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A Diphtheria Toxin-Interleukin 3 Fusion Protein Is Cytotoxic to Primitive Acute Myeloid Leukemia Progenitors But Spares Normal Progenitors[1]

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ABSTRACT

The relative cytotoxicity of a diphtheria toxin (DT) human interleukin 3 (IL3) fusion protein (DT_{388}IL3) was tested against primitive normal ($n = 3$) and acute myeloid leukemia (AML) progenitors ($n = 7$). After 24-h culture with 50 ng/ml DT_{388}IL3, the mean percentages of kill of AML colony-forming cells (CFCs), long-term culture-initiating cells (LTC-ICs), and suspension culture-ICs (SC-ICs) were 82% (range, 47–100), 56% (range, 28–91), and 74% (range, 43–87), respectively, with most surviving progenitors being cytogenetically normal. Engraftment of DT_{388}IL-3-treated AML cells in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice followed for 16 weeks was eradicated for two of these samples. In contrast, with normal bone marrow, mean percentages of CFC kill of 49 and 64% were seen with 50 or 250 ng/ml DT_{388}IL3, respectively, whereas no significant kills were observed in the LTC-IC and SC-IC assays. The NOD/SCID mouse repopulating cell (RC) frequency in normal BM cells was also not reduced by DT_{388}IL3 treatment. In subsequent experiments, NOD/SCID mice that received AML blasts i.v. followed in 24 h by 0.045 μg/g DT_{388}IL3 daily i.p. × 5 showed mean percentages of reduction in AML engraftment of 83% (range, 14–100) and 57% (range, 0–98) after 4 and 12 weeks, respectively ($n = 6$). No evidence of leukemia was detected with two of six AML samples 12 weeks after one 5-day course of DT_{388}IL3. Repeating the DT_{388}IL3 treatment every 4 weeks enhanced its effectiveness against two additional samples. Thus, DT_{388}IL3 kills primitive leukemic progenitors from a proportion of AML patients but shows no significant toxicity against equivalent normal cells.

INTRODUCTION

Although most patients with AML[4] obtain an initial complete remission with standard induction chemotherapy, the majority ultimately relapse and succumb to their leukemia (1, 2). The malignant cells in patients with refractory or relapsed AML often exhibit a highly chemotherapy drug-resistant phenotype associated with expression of the multidrug resistance-1 or related gene products (3–7). Standard induction and consolidation therapy for AML also has a relatively low selectivity for malignant rather than normal hematopoietic progenitor cells, leading to prolonged periods of pancytopenia that are responsible for substantial morbidity and occasional mortality. Thus, AML therapy could be greatly improved by the development of cytotoxic drugs with mechanisms of action that bypass the usual causes of chemotherapy drug resistance and which selectively target leukemic progenitors.

DT is a M_r 58,000 protein that consists of an A (M_r 21,000) and a B (M_r 37,000) fragment that are linked by a disulfide bond (8, 9). The B-fragment mediates binding of the toxin to the cell surface receptor, following which the bound complex is internalized by endocytosis. After translocation to the cytosol, the A-fragment catalyzes the inactivation of elongation factor 2, which causes inhibition of protein synthesis and cell death (10–13). The target cell specificity of the native DT can be altered by replacing the B-fragment with an alternative binding sequence such as a growth factor cDNA. A variety of DT-growth factor fusion proteins have been created that have specific cytotoxicity for cells expressing the relevant receptor (14–16). For example, a DT-GM-CSF fusion protein (DT_{388}GMCSF) kills most malignant CFCs (AML-CFCs) and at least some AML LTC-ICs and NOD/SCID mouse leukemia-IC (NOD/SL-IC) from AML samples expressing high-affinity GM-CSF receptors (17, 18). However, in the latter assay although an initial cytoreduction of leukemic cells in mouse BM was achieved, there was typically a subsequent regrowth of malignant blasts that could not be prevented by repeated cycles of *in vivo* therapy with the fusion toxin (18). Evaluation of the toxicity of DT_{388}GMCSF against normal CFCs and LTC-ICs showed a modest but significant toxicity against both progenitor cell types (17, 18). It thus appeared that both the efficacy and the specificity of DT_{388}GMCSF could be improved upon.

Other investigators have demonstrated that a DT-murine IL3 fusion toxin (DT_{390}-mIL-3) is cytotoxic for murine IL-3R-bearing leukemic cell lines but spares significant numbers of normal mouse BM-repopulating cells (19, 20). Most human AML blasts proliferate in response to IL-3 and express the IL-3R (21–23). The present report describes the results of studies designed to explore the possibility that substituting IL-3 for GM-CSF in the DT fusion protein would improve its specific targeting to human AML progenitors. In initial experiments, this DT-human IL-3 fusion protein (DT_{388}IL3) caused a >1 log kill of AML-CFCs from 9 of 25 patient samples tested, whereas normal BM CFCs were reduced by only 3–5-fold (24–26). We now extend this analysis by comparing the cytotoxicity of DT_{388}IL3 against more primitive leukemic and normal progenitors, including those that will sustain long-term malignant and normal lympho-myeloid hematopoiesis in NOD/SCID mice. The data demonstrate substantial killing of malignant progenitors from patients with poor-prognosis, chemotherapy-resistant AML while at the same time documenting a striking lack of toxicity against analogous normal hematopoietic cells.

