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 diphasphate-ribosylating activity of DT₃₀₀-mGM-CSF showed results that were similar to those of native DT. The DT₃₀₀ mGM-CSF immunotoxin inhibited FDCP2.1d, a murine myelomonocytic tumor line expressing the GM-CSF receptor with an IC₅₀ (concentration inhibiting 50% activity) of 5 × 10⁻¹¹ 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 DT₃₀₀ 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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From the Department of Therapeutic Radiology, Section on Experimental Cancer Immunology, the Department of Laboratory Medicine and Pathology, and the Department of Pediatrics, Division of Bone Marrow Transplantation, University of Minnesota Hospital and Clinics, Minneapolis, MN; and the National Cancer Institute, National Institutes of Health, Bethesda, MD.

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Address reprint requests to Daniel A. Vallera, PhD, Box 367, UMMC, Harvard St at E River Rd, Minneapolis, MN 55455.

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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$_{390}$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$_{390}$ 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,Ser)$_4$]$_4$ 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 a Tm restriction site and an ATG initiation codon 5' to the GM-CSF coding sequence. Primer d introduced a coding sequence for a linker [(Gly,Ser)$_4$]$_4$ directly after amino acid 389 of the mature DT molecule. The two fragments generated in the PCRs 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$_{390}$ mGM-CSF hybrid gene. The DT$_{390}$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 BL2(DE3) (Novagen, Madison, WI) and protein expression was evaluated. Briefly, recombinant bacteria were grown in 500 mL Luria broth supplemented with 100 µg/mL carbenicillin (Sigma Chemical Co, St Louis, MO), in a 2-L flask at 37°C. When the absorbance (A$_{600}$) 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$_4$. The peri-plasmic 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-PROTEAN II gel apparatus (Bio-Rad). Proteins were stained with Coomasie 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 (1% vol/vol Triton X-100, 89% vol/vol TE) for 30 minutes at room temperature after briefly homogenizing with a tissue homogenizer (Thomas Scientifics, 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

<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'AGATATACCATCGGCGTCGATGATGTTGTGATGATG'</td>
</tr>
<tr>
<td>b</td>
<td>The antisense primer introduced the codons 384 to 389 of DT and codons of a linker [(Gly,Ser)$_4$].</td>
<td>5'CGACCCACACCCGCGGGGCGGCCACGCGTGCAGACTCCGCGGCCAC</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>AAATGTTTGTGCTTCAGGAA</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'TCGTGCGGCTGGAG</td>
</tr>
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The sequence of the restriction site is in bold text. The sequence of the [(Gly,Ser)$_4$]$_4$ linker is in italic text.
tertiary 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 dialyzed 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 from 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 Has, Philadelphia, PA).

**ADP ribosylation assay.** The toxin was nicked by treating 15 μg of DT390 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 DT390 GM-CSF were examined for their ADP ribosyl transferase activity as previously described.70 Briefly, ADP-ribosylation was performed in 80-μL reaction mixtures containing 50 μL of 0.01 mol/L TRIS-HCl 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 [32P] 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 DT390 mGM-CSF, we used the murine myelomonocytic cell line FDCP2.1d (provided by Immunix 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 DT390 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 tube in complete culture media and treated with the following toxins: DT390 mGM-CSF, DT390 hIL-2, DT390 mL-4, native DT, and ricin at concentrations ranging from 1 × 10^-15 mol/L to 1 × 10^-8 mol/L. The cells were treated for 4 hours at 37°C in a 5% CO2 atmosphere. After incubation, the tubes were centrifuged for 10 minutes at 300g and the supernatant decanted. The cells were washed with complete media three times and resuspended with 600 μL media after the third wash. Cells were seeded at 3 × 10^4 cells/well in 96-well flat-bottomed
formed 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.

Colonial-forming assay. DT\textsubscript{390} mGM-CSF, DT\textsubscript{390} 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\textsuperscript{-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 \times 10\textsuperscript{5} cells/mL in complete methylcellulose media with toxin at a final concentration of either 10\textsuperscript{-4} or 10\textsuperscript{-5} mol/L and were plated and incubated in culture dishes for 14 days under fully humidified conditions in an atmosphere of 5% CO\textsubscript{2} 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 DT\textsubscript{390} mGM-CSF. The DNA fragments encoding the structural gene for DT\textsubscript{390} and mGM-CSF were obtained by separate PCRs with the 1,239 and 380 bp, respectively. After the third PCR, the resulting SOE product, DT\textsubscript{390} mGM-CSF hybrid gene, was generated with a 1,601-bp size. The DT\textsubscript{390} 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 DT\textsubscript{390}-mGM-CSF hybrid gene was cloned into the pET21d plasmid under the control of the IPTG-inducible T7 promoter to create pDT GM-CSF. Restriction endonuclease digestion and DNA sequencing analysis were used to verify that DT\textsubscript{390} mGM-CSF hybrid gene sequence had been cloned in frame (data not shown).

