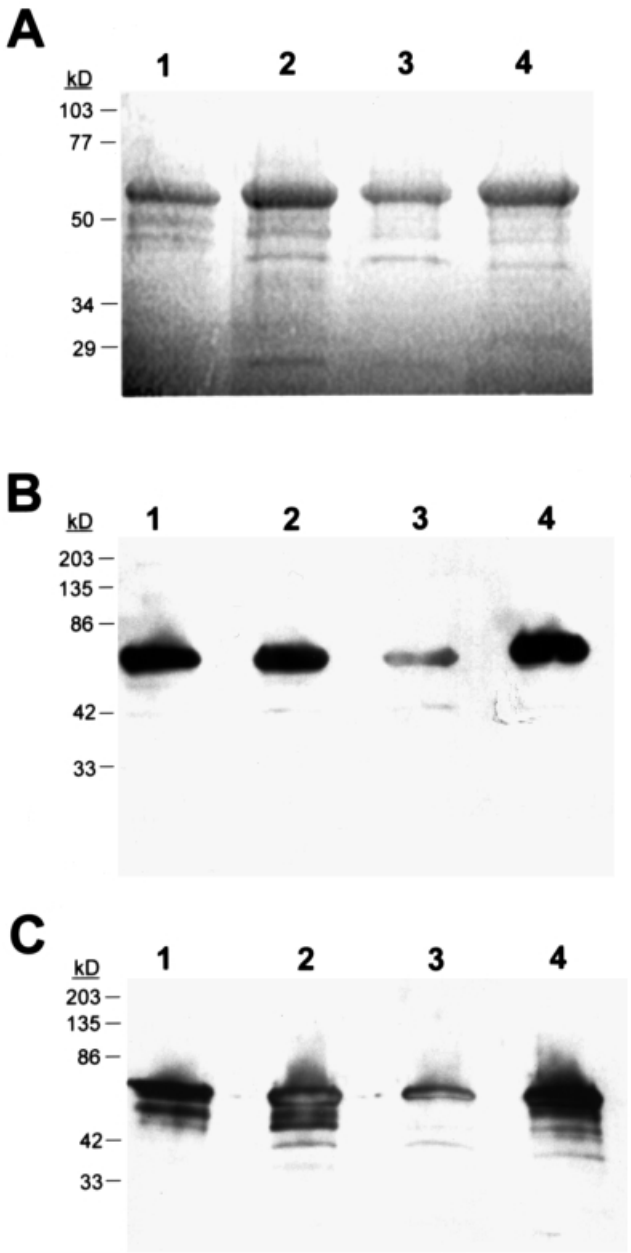

Fig. 1. 10% SDS–PAGE of DT_{388}IL3s. (**A**) Coomassie Brilliant Blue stained; (**B**) anti-IL3 immunoblot; (**C**) anti-DTA immunoblot. Molecular weight standards were run on each gel and replaced by arrows at the left of each gel. Lane 1, D; lane 2, M; lane 3, P1; lane 4, P5.

Cell cytotoxicities

TF1-ΔRaf cells were used as the IL3 receptor-positive cell line for measurements of [^{3}H]thymidine incorporation inhibition. Twelve different concentrations of DT_{388}IL3s were tested with 48 h co-incubation with cells. The concentration of fusion toxin reducing thymidine incorporation by 50% was determined for each fusion toxin. The IC_{50}s varied between the different DTIL3s (Figure 5). The most potent was D with $IC_{50} = 5 \pm 1$ pM ($n = 3$). The second most active was P1 with $IC_{50} = 9 \pm 3$ pM ($n = 2$). M was intermediate with $IC_{50} = 10 \pm 3$ pM ($n = 4$). P5 was 10-fold less active with $IC_{50} = 50 \pm 8$ pM ($n = 7$). In each experiment, DTGM was used as a control and had $IC_{50} = 1 \pm 0.2$ pM ($n = 18$). A D DT_{388}IL3 preparation was further tested on 12 different cell lines. The IC_{50}s were 5, 2, 1, 2, 2, 5, 5, 8 and 105 pM for the IL3 receptor-positive cell lines TF1/ΔRaf, TF1, TF1-Bcl2, TF1/ΔMek-LNL6, TF1/ΔMek-Bcl2, AML193, M07e, HL60 and OCI-AML, respectively. The IC_{50}s were >19 400 pM for the IL3 receptor-negative cell lines K562, CEM, P815 and TK6.