MATERIALS AND METHODS

Human Samples. Peripheral blood or BM cells were obtained from seven patients with newly diagnosed AML after informed consent and with the approval of the Clinical Research Ethics Board of the University of British Columbia. Diagnosis and classification of AML were based on the criteria of the FAB group (27). Cytogenetic analysis was performed on the BM at initial diagnosis. Mononuclear cells were isolated and cryopreserved as described previously (21). Normal human BM cells were obtained from cadaveric donors (Northwest Tissue Center, Seattle, WA). Approved institutional procedures concerning written informed consent were followed. Fresh or thawed cells were centrifuged on Ficoll/Hypaque,

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[4] The abbreviations used are: AML, acute myeloid leukemia; DT, diphtheria toxin; IL, interleukin; IL-3R, IL-3 receptor; CSF, colony-stimulating factor; G-CSF, granulocyte-CSF; GM-CSF, granulocyte/macrophage-CSF; CFC, colony-forming cell; LTC-IC, long-term culture-initiating cell; FAB, French/American/British; NOD/SCID, nonobese diabetic/severe combined immunodeficient; BM, bone marrow; FISH, fluorescence *in situ* hybridization; SF, Steel factor; SC, suspension culture; FACS, fluorescence-activated cell sorter; RC, repopulating cell.

and the low-density (<1.007 g/ml) cells were recovered. Before injection into mice, normal BM was first depleted of mature lineage-committed cells using an immunomagnetic column system as described by the manufacturer (StemSep; StemCell Technologies, Vancouver, British Columbia, Canada). Lymphocytes, including T, B, natural killer, and pre-B cells, and mature myeloid cells, including monocytes and granulocytes, were removed by a mixture of antibodies against CD2, CD3, CD19, CD24, CD56, CD14, CD16, CD66b, and glycophorin A, which results in a cell preparation highly enriched for $CD34^+$ progenitor cells.

DT_{388}IL3. DT_{388}IL3 was constructed by joining the human IL3 cDNA to a truncated DT sequence that lacks the native binding site via an intervening $(G4S)_2$ linker. Recombinant protein was prepared, purified, and stored as described previously (24). This material was found to kill IL-3R-positive cell lines (TF1 and the TF1 derivatives TF1/Bcl2, TF1 MEK+LNL6, and TF1 MEK+Bcl2) at an inhibitory concentration (IC_{50}) of 1–28 pM (0.06–1.7 ng/ml), whereas no kill of IL-3R-negative cell lines was seen (16, 24). Similar results were obtained for primary AML samples, where expression of high-affinity IL-3R was necessary to obtain >1 log AML-CFC kill (26). In addition, the cytotoxicity of the DT_{388}IL3 was blocked in a dose-dependent competitive fashion by prior incubation of IL-3R-positive cells with native human IL-3 or anti-IL3R antibodies (16, 24). The IL-3R binding affinity of this fusion toxin is similar to that of native human IL-3 (25).

Cultures of AML Cells and Normal BM Cells. In preliminary experiments, a 24-h exposure to 50 ng/ml of DT_{388}IL3 caused 100% kill of the IL-3-responsive human AML cell line MO7e (28). This concentration and duration of exposure to DT_{388}IL3 was tested in all of the *in vitro* AML and normal BM experiments unless otherwise specified.

Frozen mononuclear peripheral blood or BM cells from AML patients and normal BM cells were incubated for 24 h at 1×10^6 cells/ml in RPMI 1640 with 20% FCS and 50 ng/ml G-CSF (included to enhance cell viability without competing for IL-3R binding) with or without DT_{388}IL3. The viability of cultured cells was measured by trypan blue dye exclusion before plating in the various progenitor assays.

Assays for AML-CFCs were performed by plating cells at 0.2 to 1.0×10^5 cells/ml in growth factor-supplemented methylcellulose medium and scored for the presence of colonies after 14 days as described previously (21). In patients with a leukemia-specific clonal chromosomal abnormality, colonies were plucked onto 10-well glass slides for FISH analysis (21).

Normal BM CFCs were detected by plating cells in methylcellulose medium (Methocult H4330; StemCell Technology) supplemented with 3 units/ml human erythropoietin (StemCell), 50 ng/ml SF (Terry Fox Laboratory), and 20 ng/ml each of IL-3, GM-CSF, G-CSF (Amgen, Thousand Oaks, CA), and IL-6 (Cangene, Mississauga, Ontario, Canada). Granulopoietic, erythroid, and mixed colonies detected after 16 days of incubation at 37°C were scored as described (29).

AML LTC-IC assays were established and maintained as described previously (21). Briefly, AML cells in Myelocult LTC medium (StemCell) with 10^{-6} M Solucortef (Sigma Chemical Co.-Aldrich Canada, Oakville, Ontario, Canada) and 50 ng/ml SF were cocultured with irradiated (80 Gy) Sl/Sl-J-IL3 feeders (30). After culture for 6 weeks with weekly half-medium changes, adherent and nonadherent cells were harvested and assessed for their AML-CFC content as described above (21).

To detect normal LTC-ICs, human BM cells in Myelocult medium were plated onto preformed feeder layers of irradiated murine fibroblast cell lines genetically engineered to produce SF, G-CSF, and IL-3 (29). After 6 weeks, adherent and nonadherent cells were assessed for their CFC content as above (29).

For long-term SCs, AML cells or normal BM cells were incubated at a concentration of 1×10^6 cells/ml in serum-free medium containing 10^{-4} M β-mercaptoethanol, 2 mM glutamine, and a mixture of growth factors in Iscove's modified Dulbecco's medium with 20% BIT (10% BSA, 50 ug/ml insulin, and 1000 μg/ml transferrin; StemCell). For AML cells, 20 ng/ml each of IL-3, IL-6, G-CSF, and GM-CSF and 50 ng/ml SF were used; for normal BM cells, 100 ng/ml each of Flt-3 ligand and SF and 20 ng/ml each of IL-3, IL-6, and G-CSF were added along with 40 μg/ml low-density lipoproteins. After 6 weeks with weekly half-medium changes, cells were harvested by trypsinization and placed into AML-CFC or normal CFC assays as described previously (29, 31).

