A Murine Cytokine Fusion Toxin Specifically Targeting the Murine Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) Receptor on Normal Committed Bone Marrow Progenitor Cells and GM-CSF-Dependent Tumor Cells

By Chung-Huang Chan, Bruce R. Blazar, Cindy R. Eide, Robert J. Kreitman, and Daniel A. Vallera

A fusion protein was synthesized consisting of the murine granulocyte-macrophage colony-stimulating factor (mGM-CSF) gene spliced to a truncated form of the diphtheria toxin (DT₃₀₀) gene coding for a molecule that retained full enzymatic activity, but excluded the native binding domain. The DT₃₀₀-mGM-CSF hybrid gene was cloned into a vector under the control of an inducible promoter and the protein expressed in Escherichia coli. After induction, a protein was purified from inclusion bodies in accord with the deduced molecular weight of DT₃₀₀ mGM-CSF. Cell-free studies of the adenosine diphosphate-ribosylating activity of DT₃₀₀ mGM-CSF showed results that were similar to those of native DT. The DT₃₀₀ mGM-CSF immunotoxin FDCP2.1d, a murine myelomonocytic tumor line expressing the GM-CSF receptor with an IC₅₀ (concentration inhibiting 50% activity) of 5 × 10⁻¹⁷ mol/L. The fusion toxin was specifically cytotoxic and directed by the GM-CSF portion of the molecule because addition of a monoclonal antibody directed against GM-CSF inhibited its ability to kill the cell line. Cell lines that do not express GM-CSF receptor were not inhibited by the fusion toxin. DT₃₀₀ mGM-CSF was also able to specifically inhibit normal committed bone marrow (BM) progenitor cells as measured in clonal colony-forming unit granulocyte-macrophage assays. Together, these findings indicate that DT₂₈₉ mGM-CSF may be useful as a novel tool for purging BM of contaminating leukemia cells or in vivo for eliminating residual leukemia cells. Also, it can be used to determine whether committed and/or noncommitted BM progenitor cells express the GM-CSF receptor.

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MURINE granulocyte-macrophage colony-stimulating factor (mGM-CSF) is composed of 124 amino acids with a calculated molecular weight of 14,138 kD.¹ GM-CSF can stimulate proliferation and differentiation of colony-forming unit–granulocyte-macrophage (CFU-GM) progenitor cells as well as enhance the function of mature neutrophils, monocytes, and eosinophils.² Exposure of CFU-GM progenitors to GM-CSF causes rapid entry of cells into the cell cycle. The biologic actions of GM-CSF are mediated by binding to a specific high-affinity receptor, consisting of two components designated as α and β subunits.³ ⁴ These GM-CSF receptors are found on the surface of myeloid precursors, granulocytes, mononuclear phagocytes, and also frequently present on the myeloid malignancies.⁵ ⁶ In fact, the majority of cases of acute myeloid leukemia express high-affinity receptors for GM-CSF.

GM-CSF can interact with myeloid leukemic cells because GM-CSF, either by itself or in association with other cytokines, can induce proliferation of myeloid clonogenic blasts.¹¹ ¹² Several reports suggest that GM-CSF receptors are expressed on human nonhematopoietic tumor cell lines¹⁴ ¹⁷ and GM-CSF is capable of stimulating the growth of human solid tumor cell lines including those derived from small cell lung carcinoma, melanoma, renal carcinoma, colon carcinoma, gastric carcinoma, and ovarian carcinoma.¹⁴ ¹⁵ ¹⁶ ²⁰

The first step in the proliferative action of GM-CSF on leukemic progenitors is ligand binding to specific membrane receptors.⁵ ⁶ ¹¹ ²² The receptor is composed of two subunits and the binding of these α and β subunits together form a high-affinity (dissociation constant [kd], approximately 40 pmol/L) receptor complex.³ The binding of GM-CSF to this receptor causes rapid internalization of the ligand-receptor complex.²³ Because of the internalization of GM-CSF, we reasoned that GM-CSF could serve as a ligand for delivering a toxic molecule such as diphtheria toxin (DT) to myeloid leukemic cells.