Discussion

We felt that it was important to optimize the design and synthesis of fusion proteins prior to extensive preclinical or clinical development. Previously, DAB_{486}IL2 and LMB1 fusion proteins required further design modifications after initial clinical and preclinical studies showed difficulties in drug stability, purification and efficacy (Frankel *et al.*, 1995). This paper describes our efforts to select the best DT_{388}IL3 molecule possible for further work.

In order to accomplish this task, we studied two molecules which varied in the length of the linker between the toxin and ligand domains and two molecules which provided N-terminal or C-terminal oligohistidine tags to facilitate purification. We found that increasing the linker peptide size did not improve potency or specificity and that adding the oligohistidine tag failed to improve the yield or quality of purification significantly. Thus the DT_{388}-MH-IL3 or D protein was the best molecule for additional preclinical evaluation.

Complete sequencing of the coding regions of the M, D, P1 and P5 plasmids confirmed the intended constructions. After transformation of bacteria with the plasmids, similar growth rates were observed for each both before and after induction (~30 min doubling times), suggesting that the recombinant proteins were not toxic to the bacteria. This was expected since the enzymatic activity of the DT_{388} moiety is restricted to eukaryotic elongation factor-2 (Van Ness *et al.*, 1980). We previously observed similar excellent growth of bacteria transformed with plasmids encoding DTGM (Frankel *et al.*, 1999).

Productions of inclusion body recombinant proteins were similar to each other for D, P5 and M with about 30 mg of protein per liter of bacterial culture. These results were similar to our previous results with DTGM of 23 mg/l (Frankel *et al.*, 1999). However, the P1 transformants produced more than twice as much recombinant protein. The P1 plasmid encodes a different T7 promoter than the D or M plasmids. P1 was constructed with the use of pET21d which has a T7 promoter including a lac repressor binding site; D and M were made from the pRKDTGM plasmid, a derivative of pET3a which does not include a lac repressor site in the T7 promoter. Since P5 also has a lac repressor site in the T7 promoter and produced significantly less than P1, other unknown factors may have produced the different expression levels.

Refolding was remarkably successful with DT_{388}IL3s. Assays of cytotoxicity of recombinant proteins at the completion of refolding and after dialysis revealed full bioactivity for the 60 kDa material (unpublished data). L-Arginine redox buffers gave a refolding yield of 24% for DTGM (Frankel *et al.*, 1999), but we estimate a refolding yield of >50% for the DT_{388}IL3s. This compares favorably with other proteins and refolding environments (Buchner *et al*, 1992).

Partial purification was achieved with anion-exchange and size-exclusion chromatography for each molecule. In contrast to our previous work with DTGM, it was critically important for DT_{388}IL3 purifications to remove EDTA from the anion-exchange buffers and to raise the anion-exchange buffer pH to 8.0. Differences in p*I* or other properties of the proteins