NOD/SCID Mice. NOD/LtSz-scid/scid (NOD/SCID) mice (32) were bred and maintained under sterile conditions in the British Columbia Cancer Research Center Joint Animal Facility according to protocols approved by the Animal Care Committee of the University of British Columbia. Mice, 8–10 weeks of age, received 350 cGy from a ^{137}Cs source 24 h before injection of AML cells. 5×10^5 to 1×10^7 thawed cryopreserved, or cultured AML cells in Alpha MEM (StemCell) with 50% FCS were injected into each mouse via the tail vein. Mice treated with DT_{388}IL3 received 0.045 μg/g of body weight of the toxin daily i.p. for 5 days at intervals after injection of AML cells as specified in "Results."

NOD/SL-IC Assay. After injection of AML cells into mice, a femoral BM aspiration was performed every 4 weeks after anesthesia with Avertin (2,2,2-tribromoethanol; Aldrich, Milwaukee, WI; Ref. 18). Twelve to 16 weeks after injection of AML cells, the mice were killed by CO_2 inhalation, and BM was obtained from the four long bones by flushing with Alpha MEM with 50% FCS.

Cohorts of five or six mice were injected with cells from the same AML sample, subjected to the same experimental conditions, and analyzed in parallel. Values shown for engraftment of AML cells in mouse BM are the mean values obtained for all mice in the cohort that survived to the time of analysis.

Cells from mouse tissue were prepared for FACS analysis as described previously (33). Half of the cells were then incubated for 30 min on ice with a mouse IgG1 isotype control (Becton Dickinson Immunocytometry Systems, San Jose, CA), and the other half were incubated with fluoresceinated anti-CD45 (a human-specific pan-leukocyte marker prepared in our center from American Type Culture Collection clone HB10508, Rockville, MD) to detect human cells. Cells were washed and stained with 2 μg/ml propidium iodide. FACS analysis was performed on a Becton Dickinson FACScan or FACSort flow cytometer. The percentage of $CD45^+$ cells was determined after excluding 99.9% of cells labeled with the isotype control and nonviable cells. Nonspecific binding of CD45 on mouse BM cells is reliably ≤0.1% (33).

Human Lympho-Myeloid RC Assay. The frequency of normal human progenitors with both lymphoid and myeloid potential was measured at limiting dilution in NOD/SCID mice as described previously (34, 35). Before injection into NOD/SCID mice, normal human lineage depleted (lin^-) BM cells were incubated for 24 h with or without 50 ng/ml DT_{388}IL3 as described for the AML cells. Different numbers of cultured lin^- BM cells plus 10^6 irradiated (15 Gy) normal human BM cells as carrier cells were injected into three groups of four animals each. In previous experiments, the frequency of lympho-myeloid RCs in the lin^- BM samples used in these experiments was determined to be one per 7.5×10^5 cells and 2.5×10^5 cells for normal BM 1 and 2, respectively. To perform limiting dilution analysis, each mouse in a cohort of four animals was injected with cell numbers corresponding to 6, 1.5, or 0.25 lympho-myeloid RCs as determined in these prior experiments with untreated cells. BM aspirates were performed 4 and 8 weeks after injection of cells. When mice were killed at 12 weeks, cells were flushed from hind limb bones. BM cells from either source were stained with human-specific antibodies to determine lympho-myeloid engraftment: anti-CD45PE (Pharmingen, Mississauga, Ontario, Canada), anti-CD71PE (OKT-9, Pharmingen), anti-CD15FITC (Becton Dickinson, San Jose, CA) and anti-CD66b (Pharmingen); or anti-CD34FITC (8G12), anti-CD19PE (Becton Dickinson), and anti-CD20PE (Becton Dickinson). For human RC determinations, mice were considered negative if there were <5 $CD34^-CD19/20^+$ human B-lymphoid cells and/or <5 $CD45/71^+CD15/66b^+$ human myeloid cells per 2×10^4 viable cells analyzed (34). RC frequencies were calculated using the L-calc software program (StemCell) from the proportion of negative mice in each similarly treated group (35).

FISH. Cytospin preparations were obtained from the AML samples 24 h after incubation with or without DT_{388}IL3. Colonies from methylcellulose assay of 6-week-old LTCs and SCs were plucked onto 10-well slides and fixed in methanol:glacial acetic acid (3:1) for 10 min. To detect the 11q23 abnormality, the Quint-Essential TM 11q23 (MLL) DNA probe labeled with digoxigenin was purchased from Oncor (Gaithersburg, MD) and used as specified by the manufacturer. Probes were denatured, applied to denatured cells on slides, and hybridized overnight at 37°C. For probe detection, slides were incubated with a sheep anti-digoxigenin-FITC antibody (Boehringer-Mannheim, Mannheim, Germany) at 37°C for 1 h in the dark, washed, and then further incubated for 1 h with rabbit-antisheep FITC (Vector Labs, Burlingame, CA) and counterstained with propidium iodide as described previously (21).

Analysis of the slides was performed on a Zeiss (Oberkochen, Germany) Axioplan fluorescence microscope equipped with a double bandpass filter to allow simultaneous visualization of the FITC signal and the propidium iodide counterstain. Each colony was scored as either normal or abnormal only if a minimum of 5 cells per colony showed a clear signal and if at least 80% of the cells showed either the normal or abnormal signal.

IL-3R Density Measurements. IL-3R density measurements were performed as described previously (16, 24). Briefly, aliquots of 1×10^6 cells in RPMI

Table 1 *Patient characteristics*

Patient	Age	Sex	FAB	WBC (% blasts)	Marrow cytogenetics (% abnormal)	IL-3Rs per AML cell[a]		Response to Rx or treatment[b]	Status/Survival[c]
						High affinity	Low affinity		
1	60	F	M5a	47 (92)	46 XX	357	19390	CR	D/5
2	74	F	M4	151 (79)	46 XX	520	19190	IF	D/2
3	58	F	M4	370 (47)	47 XX + 13 (38%)	696	11140	IF	D/6
4	48	M	M4Eo	101 (71)	46 XY inv (16)(p13q22) (100%)	156	21310	CR	D/11
5	21	M	M1	320 (90)	46 XY add (6)(p23), t(6;11)(q27;q23) (100%)	379	1762	CR (2)	D/5
6	29	F	M4	87 (42)	46 XX, del (16)(q22) (32%)	88	333	CR	D/5
7	69	F	M5b-t[d]	155 (40)	46 XX, t(9;11)(p22q23) (100%)	139	279	NT	D/<1

[a] Number of IL-3 binding sites per AML blast determined as described in "Materials and Methods."
[b] CR, complete remission; CR (2), two cycles of therapy required; IF, induction failure; NT, oral hydroxyurea only.
[c] D, dead; survival, in months after diagnosis.
[d] Therapy-related leukemia developing 2 years after chemotherapy for Hodgkin's disease.