DT is a well-studied glycoprotein with a molecular weight of 58 kD. DT has potent cell-killing ability and requires internalization.²⁴ Its mechanism involves adenosine diphosphate (ADP)-ribosylation of elongation factor-2, resulting in inhibition of cellular protein synthesis and death of the cell. Investigators have shown that DT induces DNA degradation and morphologic changes consistent with apoptosis.²⁵ Delivering a single DT molecule into the cytoplasm is sufficient to kill a cell.²⁶ Native DT contains three domains: the cell-binding domain, the translocation domain, and the enzymatic cytotoxic domain.²⁶ ²⁸ The cell-binding domain of the DT gene can be replaced by a growth factor gene, resulting in a toxin-growth factor hybrid gene, whose protein product is targeted to a specific growth-factor receptor. Fusion toxins have been reported that specifically target DT to cytokine receptors including interleukin-2 (IL-2), IL-4, IL-6, and G-CSF receptors.²⁹ ³³

We constructed a fusion toxin targeting cells bearing the mGM-CSF receptor (1) to devise a reagent that is potentially useful in destroying the residual myeloid leukemic cells dur-
ing bone marrow (BM) purging or that could be used for in vivo therapy of residual disease or (2) to explore the expression of GM-CSF receptor on noncommitted and committed BM progenitor cells. In this report, we describe the construction of the novel DT<sub>30</sub> mGM-CSF fusion toxin consisting of the murine GM-CSF gene spliced to a truncated form of the DT gene that retains enzymatic activity, but excludes the native binding domain. We investigated the potency and specificity of our construct in its reactivity with murine GM-CSF receptor-expressing cell lines and normal granulocyte-macrophage BM progenitor cells.

**MATERIALS AND METHODS**

**Construction of hybrid gene and plasmid.** Oligonucleotides were synthesized using cyanomethyl phosphoramidite chemistry on an Applied Biosystems model 380 A DNA synthesizer and purified by chromatography on Oligonucleotide Purification Cartridges (Applied Biosystems, Foster City, CA) as recommended by the manufacturer. Purified oligonucleotides were resuspended in TE buffer (10 mmol/L TRIS base, 1 mmol/L EDTA, pH 8.0). The sequences of oligonucleotides used in this study are listed in Table 1. The hybrid gene encoding DT<sub>30</sub> mGM-CSF was constructed by the method of gene splicing by overlap extension (SOE) as described. Briefly, a DT gene fragment was generated in the first polymerase chain reaction (PCR) by using 5.5 ng plasmid containing the cDNA of DT mutant CRM107 as a template with primers a and b (Table 1). Primer a created an Nco I restriction site and an ATG initiation codon 5' to the DT coding sequence. Primer b introduced a coding sequence for a linker ([Gly<sub>4</sub>Ser<sub>14</sub>]) directly after amino acid 389 of the mature DT molecule. A murine GM-CSF gene fragment was generated in the second PCR by using 1.8 ng plasmid containing the cDNA of murine GM-CSF as a template with primers c and d. Primer c created the restriction site and the last 7 codons of GM-CSF and codons 384 to 389 of DT (Table 1). Primer d introduced an Nco I restriction site at the end of the GM-CSF molecule. The two fragments generated in the PCR's described above were then purified and used as templates in a SOE reaction using primers a and d. This SOE formed the full-length DT<sub>30</sub>-mGM-CSF hybrid gene. The DT<sub>30</sub>-mGM-CSF hybrid gene was digested with restriction enzymes Nco I and Xho I (GIBCO BRL, Gaithersburg, MD) and ligated into the Nco I and Xho I cloning sites in the pET21d plasmid (Novagen, Madison, WI). The assembly of plasmid PDTGM-CSF is shown in Fig 1.