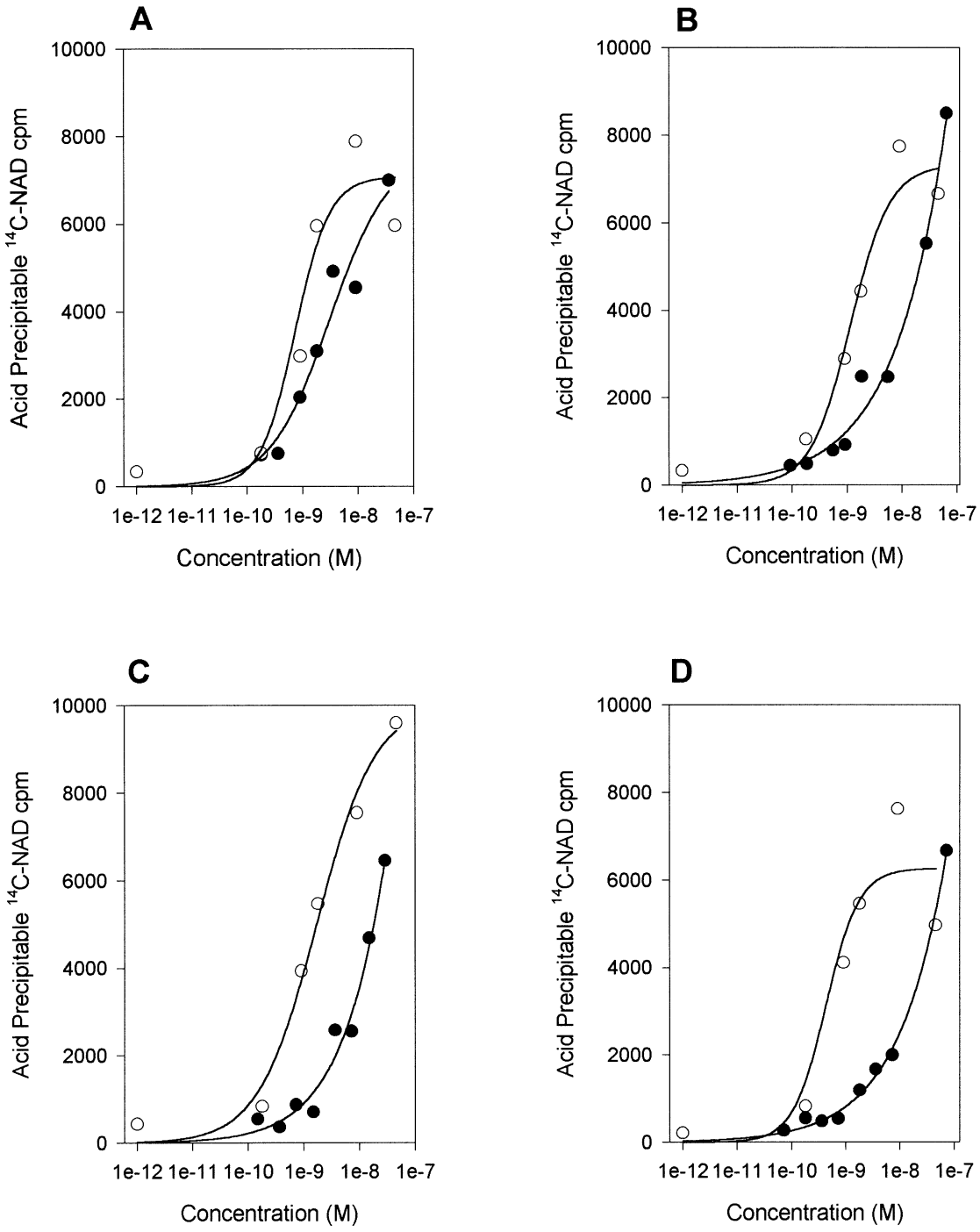

Fig. 2. ADP-ribosylating activity of DT_{388}IL3s. DAB_{389}EGF (Seragen, Hopkinton, MA) or DT_{388}IL3s in varying concentrations was mixed in DTEB buffer (0.2% bovine serum albumin, 40 mM dithiothreitol, 1 mM EDTA, 50 mM Tris, pH 8.0, in a total volume of 450 µl. To this, we added 10 µl of [^{14}C]NAD and 20 µl of wheat germ extract. After incubation at 37°C for 120 min, protein was TCA precipitated and counted. Molarity of DT_{388} versus acid-precipitable c.p.m. was plotted. ○, DAB_{389}EGF; ●, DT_{388}IL3s. (**A**) D; (**B**) M; (**C**) P1; (**D**) P5.

may be responsible. The order of purity achieved decreased in the order P5 > P1 = D > M. Improved purifications can be achieved by more narrowly collecting the monomer peak. With D DT_{388}IL3, we have recently increased the purity to >90% simply by modifying the peak collection (unpublished data). The addition of Ni^{2+} affinity chromatography for the molecules with oligohistidine tags (P5 and P1) increased the purity to >97%. The major contaminants appear to be fragments of DT_{388}IL3 with loss of the C-terminal IL3 peptide. This probably occurs in the bacteria since the fragments were observed after cell pelleting prior to extraction and processing (unpublished data).

Biochemical characterization revealed the correct mass, free thiols and domain functions. Since the structure of DT reveals physically separate domains for enzyme, translocation and receptor binding activities (Choe *et al.*, 1992), the replacement of the normal tissue binding domain with human IL3 was not expected to alter the non-cell binding functions of the molecule. We and others have observed similar results with other DT fusions (Foss *et al.*, 1998; Frankel *et al.*, 1999).