1640 plus 2.5% BSA, 20 mM HEPES, and 0.2% sodium azide were mixed with various amounts of ^{125}I-labeled Bolton-Hunter SC-65461 (Pharmacia, St. Louis, MO; specific activity, 75–80 μCi/μg) as described previously with or without excess (2 μg) unlabeled IL-3 (SC-50341; Pharmacia) in a total volume of 170 μl in 1.5-ml Eppendorf tubes (16). Cells were incubated at 37°C for 40 min and then layered over a 200-μl oil phthalate mixture (1 part dioctylphthalate and 1.5 parts dibutylphthalate; Aldrich, Milwaukee, WI). After centrifugation at 14,000 rpm for 2 min at room temperature, the radioactivity in both pellets and supernatants were counted in a Packard Auto-Gamma 5650 gamma counter gated for ^{125}I with 50% counting efficiency. Background cpm were calculated by linear extrapolation from incubations with excess unlabeled IL-3. Binding saturation curves were made using Graph Pad Prism (Graph Pad Software, Inc.) and Microsoft Excel (Microsoft Corp.). Nonlinear regression analysis was used to calculate K_d and B_{max} of the high- and low-affinity receptors.

Statistical Analysis. Comparison of levels of AML cell engraftment between cohorts of NOD/SCID mice was performed using the Student's *t* test. $P < 0.05$ was considered significant.

RESULTS

Patient Sample Characteristics. Leukemic blast samples from seven patients with newly diagnosed AML were studied. All seven patients had poor prognosis and/or chemotherapy refractory AML. Five presented with WBC counts greater than 100×10^9/l. Six patients received conventional or high dose 1-β-D-arabinofuranosylcytosine-based chemotherapy regimes for remission induction, whereas the seventh patient elected to receive palliative therapy with oral hydroxyurea only. Among the six patients where remission induction was attempted, two were induction failures, two had remissions lasting <2 months, one required two cycles of high dose 1-β-D-arabinofuranosylcytosine and anthracycline therapy to enter remission and proceeded to unrelated donor BM transplant with a hypocellular marrow and low peripheral blood counts, and the sixth patient had a remission of 10 months duration before a central nervous system relapse of his leukemia. All patients were dead within 11 months of the diagnosis of AML (Table 1). These AML samples were chosen based on our previous knowledge of their ability to grow well in the *in vitro* assays and engraft to high levels in NOD/SCID mice. The FAB subtype of the leukemias was M4 and M1, M4Eo, M5a or M5b for 1 patient each. BM cytogenetics was abnormal in five of the patient samples (Table 1). IL-3Rs were detected on AML cells from all seven samples. The number of high-affinity human IL-3 binding sites per AML blast varied from 88 to 696. A much larger number of low-affinity binding sites were detected on cells from most samples, consistent with published data demonstrating high-level expression of the IL-3R α-subunit on most AML blasts (Ref. 36; Table 1).

Sensitivity of AML-CFCs, LTC-ICs, and SC-ICs to DT_{388}IL3. The seven AML samples were cultured for 24 h with or without 50 ng/ml DT_{388}IL3 and then plated into AML-CFC, LTC-IC, and SC-IC assays. When total nucleated cell numbers were evaluated at the end of the initial 24-h culture period, a median 25% (range, 0–75%) reduction in total viable AML cells was observed in the toxin-treated as compared with untreated cultures by trypan blue dye exclusion.

AML-CFCs were detected in control (untreated) cultures of all AML samples (range, 3–1480 AML-CFC/10^5 cells). After 24-h exposure to DT_{388}IL3, the mean percentage of kill of AML-CFCs was 82% (range, 47–100%) with three samples showing >90% kill (Table 2).

The frequency of AML LTC-ICs or SC-ICs detected in six samples varied from 35 to 15,137 and from 9 to 8,100 per 10^6/cells, respectively, after 24-h culture without DT_{388}IL3 (Table 2). The remaining sample (no. 4) showed no colony growth in control LTC-IC or SC-IC assays. Exposure to DT_{388}IL3 produced a mean percentage of kill of 56% (range, 28–91%) and 74% (range, 43–87%) for AML LTC-ICs and SC-ICs, respectively, among the former six samples (Table 2).

The effect of increasing the concentration of DT_{388}IL3 5-fold to 250 ng/ml was tested with three patient samples. Although there was evidence of increased cytotoxicity from the higher dose of DT_{388}IL3 in the different progenitor assays from all three patient samples, killing was still typically incomplete, particularly in the LTC-IC assay (Table 2).

FISH Analysis of Cultured AML Cells. FISH analysis was performed on AML cells recovered after 24-h incubation with or without

Table 2 *AML progenitor growth in vitro after 24-h incubation with or without DT_{388}IL3*

Patient	CFC/10^5 cells (% kill)			LTC-IC/10^6 cells (% kill)			SC-IC/10^6 cells (% kill)		
		DT-IL3 ng/ml			DT-IL3 ng/ml			DT-IL3 ng/ml	
	Control	50	250	Control	50	250	Control	50	250
1	7	1 (86)		196	33 (83)		146	45 (70)	
2	282	0 (100)	0 (100)	1069	651 (39)	354 (67)	280	161 (43)	1 (99)
3	39	6 (85)		15137	7463 (51)		3201	560 (84)	
4	3	1 (67)		ND[a]	ND		ND	ND	
5	1480	96 (94)	43 (97)	35	20 (43)	4 (89)	9	2 (78)	0.5 (94)
6	730	385 (47)		920	79 (91)		8100	1744 (79)	
7	265	13 (95)	7 (97)	2247	1610 (28)	948 (58)	909	117 (87)	44 (95)

[a] ND, none detected.