**Expression and localization of fusion proteins.** Plasmid, pDT GM-CSF, was transformed into the E. coli strain BL21(DE3) (Novagen, Madison, WI) and protein expression was evaluated. Briefly, recombinant bacteria were grown in 500 mL Luria broth, supplemented with 100 µg/mL carbencillin (Sigma Chemical Co, St Louis, MO), in a 2-L flask at 37°C. When the absorbance (A<sub>600</sub>) of culture reached 0.6, expression of the hybrid gene was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (GIBCO BRL). Three hours after induction, the bacteria were obtained by centrifugation at 5,000g for 10 minutes. To determine the localization of expressed protein, an aliquot of bacterial pellet was resuspended in 30 mmol/L TRIS, pH 7.5, 20% sucrose, 1 mmol/L EDTA and osmotically shocked by placing in ice-cold 5 mmol/L MgSO<sub>4</sub>. The periplasmic fraction (supernatant) was obtained by centrifugation at 8,000g for 10 minutes. Another aliquot of bacterial pellet was resuspended in sonication buffer (50 mmol/L sodium phosphate, pH 7.8, 300 mmol/L NaCl). After incubation at −20°C for 16 hours, the resuspended sample was sonicated for 5 minutes. The spheroplast fraction (pellet) and cytosolic fraction (supernatant) were collected separately by centrifugation at 10,000g for 20 minutes.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.** Crude and purified fusion proteins were analyzed on SDS-PAGE. SDS-PAGE was performed using 4% to 20% gradient gels (Bio-Rad, Richmond, CA) and a Mini-Protein II gel apparatus (Bio-Rad). Proteins were stained with Coomassie brilliant blue. For immunoblotting, electrophoresed proteins were transferred to nitrocellulose membranes. Membranes were blocked with 3% gelatin-containing TBS (20 mmol/L TRIS, 500 mmol/L NaCl, pH 7.5) and washed with TTBS (TBS, 0.05% Tween-20, pH 7.5). Horse anti-DT antisera (Connaught Lab, Switwater, PA) and anti-GM-CSF (a rat IgG monoclonal antibody from clone 22E9.11 generously supplied by Dr John Abrams (DNAX, Palo Alto, CA) were used as a source of primary antibodies. The blots were processed using horseradish peroxidase-conjugated protein G (protein G-HRP) and developed using HRP color reagents (Bio-Rad, Richmond, CA).

**Isolation of inclusion bodies and purification of the fusion proteins.** To isolate expressed protein from inclusion bodies, a bacterial pellet was resuspended in TE buffer (50 mmol/L TRIS, pH 8.0, 20 mmol/L EDTA, 100 mmol/L NaCl) and treated with 5 mg/mL lysozyme for 30 minutes. The pellet was then incubated in Triton X-100 buffer (11% vol/vol Triton X-100, 89% vol/vol TE) for 30 minutes at room temperature after briefly homogenizing with a tissue homogenizer (Thomas Scientific, Staufen, Germany). Inclusion bodies were collected by centrifugation at 24,000g for 50 minutes. Solubilization of the inclusion body pellet was achieved in the presence of strong denaturants and reducing agents in a buffer consisting of 7 mol/L guanidine, 0.1 mol/L TRIS, pH 8.0, 2 mmol/L EDTA, and 65 mmol/L dithioerythritol. The solution was incubated at room temperature for 16 hours. To remove insoluble material, the solution was centrifuged at 40,000g for 10 minutes. Protein concentrations were determined according to Bradford method. To ensure proper

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**Table 1. Sequence of Oligonucleotides Used in This Study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Characteristics</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>a</td>
<td>The sense primer introduced an Nco I site with an initiation codon ATG and the initial 7 codons of DT.</td>
<td>5'AGATATACACATGGGCGCCTGATGATGTGGTTGAGTGA3'</td>
</tr>
<tr>
<td>b</td>
<td>The antisense primer introduced the codons 384 to 389 of DT and codons of a linker ([Gly&lt;sub&gt;4&lt;/sub&gt;Ser&lt;sub&gt;14&lt;/sub&gt;]4.</td>
<td>5'CAACACCCACACCGCCGCCGACCGACACACCGCCGCCGCAGACTTTTGATGGCGGAGGATGATGGTTGAGTGA3'</td>
</tr>
<tr>
<td>c</td>
<td>The sense primer introduced the codons 26 to 31 of GM-CSF and codons of part of the linker.</td>
<td>5'CCAACCCACACCGCCGCCGACCGACACACCGCCGCCGCAGACTTTTGATGGCGGAGGATGATGGTTGAGTGA3'</td>
</tr>
<tr>
<td>d</td>
<td>The antisense primer introduced an Xho I site and the last 7 codons of GM-CSF.</td>
<td>5'CCAACCCACACCGCCGCCGACCGACACACCGCCGCCGCAGACTTTTGATGGCGGAGGATGATGGTTGAGTGA3'</td>
</tr>
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</table>