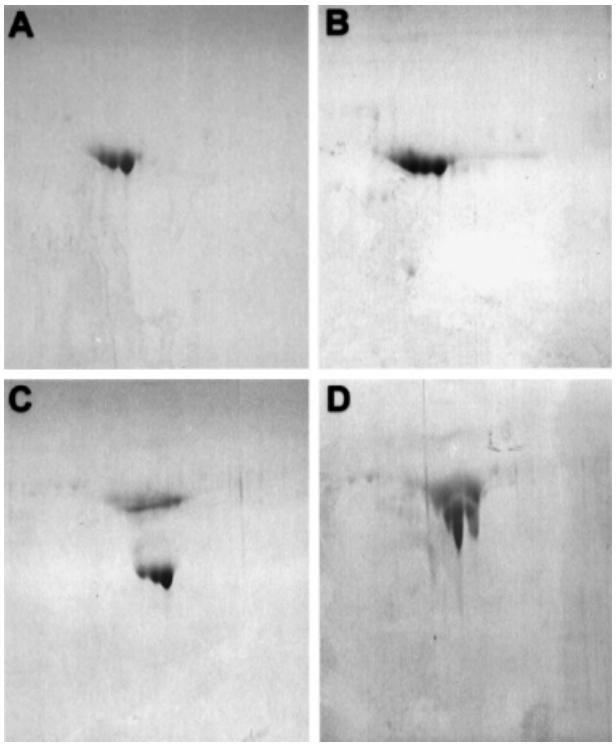

Fig. 3. Two-dimensional PAGE with isoelectric focusing. Gel stained with Commassie Brilliant Blue with pH 3.5–10 ampholines used. (**A**) D; (**B**) M; (**C**) P1; (**D**) P5.

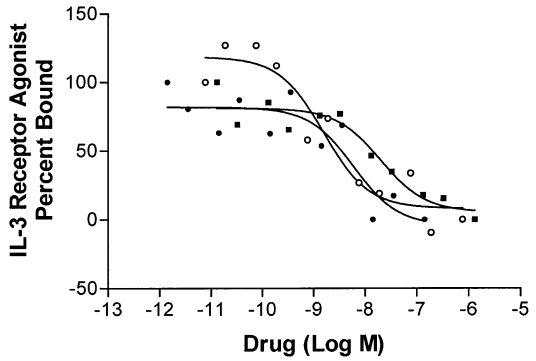

Fig. 4. Cold competition experiments for [^{125}I]SC65461 binding to TF1 cells. Increasing amounts of unlabeled IL3 (SC50341) or DT_{388}IL3s were added to a constant amount of radiolabeled SC65461 high-affinity IL3 receptor agonist in order to measure the K_d of IL3. Background binding was determined by vast excess unlabeled IL3 and subtracted from each point. The percentage of [^{125}I]SC65461 bound is plotted against the logarithm of the concentration of unlabeled IL3. ○, IL3; ●, M; ■, P5. Half-maximum inhibition = K_d was 1.6 nM, R^2 = 0.9038 for IL3, 5.9 nM, R^2 = 0.8227 for M and 20 nM, R^2 = 0.9176 for P5 for the assays shown.

Full IL3 receptor binding affinities for the M and D DT_{388}IL3s were unexpected. Two other laboratories have constructed DT_{388} fusions with mouse IL3 (Chan *et al*, 1996; Liger *et al*, 1997) and one group (Liger *et al.*, 1997) reported that a 10 amino acid linker peptide between the DT_{388} and mIL3 moieties was critical for full DT_{388}mIL3 bioactivity and binding. The DT_{388} fusions with human IL3 did not require a large linker peptide for retention of IL3 receptor binding. Differences between the human and mouse IL3 receptors (Orban *et al.*, 1999) or differences in refolding/purification may be responsible. Remarkably, the eight amino acid addition to the C-terminus of IL3 only marginally impaired (less than