Table 3 *FISH on AML blasts and progenitors*[a]

Patient	Cytogenetic marker in diagnostic marrow (% abnormal)	Total nucleated cells		AML-CFC		AML LTC-IC		SC-IC	
		Control	DT_{388}IL3	Control	DT_{388}IL3	Control	DT_{388}IL3	Control	DT_{388}IL3
5	46 XY add (6)(p23), t(6;11)(q27;q23) (100%)	172/180[b] (96)	12/95 (13)	8/8 (100)	1/8 (13)	4/7 (57)	2/10 (20)	6/8 (75)	0/6 (0)
7	46 XX, t(9;11)(p22q23) (100%)	81/103 (79)	5/40 (13)	1/10 (10)	0/9 (0)	4/9 (44)	0/9 (0)	5/9 (56)	0/10 (0)

[a] FISH analysis from cells studied directly after 24-h culture with or without DT_{388}IL3 or colonies derived from AML-CFCs, LTC-ICs, and SC-ICs (see Table 2 for total absolute and relative progenitor numbers in the cultures, respectively).
[b] No. abnormal/no. analyzed (% abnormal).

DT_{388}IL3, on colonies derived from AML-CFC assays and from methylcellulose assays of cells harvested from LTCs and SCs from two patients (nos. 5 and 7) with known karyotypic abnormalities. The expected cytogenetic change was detected in AML blasts recovered after 24 h in culture. However, the proportion of cytogenetically abnormal cells was significantly reduced after exposure to the toxin (Table 3).

FISH analysis showed cytogenetically abnormal progenitors among cells cultured for 24 h without DT_{388}IL3. After toxin treatment, no abnormal colonies were detected in AML-CFC, LTC-IC, or SC-IC assays from patient 7, whereas the proportions of abnormal CFCs and LTC-ICs were reduced, and no abnormal SC-ICs were detected in cultures of cells from patient 5 (Table 3).

Sensitivity of AML NOD/SL-ICs to *ex Vivo* Incubation with DT_{388}IL3. AML cells (5×10^5 to 10^7) from the seven patient samples were transplanted into cohorts of five NOD/SCID mice after 24-h incubation with or without 50 ng/ml DT_{388}IL3. Among mice injected 4 weeks previously with cells from control cultures, engraftment of human cells was easily detectable at 2–80% of cells in mouse BM for five of seven samples. Control cells from patients 4 and 6 did not show evaluable engraftment until weeks 8 and 12, respectively. In comparison with these controls, at week 4 a >90% reduction in the proportion of human cells detected was seen among mice receiving cells treated with DT_{388}IL3 from the five samples evaluable at that time point. Complete eradication of NOD/SL-IC appeared to have been accomplished with two of these samples (nos. 5 and 7) because AML cells could not be detected in mouse marrow on repeated testing until week 16. However, for the remaining samples either regrowth of DT_{388}IL3-treated leukemic cells (nos. 1, 2, and 3) or no consistent difference between mice receiving treated or untreated cells (nos. 4 and 6) was detected at the later time points (Table 4).

***In Vivo* Treatment of NOD/SL-IC with DT_{388}IL3.** Nonspecific dose-limiting toxicity had been observed previously when large doses of DT_{388}GMCSF were injected into mice and *in vitro* when IL-3R-negative cell lines were exposed to very high concentrations of DT_{388}IL3 (18, 26). Thus, in preliminary experiments cohorts of otherwise unmanipulated 8–10-week-old NOD/SCID mice received doses of DT_{388}IL3 ranging from 0.030 to 0.083 μg/g of body weight daily $\times$ 5 i.p. At doses >0.05 μg/g/day, the majority of mice died of apparent drug toxicity, whereas at or below that dose >90% of treated mice survived with no apparent ill effects (data not shown). Thus, the maximum tolerated dose was considered to be 0.045 μg/g/day $\times$ 5 i.p. This dose and schedule of DT_{388}IL3 was used to treat cohorts of five to six mice that had been injected 24 h previously with AML cells. Engraftment of all six AML patient samples was detected in the BM of untreated mice after 4 and 12 weeks (Table 5). As compared with these controls, at weeks 4 and 12 there was a mean percentage of reduction of leukemic engraftment in DT_{388}IL3-treated mice of 83% (range, 14 to >99%) and 57% (0 to >99%) at the two time points, respectively. DT_{388}IL3-treated mice engrafted with AML cells from patients 1 and 2 showed little evidence of leukemia at week 4 after treatment but substantial regrowth at week 12. Two additional patient samples (nos. 3 and 5) showed >95% reduction in engraftment of AML cells at week 4 that was maintained or improved over time so that complete elimination of detectable leukemic cells was seen in all treated mice at week 12 (Table 5).

To attempt to improve upon these results, cohorts of five mice were treated more intensively with two or three 5-day cycles of i.p. DT_{388}IL3 injections every 4 weeks, beginning 24 h after injection of cells from the same six AML patients. As shown on Fig. 1, repeated DT_{388}IL3 treatments increased the proportion of AML cells from patients 1 and 2 killed with no detectable malignant cells remaining at week 12 in treated mice injected with cells from patient 1.