| The sequence of the restriction site is in bold text. The sequence of the ([Gly<sub>4</sub>Ser<sub>14</sub>]4 linker is in italic text. | 5'CCAACCCACACCGCCGCCGACCGACACACCGCCGCCGCAGACTTTTGATGGCGGAGGATGATGGTTGAGTGA3' |
ternary folding, renaturation was initiated by a rapid 100-fold dilution of the denatured and reduced protein into refolding buffer consisting of 0.1 mol/L TRIS, pH 8.0, 0.5 mol/L L-arginine, 0.9 mmol/L oxidized glutathione, and 2 mmol/L EDTA. The samples were incubated at 10°C for 48 hours. The refolded protein was diafiltrated and ultrafiltrated against 20 mmol/L TRIS, pH 7.8, using a spiral membrane ultrafiltration cartridge on Amicon’s CH2 system (Amicon, Beverly, MA). Samples were loaded on a Q-Sepharose (Sigma) column and eluted with 0.3 mol/L NaCl in 20 mmol/L TRIS, pH 7.8. The protein was diluted fivefold and subsequently applied to another Q-Sepharose column and eluted with a linear salt gradient form 0 to 0.4 mol/L NaCl in 20 mmol/L TRIS, pH 7.8. The main peak from the second Q-Sepharose column was purified by size-exclusion chromatography on a TSK 250 column (Toso Hase, Philadelphia, PA).

**ADP ribosylation assay.** The toxin was nicked by treating 15 μg of DT300 mGM-CSF with 0.04 μg of trypsin for 15 minutes at 37°C. The reaction was stopped with soybean inhibitor. Duplicate samples of nicked DT and DT300 GM-CSF were examined for their ADP ribosyl transferase activity as previously described. Briefly, ADP-ribosylation was performed in 80-μL reaction mixtures containing 50 μL of 0.01 mol/L TRIS-Cl buffer with 1.0 mmol/L dithiothreitol, pH 8.0, 10 μL of rabbit reticulocyte lysate (containing the elongation factor 2, EF-2), and 10 μL of toxin sample. The reaction was initiated by the addition of 10 μL of 0.57 mmol/L [3H] nicotinamide adenine dinucleotide (ICN Biomedicals, Irvine, CA). Reaction mixtures were incubated at room temperature for 1 hour and the reaction was stopped by the addition of 1 mL 10% trichloroacetic acid (TCA). The precipitate was collected by centrifugation and washed with 1 mL 10% TCA. The radioactivity was counted by standard scintillation techniques.

**Cytotoxicity assay.** To characterize the cytotoxic activity of DT300 mGM-CSF, we used the murine myelomonocytic cell line FDCP2.1d (provided by Immunex Inc, Seattle, WA), which is dependent on mGM-CSF for proliferation. Cultured FDCP2.1d cells were maintained in complete culture media consisting of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1.5% L-glutamine, 2% penicillin/streptomycin, 2% HEPES, 0.8% L-arginine, 2% folic acid and L-asparagine, and exogenous mGM-CSF at a final concentration of 1 ng/mL. We assayed the cytotoxic activity by measuring the ability of DT300 mGM-CSF to inhibit the proliferation of FDCP2.1d cells. Cells were initially washed twice with plain DMEM to remove the exogenous cytokine followed by a 1-hour incubation at 37°C. A third wash was performed and the cells were resuspended. Cells were seeded at a concentration of 9 × 10^4 cells per well in 96-well flat-bottomed

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Fig 1. Assembly of the DT300 mGM-CSF gene in the pET2ld vector. The hybrid gene encoding DT300 mGM-CSF was constructed by the method of gene splicing by SOE. The DT300 mGM-CSF hybrid gene was digested with restriction enzymes NcoI and XhoI and ligated into the NcoI and XhoI cloning sites of a pET2ld plasmid under the control of a T7 promoter.
plates in a volume of 200 µL. One microliter [3H]-thymidine and exogenous mGM-CSF at final concentration of 1 ng/mL were added into each well. After a 24-hour incubation, the cells were procured on glass fiber filters. Filters were washed, dried, and counted according to standard methods suggested by manufacturer. Cells cultured with media alone served as the control. All assays were performed in triplicate. Two additional control murine cell lines EL4, a T-cell leukemia/lymphoma and the myeloid leukemia C1498 (American Type Culture Collection, Rockville, MD) were used that did not respond to mGM-CSF.