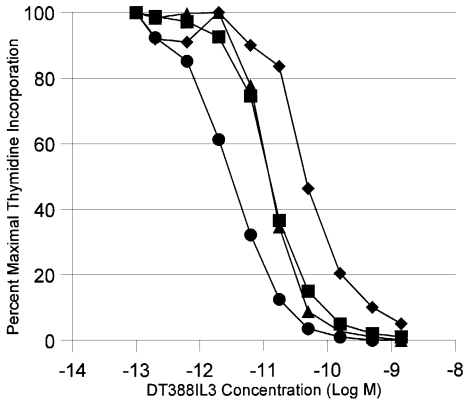

Fig. 5. TF1-ΔRaf cell cytotoxicity. Cells were incubated for 48 h with 12 different concentrations of fusion proteins and then pulsed for 18 h with [^{3}H]thymidine and then harvested on to glass-fiber mats and counted. Percentage maximum thymidine incorporation versus fusion toxin concentration was plotted for each representative assay. ▲, P1; ●, D; ◆, P5; ■, M. For the experiments shown, the IC_{50}s were 3, 7, 9 and 47 pM for D, M, P1 and P5, respectively.

3-fold) its binding affinity to its receptor. Based both on the theoretical docking of human IL3 to its receptor (Lyne *et al.*, 1995) and tolerant positions for single-site and region-restricted mutagenesis (Olins *et al.*, 1995; Klein *et al.*, 1999), the C-terminal 16 amino acids may not directly participate in receptor binding. The dramatic alteration in affinity for the P5 molecule was also unexpected. This molecule has a 24 amino acid peptide added to the N-terminus. Since the addition is to the DT_{388} moiety, we did not expect any effect on ability of this IL3 fusion protein to bind its receptor. However, a 10-fold reduction in IL3 receptor affinity occurred. This change may have resulted from improper P5 folding or steric effects from the freely mobile N-terminal peptide.

For the evaluation of cell cytotoxicity, we employed the TF1-ΔRaf cell line, because it had a reduced growth requirement for cytokines in the medium owing to the constitutively active Raf transgene (McCubrey *et al.*, 1998). Consequently, the co-incubation with fusion toxins could be done in the absence of human GM-CSF or IL3, which has been shown to compete for cell binding (Frankel *et al.*, 2000b). All the DT_{388}IL3 fusion proteins were cytotoxic to IL3 receptor-positive leukemic cell lines. The order of potency was D > P1 = M >> P5. The efficiency of cell kill is similar to that observed with DTGM. The lower potency for the P5 DT_{388}IL3 parallels its lower receptor affinity. The cell sensitivity of a panel of malignant cell lines corresponds to the presence of human IL3 receptor and matches closely our previous results with an early M DT_{388}IL3 preparation (Frankel *et al.*, 2000b). The *in vitro* therapeutic window is >10 000.

We originally sought to construct an additional fusion toxin for myeloid leukemias because our first generation fusion toxin, DTGM, was toxic to blasts from only a fraction of patients with acute myeloid leukemia (AML) and none of the patients with chronic myeloid leukemia (CML). Further, we wanted to avoid the cytokine release observed with the first-generation fusion protein. In an initial study of patient leukemic progenitors, 3/9 AML patient blasts and 4/11 acute phase CML blasts had a >1 log colony inhibition with M DT_{388}IL3 (Frankel *et al*, 2000b). When tested on human mononuclear cells, P1 DT_{388}IL3 did not trigger TNFα release (A.E.Frankel, in preparation). Thus, DT_{388}IL3 appears to satisfy all our

criteria for a second-generation myeloid leukemia-directed fusion toxin.

Although difficult to predict from tissue culture data, the similar potency and specificity of DT_{388}IL3 to DTGM on cell lines with both receptors are encouraging. Since DTGM has shown anti-leukemic activity in an ongoing phase I clinical trial in patients with chemotherapy refractory AML (unpublished data), DT_{388}IL3 appears to be a reasonable candidate for further clinical development.

Acknowledgements

We are grateful to Dr James McCubrey for providing AML cell lines and Dr Barbara Klein for recombinant IL3 proteins. We appreciate the advice and guidance of Dr Robert Kreitman. This work was supported by Leukemia and Lymphoma Society grant 6114-98 (A.E.F.) and National Institutes of Health grant R01CA76178 (A.E.F.).

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Received March 15, 2000; revised April 29, 2000; accepted May 2, 2000