Cytotoxicity of DT_{388}IL3 on Normal BM CFCs, LTC-ICs, and SC-ICs. Three normal BM samples were incubated for 24 h with or without the two concentrations of DT_{388}IL3 that had been tested on the AML samples and then plated in the *in vitro* progenitor cell assays. As shown on Table 6, a mean of 49% (range, 43–56%) of normal CFCs were eliminated after incubation with 50 ng/ml DT_{388}IL3 compared with 64% (range, 52–82%) when the concentration was increased to 250 ng/ml. In the LTC-IC assay, no progenitor kill was observed at either toxin concentration, whereas in the SC-IC assay, no kill was observed after incubation with 50 ng/ml DT_{388}IL3 and 0–30% kill at 250 ng/ml.

Cytotoxicity of DT_{388}IL3 on Normal RC. Twenty-four h after incubation without 50 ng/ml DT_{388}IL3, the frequencies of normal human lympho-myeloid RCs detected among control cells did not

Table 4 Cytotoxicity of ex vivo incubation with 50 ng/ml DT_{388}IL3 against NOD/SL-ICs[a]

Patient	Week 4		Weeks 12–16	
	Control	DT_{388}IL3	Control	DT_{388}IL3
1	48 ± 6.8 (5)	3.5 ± 0.89 (5)	87 ± 8.5 (5)	68 ± 7.8 (5)
2	80 ± 4.0 (5)	3.8 ± 1.6 (5)	17 ± 12 (5)	42 ± 5.4 (5)
3	46 ± 9.9 (5)	0.6 ± 0.16 (5)	93 ± 1.6 (5)	32 ± 17 (5)
4	0.3 ± 0.15 (5)	1.2 ± 0.25 (5)	14 ± 7.5 (5)	35 ± 3.0 (5)
5	70 ± 8.7 (4)	0.1 ± 0.06 (4)[b]	53 ± 19 (3)	0.03 ± 0.02 (4)[b]
6	0.03 ± 0.02 (6)	0.1 ± 0.03 (5)	14 ± 12 (5)	0.6 ± 0.18 (3)
7	2.1 ± 1.0 (4)	0.01 ± 0.01 (5)[b]	17 ± 12.5 (3)	0 ± (5)[b]

[a] Mean ± SE percentage of $CD45^+$ cells in NOD/SCID mouse BM 8–12 weeks after injection of AML cells cultured for 24 h without (control) or with exposure to DT_{388}IL3 (numbers of mice analyzed).
[b] $P < 0.05$ for comparison between treated and untreated AML cells.

Table 5 *Effect of one 5-day course of DT_{388}IL3 i.p. on detection of AML cells in NOD/SCID mouse BM*[a]

Patient	Week 4		Week 12	
	Control	DT_{388}IL3	Control	DT_{388}IL3
1	23 ± 6.7 (5)	0.1 ± 0.09 (5)	92 ± 1.8 (5)	11 ± 6.3 (5)
2	25 ± 14 (4)	0.04 ± 0.02 (5)	15 ± 2.8 (4)	27 ± 12 (5)
3	18 ± 2.7 (5)	0.9 ± 0.1 (5)	0.5 ± 0.3 (5)	0.07 ± 0.03 (5)
4	3.8 ± 1.0 (5)	3.3 ± 1.1 (5)	18 ± 9.4 (5)	7.8 ± 2.4 (5)
5	1.5 ± 0.3 (5)	0.03 ± 0.02 (5)	0.7 ± 0.5 (5)	0.01 ± 0.005 (5)
6	8 ± 0.9 (5)	0.8 ± 0.6 (4)	36 ± 1.7 (3)	31 ± 4.5 (5)

[a] Data are shown as mean percentage of $CD45^+$ cells in mouse BM ± SE (number of mice analyzed).

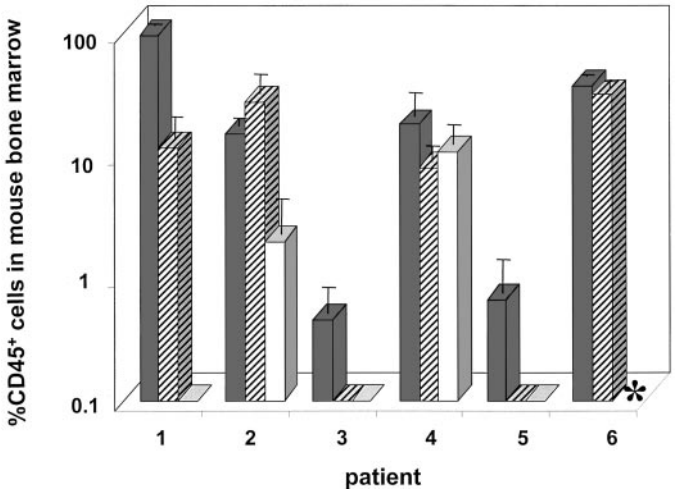

Fig. 1. The effect of *in vivo* treatment with DT_{388}IL3 on NOD/SCID mice engrafted with AML cells from six different patients. Cohorts of five or six mice were either untreated (*gray columns*) or received 0.045 μg/g DT_{388}IL3 i.p. daily $\times$ 5 days beginning 24 h after injection of AML cells (*hatched columns*). A third cohort of mice received an additional one or two 5-day DT_{388}IL3 treatments at 4-week intervals after the first treatment (*white columns*). The height of the columns represents the mean percentage of $CD45^+$ cells detected in mouse BM 12 weeks after injection of AML cells when all mice were sacrificed and analyzed. *Bars,* 1 SE. All mice engrafted with AML cells from patient 6 that received repeated cycles of DT_{388}IL3 treatment died before week 12 and had extensive infiltration with human leukemia on their week 8 BM aspiration (*). The changes in AML cell engraftment between treated and untreated mice reached statistical significance ($P < 0.05$) for both single and repeated DT_{388}IL3 treatments for patient samples 1, 3, and 5.

change significantly from weeks 4 to 12 after transplant and was very similar to the frequency of RCs detected in uncultured cells, *i.e.,* 1.3 and 4 RCs per 10^6 cells from normal BM 1 and 2, respectively. Interestingly, the frequency of RCs detected among DT_{388}IL3-treated cells was consistently, but not significantly, higher than that detected among untreated control cultured (or uncultured) cells with both normal BMs at all three time points (Table 7). The proportion of human cells detected in mouse BM also did not differ for mice receiving control untreated *versus* DT_{388}IL3-treated normal BM (mean $\pm$ SD % $CD45^+$ cells detected 12 weeks after injection of cells containing the equivalent of six RCs was 0.8% $\pm$ 1.0 *versus* 4.4% $\pm$ 1.9 and 12.1% $\pm$ 4.9 *versus* 5.7% $\pm$ 3.2 for normal BM 1 and 2, respectively). Thus, there was no evidence of cytotoxicity from DT_{388}IL3 against normal, multipotential BM RCs.