Colony-forming assay. DT390 mGM-CSF, DT390 mIL-2, and native DT were examined for their effects on BM cells in a colony-forming assay, which was performed by short-term culture of toxin-treated murine BM cells in complete methylcellulose media (30% fetal calf serum, 1% pokeweed mitogen-stimulated murine spleen cell conditioned medium, 1% bovine serum albumin (BSA), 0.9% methylcellulose, 10^-4 mol/L 2-mercaptoethanol, and 3 U/mL erythropoietin) (StemCell Technologies, Vancouver BC, Canada). Briefly, BM cells were collected into RPMI 1640 media by flushing the shafts of femora and tibia of C57BL/6 mice. Cells were resuspended at 5 x 10^4 cells/mL in complete methylcellulose media with toxin at a final concentration of either 10^-8 or 10^-7 mol/L and were plated and incubated in culture dishes for 14 days under fully humidified conditions in an atmosphere of 5% CO2 at 37°C. Under an inverted microscope, colonies of greater than 50 cells were scored as CFU-GM according to their morphology. All assays were performed in duplicate.

RESULTS

Genetic construction of DT390 mGM-CSF. The DNA fragments encoding the structural gene for DT390 and mGM-CSF were obtained by separate PCRs with the 1,239 and 380 bp, respectively. After the third PCR, the resulting SOE product, DT390 mGM-CSF hybrid gene, was generated with 1,601-bp size. The DT390 mGM-CSF hybrid gene encodes an Nco I restriction site, an ATG initiation codon, the first 389 amino acids of DT, a 20-amino acid interchain spacer, the mature murine GM-CSF polypeptide, and an Xho I restriction site. After digestion, the DT390-mGM-CSF hybrid gene was cloned into the pET2ld plasmid under the control of the IPTG-inducible T7 promoter to create PDT hybrid gene. The plasmid was sequenced by the University of Minnesota Microchemical Facility (University of Minnesota, Minneapolis). The junctional region was found to encode for amino acids 333 to 389 of DT with one substitution of alanine at position 356 by serine. The linker was found to encode for the following amino acids, Gly-Ala-Gly-Ser-Phe joined by a sequence encoding amino acids 27 through 175 of mGM-CSF.

Expression and purification of DT390-mGM-CSF fusion protein. Expression of the fusion protein in E coli was induced with IPTG at 37°C. Coomassie blue-stained SDS-polyacrylamide gel of whole bacterial lysate post-IPTG induction showed a protein migrating at 58 kD, which corresponds to the expected size for DT390-mGM-CSF protein. The localization study of the expressed fusion protein showed that DT390 mGM-CSF was retained in the inclusion bodies (Fig 2). To extract the DT390-mGM-CSF protein, the inclusion bodies were isolated, denatured, and refolded as described in Materials and Methods. After the renaturation procedure, the crude DT390 mGM-CSF was purified by sequential chromatography. The elution from the anion-exchange Q-sepharose column showed an enrichment of a protein with an electrophoretic mobility corresponding to an apparent molecular mass of 58 kD (Fig 2, lane 6). To further purify this fusion protein, pooled peak fractions from the anion-exchange Q-sepharose column were subjected to high-performance liquid chromatography (HPLC) using a TSK 250 sizing column (Fig 2, lane 7). The final product was 80% pure. To prove that the protein was indeed a fusion of of this DT390 spliced to mGM-CSF, further analysis was performed by immunoblotting. Anti-DT antiserum was able to recognize the DT390 mGM-CSF, both in the crude bacterial lysate and after renaturation (Fig 3). It is of interest to note that anti-mGM-CSF antiserum was able to recognize the DT390 mGM-CSF only after proper renaturation.