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)\textsubscript{3}Ser]GlyAla(Gly)\textsubscript{2}Ser-(Gly)\textsubscript{2}SerPhe joined by a sequence encoding amino acids 27 through 175 of mGM-CSF.

Expression and purification of DT\textsubscript{390}-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 DT\textsubscript{390}-mGM-CSF protein. The localization study of the expressed fusion protein showed that DT\textsubscript{390} mGM-CSF was retained in the inclusion bodies (Fig 2). To extract the DT\textsubscript{390}-mGM-CSF protein, the inclusion bodies were isolated, denatured, and refolded as described in Materials and Methods. After the renaturation procedure, the crude DT\textsubscript{390} 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 DT\textsubscript{390} and mGM-CSF, further analysis was performed by immunoblotting. Anti-DT antiserum was able to recognize the DT\textsubscript{390} 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 DT\textsubscript{390} 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 DT\textsubscript{390}-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 DT\textsubscript{390} mGM-CSF in the presence of [\textsuperscript{32}P]–nicotinamide adenine dinucleotide. Incubation with either toxin showed a similar dose-dependent increase in [\textsuperscript{32}P] incorporation into the TCA-precipitable fraction (Fig 4).
This result confirmed that DT₃₉₀ mGM-CSF possesses ADP-ribosyl transferase activity.

To characterize the cytotoxic activity of DT₃₉₀ 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 DT₃₉₀ mGM-CSF to inhibit the proliferation of FDCP2.1d cells was examined. FDCP2.1d cells were inhibited by DT₃₉₀ mGM-CSF in a dose-dependent manner with an IC₅₀ of 5 × 10⁻⁸ mol/L or 3 ng/mL (Fig 5). To determine if the cytotoxic activity of DT₃₉₀ mGM-CSF on FDCP2.1d cells was mediated by the binding of the mGM-CSF moiety, several other toxins including DT₃₉₀ hIL-2, DT₃₉₀ mIL-4, and native DT were also assayed for their activity against FDCP2.1d. In contrast with DT₃₉₀ mGM-CSF, FDCP2.1d cells were resistant to DT₃₉₀ hIL-2, DT₃₉₀ mIL-4, and native DT up to a concentration of 1 × 10⁻⁸ mol/L. Furthermore, anti-mGM-CSF antibodies blocked the cytotoxic effect of DT₃₉₀ mGM-CSF in a dose-dependent manner. Without addition of anti-mGM-CSF antibodies, DT₃₉₀ mGM-CSF at concentration of 1 × 10⁻⁸ mol/L produced an 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 DT₃₉₀ also enhanced the ADP-ribosyl transferase activity of DT₃₉₀ mGM-CSF. Trypsin-nicked DT₃₉₀ 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 (EF2).
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 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 species of GM-CSF, 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, DAB389 IL-2 and DAB4Xh 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 dependent cell line FDCP2.1d, but not the 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. Our findings support this.

One major problem in myeloid leukemia is purging occult leukemia cells from patient BM before autologous BM transplant. Complete remission can be induced by intensive chemotherapy, but remission can be short-lived and the patient frequently experiences relapse of their underlying disease. Autologous BM transplantation is being explored as treatment modality designed to improve relapse-free survival. The patient’s own BM is removed and then returned as a hematopoietic rescue procedure after aggressive chemotherapy and irradiation therapy. Because the procedure is complicated by metastatic leukemia cells infiltrating the BM, one
of the most commonly used techniques involve chemical purging of the BM to eliminate leukemia cells. However, one drawback is that clinical chemical purging has a broad spectrum of depletion and eliminates beneficial cells including lymphocytes. An advantage of using mGM-CSF to direct toxin is that GM-CSF would bind to myeloid leukemia cells 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, DT₃⁹₀ 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. Although the in vivo efficacy of this agent will depend on its ability to access leukemia cells, there 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. 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 DT₃⁹₀ mGM-CSF resulting in life-threatening myelosuppression. The GM-CSF receptor is expressed on committed murine progenitor cells. In fact, studies in humans show that GM-CSF is active in stimulating CD34⁺ human progenitor cells. In this paper, DT₃⁹₀ mGM-CSF was reactive against committed myeloid BM progenitor 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. Thus, expression of mGM-CSF receptors occurs between CFU-S and CFU-GM stages of development. In future studies, DT₃⁹₀ 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. GM-CSF stimulates the proliferation of endothelial cells. However, our histopathologic studies of mice given in vivo DT₃⁹₀ mGM-CSF showed endotheliitis 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 DT₃⁹₀ 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 kₐ of 20 to 60 pmol/L and 700 to 1,200 pmol/L, respectively. 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 DT₃⁹₀ 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. Thus, these tumors also may be effectively targeted.

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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

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