DISCUSSION

Primitive normal and leukemic hematopoietic progenitors share many cell surface phenotypic characteristics (37–40). However, some differences between these cells have been identified that allow them to be separated by flow cytometry (41, 42). It seems possible that such unique characteristics of AML progenitors could be targeted for therapeutic purposes.

The human IL-3R is a heterodimeric structure in which the α subunit, directly binds IL-3, whereas the β chain (β_c) is the primary signaling subunit (43). Coexpression of the α and β chains is required for high-affinity IL-3 binding and receptor internalization (43, 44). The IL-3R α subunit is highly expressed on AML cells, including the $CD34^+CD38^-$ subpopulation that is enriched for AML LTC-ICs and NOD/SL-ICs, whereas the β_c molecule is typically expressed at much lower levels (36–38, 44). The presence of the IL-3R on normal progenitor cells has been more difficult to demonstrate. However, using reverse transcription-PCR expression of both subunits has been detected in $CD34^+$ normal hematopoietic cells including the $CD38^-$ subpopulation (45).

Both high- and low-affinity IL-3R were detected on AML blasts from each of the seven samples analyzed for DT_{388}IL3 sensitivity in the current study (Table 1). Because high-affinity receptor binding would be necessary to mediate internalization of the toxin, it is not surprising that there was no direct correlation between the number of low-affinity receptors detected and the degree of DT_{388}IL3 cytotoxicity observed in any of the leukemic progenitor assays. We did observe a direct ($r = 0.55$), but not statistically significant, correlation between the number of high-affinity IL-3Rs per cell and the percentage of kill of AML-CFCs after *ex vivo* exposure to the toxin. This result, combined with our previous observation of a lack of DT_{388}IL3 cytotoxicity against IL-3R-negative cells, is consistent with our expectation that the toxin would specifically target high-affinity IL-3R-expressing cells (24). The correlation between receptor numbers and malignant progenitor kill was much weaker for cells detected in all of the other assays used including NOD/SL-ICs. In particular, 50 ng/ml of DT_{388}IL3 was relatively ineffective in killing AML LTC-ICs, SC-ICs, and/or NOD/SL-ICs from patients 1–4, although high-affinity IL-3Rs were easily detected on unfractionated blasts from these samples (Tables 1 and 2). These results suggest that in some cases, either the high-affinity IL-3R was not expressed in sufficiently large numbers or that the toxin did not efficiently enter the cytoplasm after ligand binding on the progenitors detected in the long-term *in vitro* and *in vivo* assays. However, the fact that increasing the concentration of DT_{388}IL3 to 250 ng/ml improved the cytotoxicity of the fusion protein against LTC-ICs and SC-ICs from patient 2 (Table 2) suggests that the higher concentration might also have been effective in eliminating NOD/SL-ICs from this, and perhaps other, samples.

After exposure to a standard dose of 50 ng/ml of DT_{388}IL3, five of the seven AML samples appeared to be highly sensitive to this toxin in the AML-CFC assay, and for two of these (nos. 5 and 7), the cytotoxicity in this assay was similar to that seen against LTC-ICs,

Table 6 *Cytotoxicity of DT_{388}IL3 on normal BM progenitors*

		CFC		LTC-IC		SC-IC	
Experiment	DT_{388}IL3 ng/ml	per 10^5 cells	% kill	per 10^6 cells	% kill	per 10^6 cells	% kill
1	0	142		23		2822	
	50	81	43	98	0	3274	0
	250	58	59	95	0	1976	30
2	0	205		82		3598	
	50	91	56	244	0	4536	0
	250	99	52	111	0	3203	11
3	0	81		29		6368	
	50	42	48	51	0	8118	0
	250	15	82	33	0	10920	0

Table 7 *Cytotoxicity of DT_{388}IL3 against normal BM RCs*

	Week 4 RCs/10^6 cells[a]		Week 8 RCs/10^6 cells[a]		Week 12 RCs/10^6 cells[a]	
BM	Control	DT_{388}IL3	Control	DT_{388}IL3	Control	DT_{388}IL3
1	0.6 (0.2–1.5)[b]	4.7 (1.4–16.1)	1.8 (0.7–5.1)	4.7 (1.4–16.1)	0.7 (0.3–1.8)	2.9 (0.9–8.6)
2	3.9 (0.9–15.7)	11 (3–36)	4.5 (0.9–21.9)	4.7 (1.6–13.8)	5.5 (0.17–17.8)	7.3 (1.4–38.7)

[a] RC frequency determined as described in "Materials and Methods" and expressed per 10^6 lin^- BM cells initiating 24-h cultures $\pm$ 50 ng/ml DT_{388}IL3.
[b] 95% confidence interval.

SC-ICs, and NOD/SL-ICs (Tables 2 and 4). However, for two other samples (nos. 1 and 2), AML-CFC kill did not predict for progenitor kill in the long-term *in vitro* or *in vivo* assays. It is tempting to speculate that elimination of progenitors detected after 6 or more weeks in culture or in mice is more clinically relevant than toxicity detected against leukemic blasts or direct colony-forming cells. However, only direct comparison between the antileukemic effects of DT_{388}IL3 in human AML patients and the results obtained when treated cells from the same patients are evaluated in the different progenitor assays will establish which, if any, of these tests has predictive value for clinical efficacy.