Enzymatic activity and in vitro cytotoxicity. Protein synthesis inhibition by DT is caused by fragment A-catalyzed ADP-ribosylation of cytoplasmic EF-2. To determine whether the DT390-mGM-CSF protein also displays such enzymatic activity, a cell-free assay system was used, in which rabbit reticulocyte lysate, a source of EF-2, was exposed to either native DT or DT390 mGM-CSF in the presence of [32P]-nicotinamide adenine dinucleotide. Incubation with either toxin showed a similar dose-dependent increase in [32P] incorporation into the TCA-precipitable fraction (Fig 4).
Fig 3. Western blot analysis of DT<sub>390</sub>-mGM-CSF protein. (A) Nitrocellulose probed with polyclonal anti-DT serum. Lane 1, renatured protein after anion-exchange column; lane 2, induced total bacteria lysate; lane 3, uninduced total bacteria lysate; and lane 4, molecular weight standards. (B) Nitrocellulose probed with anti-mGM-CSF antibody. Lane 1, renatured protein after anion-exchange column; lane 2, induced total bacteria lysate; lane 3, uninduced total bacteria lysate; and lane 4, molecular weight standards.

This result confirmed that DT<sub>390</sub> mGM-CSF possesses ADP-ribosyl transferase activity.

To characterize the cytotoxic activity of DT<sub>390</sub> mGM-CSF, a bioassay was devised using the mGM-CSF–dependent myelomonocytic leukemia cell line FDCP2.1d. The cytotoxicity was evaluated by measuring the inhibition of cellular proliferation. The ability of various concentrations of DT<sub>390</sub> mGM-CSF to inhibit the proliferation of FDCP2.1d cells was examined. FDCP2.1d cells were inhibited by DT<sub>390</sub> mGM-CSF in a dose-dependent manner with an IC<sub>50</sub> of 5 × 10<sup>-11</sup> mol/L or 3 ng/mL (Fig 5). To determine if the cytotoxic activity of DT<sub>390</sub> mGM-CSF on FDCP2.1d cells was mediated by the binding of the mGM-CSF moiety, several other toxins including DT<sub>390</sub> hIL-2, DT<sub>390</sub> mIL-4, and native DT were also assayed for their activity against FDCP2.1d. In contrast with DT<sub>390</sub> mGM-CSF, FDCP2.1d cells were resistant to DT<sub>390</sub> hIL-2, DT<sub>390</sub> mIL-4, and native DT up to a concentration of 1 × 10<sup>-9</sup> mol/L. Furthermore, anti-mGM-CSF antibodies blocked the cytotoxic effect of DT<sub>390</sub> mGM-CSF in a dose-dependent manner. Without addition of anti-mGM-CSF antibodies, DT<sub>390</sub> mGM-CSF at concentration of 1 × 10<sup>-9</sup> mol/L produced a 84% inhibition of cellular proliferation of FDCP2.1d. The addition of 1 nmol/L anti-mGM-CSF antibodies partially neutralized this cytotoxic effect. The addition of 10 nmol/L or 100 nmol/L anti-mGM-CSF antibodies completely neutralized this cytotoxic effect (Fig 6). The addition of 10 nmol/L GM-CSF to 1 nmol/L DT<sub>390</sub> mGM-CSF also caused a complete neutralization of its cytotoxic effect.

Fig 4. ADP ribosyl transferase activity of DT<sub>390</sub> mGM-CSF. Trypsin-nicked DT<sub>390</sub> mGM-CSF, trypsin-nicked native diphtheria toxin, or BSA was studied in cell-free assay. Protein was added at various concentration to the reaction system. The activity was measured as the count of bound ³²P-ADP-ribose to rabbit reticulocyte lysate (EF-2).
mGM-CSF inhibited the response 25%, but the addition of IL-3 did not inhibit. The effect of DT₃₉₀ mGM-CSF on two GM-CSF nonresponsive cell lines was performed to further examine the specificity of the cytotoxic effect. DT₃₉₀ mGM-CSF did not inhibit the T-cell line EL4 or leukemia cell line C1498 (Table 2). Together these data indicate that DT₃₉₀ mGM-CSF is specifically cytotoxic to cells via the GM-CSF ligand-receptor complex.