The outgrowth of cytogenetically normal LTC-ICs and SC-ICs from cultures of DT_{388}IL3-treated AML cells suggested that the fusion toxin might be selectively toxic for leukemic progenitors. This impression was confirmed when normal BM cells were exposed to DT_{388}IL3. Although a substantial number of normal CFCs were eliminated by the toxin, normal LTC-ICs and SC-ICs were resistant to it (Table 7). Even increasing the concentration of DT_{388}IL3 to 250 ng/ml did not result in a decrease in the formation of LTC-IC-derived colonies and resulted in very little toxicity to progenitors detected using the SC assay. Limiting dilution analysis in NOD/SCID mice allows the quantitative detection of human BM RCs with both lymphoid and myeloid potential (35, 46). Analysis of the effect of a 24-h exposure to 50 ng/ml DT_{388}IL3 on these primitive progenitors demonstrated no evidence of toxicity, regardless of the time point (up to 12 weeks) chosen for analysis. It seems unlikely that increasing the concentration of DT_{388}IL3 would have changed these results, given that this maneuver did not substantially increase toxicity against normal LTC-ICs or SC-ICs. This surprising lack of toxicity for DT_{388}IL3 against normal progenitors suggests that IL-3Rs are expressed at a much lower level on these cells than on equivalent leukemic progenitors or that events that take place after ligand binding differ between normal and malignant cells. In studies analyzing IL-3-IL-3R binding and signaling, it has been shown that ligand binding to the α chain receptor subunit is the rate-limiting step in formation of the α/β signaling complex (47, 48). It seems feasible that enhanced association of DT_{388}IL3 with the IL-3R α chain molecules, which are highly expressed on leukemic cells, might accelerate the formation and internalization of the α/β receptor complex (Table 1; Ref. 36). Such events could result in more efficient delivery of DT molecules to the cytoplasm of AML progenitors. Although it is known that a single DT molecule is sufficient to kill a cell, it has also been shown that when DT concentration is low, a prolonged exposure to the toxin is required for cytotoxicity (13, 49). Thus, the kinetics of ligand binding, internalization, and toxin delivery may be insufficient to result in cell death in normal progenitors, although high-affinity IL-3R can be detected on these cells (45). Because we have observed significant toxicity against normal LTC-ICs with the DT_{388}GMCSF fusion protein, it seems unlikely that more efficient degradation of DT in normal as compared with leukemic cells explains the reduced toxicity of DT_{388}IL3 against the former (18).

In a separate set of experiments, we used NOD/SCID mice engrafted with human AML cells from six different patients as a preclinical model in which to test the effectiveness and toxicity of *in vivo* administration of DT_{388}IL3. A single 5-day course of maximally tolerated doses of the drug completely eradicated detectable leukemia from all mice injected previously with cells from patients 3 and 5 with no evidence of regrowth of malignant cells when the animals were sacrificed for analysis on week 12. Among mice injected with cells from three of the remaining four patients, >90% reduction of leukemic cell burden in mouse BM was observed 4 weeks after toxin treatment. Although the benefit of a single course of treatment was not sustained at later time points in these animals, repeating the 5-day cycle of DT_{388}IL3 treatment eliminated all evidence of human leukemia from a cohort of mice injected with cells from patient 1 and substantially reduced the malignant cells detected in mice injected with cells from an additional patient (no. 2). Thus, overall the *in vivo* DT_{388}IL3 treatments were able to "cure" mice injected with cells from three of the six poor-prognosis AML patient samples tested (Fig. 1). However, it is worth noting that in two of these cases (nos. 3 and 5), the level of AML cell engraftment in control mice was quite low. This suggests that this fusion toxin may be most effective when used in a setting of minimal residual disease or in combination with other agents. In preliminary experiments, we have observed synergistic toxicity for the DT_{388}GMCSF fusion protein and 1-β-D-arabinofuranosylcytosine against human AML cell lines (50). These results and data from other systems where targeted therapies have been used with conventional chemotherapy drugs suggest that it may be possible to further improve the effectiveness of DT_{388}IL3 for AML treatment by incorporating it into such regimens (51–54).

In a Phase I trial of DT_{388}GMCSF in relapsed or refractory AML patients, most patients were found to have low titer, nonneutralizing but detectable anti-DT antibodies as a consequence of prior immunization with diphtheria toxoid. After treatment with DT_{388}GMCSF, patients typically showed an increase in antibody titer, but in only 5 of 22 individuals did neutralizing antibody develop. Thus, although it is expected that DT_{388}IL3 will react with preexisting DT antibodies in most patients' serum, it is likely that titers will not usually be high enough or the antibody avid enough so as to preclude a first or even second course of fusion toxin therapy (55).

In summary, DT_{388}IL3 can target leukemic cells from some patients with poor prognosis AML, including progenitors that will maintain the leukemic clone for several months *in vivo*, while demonstrating minimal toxicity against normal hematopoietic precursors. Expression of the IL-3R has been demonstrated on central cholinergic neurons, the testis, and vascular endothelium, raising concerns about the potential toxicity of DT_{388}IL3 on nonhematopoietic tissues (56–58). In this regard, it is interesting that the dose-limiting toxicity observed for a diphtheria toxin-murine IL-3 fusion protein in mice was diffuse hemorrhage, possibly because of the expression of the IL-3R on megakaryocyte progenitors and vascular endothelial cells (20). However, studies in nonhuman primates, which are currently ongoing, have thus far failed to demonstrate such toxicity. If such data continue to suggest that DT_{388}IL-3 has a high degree of selective toxicity for leukemic cells, this agent will warrant clinical testing in patients with relapsed or refractory AML.

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