The effect of DT₃₉₀ mGM-CSF on myeloid progenitor stem cells. It has been well documented that GM-CSF plays a role in the development of the myeloid lineage in hematopoiesis. To test the effect of DT₃₉₀ mGM-CSF on committed myeloid progenitor cells, a colony-forming assay was performed by incubating murine BM cells with toxins including DT₃₉₀ mGM-CSF, DT₃₉₀ hIL-2, and native DT. The DT₃₉₀ mGM-CSF inhibited the formation of CFU-GM up to 90% at the toxin concentrations of 1 nmol/L and 10 nmol/L. In contrast, DT₃₉₀ mIL-2 and native DT had little inhibitory effect on CFU-GM (Fig 7). From these data, we conclude that DT₃₉₀ mGM-CSF has activity against committed myeloid progenitor cells in vitro.

DISCUSSION

The unique contribution of this work is the construction and description of a fusion toxin, DT₃₉₀ mGM-CSF by genetically splicing the DNA segment encoding the ADP-ribosyl transferase enzymatic and hydrophobic translocation enhancing region of DT, but not the native binding site to the DNA segment encoding the amino acids of the mature mGM-CSF molecule. Our data show that the selective binding of this chimeric protein to GM-CSF receptor expressing myelomonocytic leukemia cell lines results in the delivery of a potent and fatal signal that precipitously decreases the proliferation of these cells.

The potency of DT₃₉₀ mGM-CSF was high because we measured an IC₅₀ of about 5 × 10⁻¹¹ mol/L against the cell line FDCP2.1d. Recently, Lappi et al has chemically conjugated human GM-CSF to the ribosome-inactivating protein saporin (SAP). The hGM-CSF-SAP showed an IC₅₀ of about 3 to 4 × 10⁻¹² mol/L on cell lines transfected with both subunits of GM-CSF receptors. Although these studies involved different toxin moieties, different subunits of GM-CSF receptors, and different receptor numbers on different cell lines, DT₃₉₀ mGM-CSF was comparable in cytotoxicity to this and other diphtheria-based fusion toxins. For example, DAB₃₈₀ IL-2 and DAB₃₈₅ IL-2 usually show potency with IC₅₀ ranging from 2 × 10⁻¹¹ to 1 × 10⁻¹⁰ mol/L. DT₃₉₀ mGM-CSF was specific because it was cytotoxic to mGM-CSF nonresponsive cell lines C1498 and EL4. DT₃₉₀ mGM-CSF activity was inhibited by an excess of anti-mGM-CSF antibody and, thus, was directed by the GM-CSF portion of the molecule. The IC₅₀ of the fusion protein (50 pmol/L) corresponded to the reported dissociation constant for high-affinity GM-CSF binding sites (20 to 60 pmol/L).

The study was initiated because alternative therapies are still a priority for the treatment of acute nonlymphocytic leukemias, which are still a serious clinical problem. GM-CSF was selected as a ligand because GM-CSF receptor is expressed on these myeloid leukemias. It has been postulated that GM-CSF is involved in malignant transformation and metastases because of its expression on neoplastic cells. Binding GM-CSF causes internalization of GM-CSF receptor in a variety of murine cells at 37°C by receptor-mediated endocytosis. Thus, we expected that the GM-CSF component of the fusion toxin would be able to bind to the GM-CSF receptor, resulting in the internalization of the GM-CSF receptor-fusion toxin complex into the endocytic vesicles in a fashion analogous to diphtheria toxin itself.

| Table 2. Sensitivity of Various Cell Lines to DT₃₉₀-mGM-CSF |
|----------------|----------------|----------------|
| Cell Line | Origin | mGM-CSF | Response | IC₅₀ (mol/L) |
| FDCP2.1d | Mouse myelomonocytic leukemia | mGM-CSF | dependent | 5 × 10⁻¹¹ |
| C1498 | Mouse lymphoma | Negative | 1 × >10⁻⁸ |
| EL4 | Mouse T-cell lymphoma | Negative | 1 × >10⁻⁸ |

Cell lines were incubated with DT₃₉₀-mGM-CSF for 4 hours, washed, pulsed with tritiated thymidine, and then incubated for 24 hours. Cells were harvested onto filters and then counted. Data were plotted as percent control inhibition versus increasing concentration of fusion toxin. IC₅₀ dosage (the dose at which 50% of the total response was inhibited) was determined from the curves.
of the most commonly used techniques involve chemical purging of the BM to eliminate leukemia cells.\textsuperscript{43} However, one drawback is that chemical purging has a broad spectrum of depletion and eliminates beneficial cells including lymphocytes.\textsuperscript{44} An advantage of using mGM-CSF to direct toxin is that GM-CSF would bind to myeloid leukemia cells\textsuperscript{45} and not to cells that do not express the GM-CSF receptor.

Because residual metastatic leukemia cells that survive the preparative regimen can also lead to relapse and transplantation failure, DT\textsubscript{390} mGM-CSF could be used for in vivo therapy, especially because there is clinical precedence for the use of DT-based fusion toxins for therapy of leukemia.\textsuperscript{46} Although the in vivo efficacy of this agent will depend on its ability to access leukemia cells, their are several complicating issues that must also be explored. These issues can best be studied, and in some cases can only be studied, in animal models.

The issues are as follows: (1) In vivo depletion of myeloid cells might result in immunosuppression. Cells of myeloid origin participate as antigen presenting cells in generating optimal T-cell responses.\textsuperscript{47} A fusion toxin directed against these cells might either reduce the immune response to tumor or render the host susceptible to secondary infections. (2) GM-CSF receptor expression on stem cells might limit the in vivo antileukemia effectiveness of DT\textsubscript{390} mGM-CSF resulting in life-threatening myelosuppression. The GM-CSF receptor is expressed on committed murine progenitor cells.\textsuperscript{48}

In fact, studies in humans show that GM-CSF is active in stimulating CD34\textsuperscript{+} human progenitor cells.\textsuperscript{49} In this paper, DT\textsubscript{390} mGM-CSF was reactive against committed myeloid BM progenitors in in vitro CFU-GM assays. In separate studies (data not shown), we found that the fusion toxin had little effect against day 8 CFU-S, which measures earlier erythroid and myeloid stem cells, and day 12 CFU-S, which measures multilineage progenitor cells.\textsuperscript{50,51} Thus, expression of mGM-CSF receptors occurs between CFU-S and CFU-GM stages of development. In future studies, DT\textsubscript{390} mGM-CSF can be used in murine in vivo adoptive transfer experiments designed to directly determine stem cell expression of the GM-CSF receptor. Such experiments cannot be performed in humans. (3) Some believe that GM-CSF expression on endothelial cells could result in fusion toxin injury to the vasculature causing vascular leak syndrome, which has been problematic in the use of other immunotoxins.\textsuperscript{52} GM-CSF stimulates the proliferation of endothelial cells.\textsuperscript{53} However, our histopathologic studies of mice given in vivo DT\textsubscript{390} mGM-CSF showed endothelitis in some larger vessels, but no evidence of endothelial cell destruction (data not shown). (4) GM-CSF receptors are heterogeneously expressed which might effect the efficacy of DT\textsubscript{390} mGM-CSF against leukemic targets in vivo. Myeloid cells in mice appear to express two distinct types of GM-CSF receptors with high and low affinities, with kd of 20 to 60 pmol/L and 700 to 1,200 pmol/L, respectively.\textsuperscript{40} The onset of proliferation of GM progenitor cells remains highly asynchronous, which may result from this heterogeneous expression of either high or low affinity GM-CSF receptors.

In conclusion, these findings describe a new fusion protein with high potential to eradicate tumor cells of myeloid lineage. There is potential for using this agent to purge BM contaminated with leukemia or against minimal residual disease surviving current clinical conditioning regimens and causing relapse. The agent can be used to study the role of GM-CSF receptor expressing cells in lymphohematopoiesis. It is also noteworthy that the potential of DT\textsubscript{390} mGM-CSF is not limited to myeloid leukemia because high-affinity GM-CSF receptors have been detected on solid tumor cell lines including those of lung cell or colon origin.\textsuperscript{14-17} Thus, these tumors may also be effectively targeted.

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CH Chan, BR Blazar, CR Eide, RJ Kreitman and DA Vallera