Recombinant polypeptide cytotoxins are provided which comprise a cytokine or cytokine subunit or a single chain antibody subunit fused to a polypeptide toxin which suppress the proliferation of a population of mammalian cells by causing apoptotic cell death. The invention further provides a method which utilizes the disclosed recombinant polypeptide cytotoxins to systemically treat cancer patients.
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Recombinant Polypeptide Cytotoxins for Cancer Treatment

Background of the Invention

Acute Myeloid Leukemia

An estimated 26,000 Americans will develop leukemia in 1995, and over 20,000 will succumb to their disease because of the lack of adequate therapies. Wingo et al., CA, 45, 8 (1995). Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults and the second most frequent leukemia in children, accounting for 20-25% of acute childhood leukemias. Priesler et al., Blood, 80, 2600 (1992). Though the majority of patients with myeloid leukemias initially respond to intensive chemotherapy regimens, most will relapse and eventually succumb to their disease. Additionally, attempts to identify useful and specific prognostic factors to effectively stratify good and poor outcome AML patients have generally not been successful, with the result that all patients receive very intensive therapy at the price of great morbidity. Furthermore, contemporary multiagent chemotherapy regimens for AML fail to cure more than half of the patients because of multidrug resistance of leukemia cells and often lead to potentially fatal systemic toxicity. Gale et al., Sem. Hematol., 24, 40 (1987). Finally, although allogeneic bone marrow transplantation has been demonstrated to be an effective therapy for many patients with myeloid leukemia, its application is limited by the availability of suitable HLA-matched and MLC-unreactive donors. Woods et al., J. Clin. Oncol., 11, 1448 (1993). In autologous bone marrow transplantation for childhood acute myeloid leukemia, gene marker studies have indicated that subclinical disease in unpurged “remission” marrow harvested for transplantation contributes significantly to disease recurrence. Brenner et al., Lancet, 341, 85 (1993). Myeloablative chemotheraphy or supralethal radiochemotherapy followed by allogeneic or autologous bone marrow transplantation are associated with considerable morbidity and mortality and fail to substantially improve the overall survival of AML patients, underscoring the need for rational, drug design-based therapies for AML. Yeaper et al., New Engl. J. Med., 315, 141 (1986); Woods et al., cited supra.
t(4;11) Mixed Lineage Leukemia

The t(4;11)(q21;q23) chromosomal translocation is the most common clonal chromosomal abnormality in leukemic blasts from infants with acute lymphoblastic leukemia (ALL). The translocation breakpoint on 11q23 occurs within the Mixed Lineage Leukemia (MLL) gene, which encodes a 431 kDa protein with features characteristic of minor groove DNA binding proteins as well as DNA methyltransferases. Since 80% of the infants and children with ALL relapse or do not enter remission despite intensive therapy, the t(4;11)(q21;q23) chromosomal translocation confers a dismal prognosis on these children. Pui et al., Blood, 77, 440 (1991).

B-cell Precursor Leukemia

B-cell precursor (BCP) leukemia is the most common form of childhood cancer and represents one of the most radiation-resistant forms of human malignancy. Rivera et al., New Engl. J. Med., 329, 1289 (1993); Uckun et al., International Journal of Radiation Oncology Biology Physics, 27, 899 (1993). Recent studies demonstrated that >75% of clonogenic BCP leukemia cells from more than one-third of the newly diagnosed patients and virtually all of the relapsed patients are able to repair potentially lethal or sublethal DNA damage induced by radiation doses which correspond to the clinical TBI dose fractions (i.e., 2-3 Gy). Uckun et al., cited supra. Despite the clinical radiation resistance of BCP leukemias, bone marrow transplantation remains the best prospect for survival of high risk BCP leukemia patients. Barrett et al., N. Engl. J. Med., 331, 1253 (1994). However, the vast majority of high-risk BCP leukemia patients undergoing total body irradiation (TBI) in the context of bone marrow transplantation (BMT) relapse within the first 12 months and only 15-20% survive disease-free beyond the first 2 years. Uckun et al., International J. Radiation Biol, 56, 611 (1989). Combined or adjunctive therapies that exploit diverse cytotoxic mechanisms offered by biotherapy and chemotherapy may assist in the elimination of radiation resistant BCP leukemia cells.
Immunotoxins

Immunotoxins embody a new approach to killing malignant cells, while leaving normal tissues unharmed. To construct immunotoxins, cell targeting proteins are linked to cytotoxic agents, combining the selectivity of the carrier moiety with the potency of the cytotoxic moiety. The choice of carrier moiety can be based on the surface antigen profile of a given malignant cell determined by reaction with an enzyme or fluorescently labelled antibody.

For the past decade, immunotoxins have been under investigation for the treatment of various cancers, and more recently, for the treatment of immunological disorders such as rheumatoid arthritis and acquired immune deficiency syndrome (AIDS). Uckun, Brit J. Hematol., 85, 435 (1993); Perentesis et al., Biofactors, 3, 173 (1992). Although these agents have shown some potential to provide safe and effective therapy for certain human pathologies, many difficulties remain. For example, native toxin has a limited supply. Furthermore, the production of immunotoxins by chemical coupling methods is expensive because it requires large amounts of antibody and toxin. Additionally, immunotoxins prepared by chemically linking a whole antibody to a toxin are large in size, typically having a molecular mass of 210,000 kDa. Because of the large size, these immunotoxins have limited vascular and tissue penetration; particularly, they cannot pass the blood-brain barrier. Finally, because the chemical conjugation methods used produce heterogeneous products, immunotoxins tend to illicit significant non-specific systemic toxicity. Also contributing to this non-specific systemic toxicity is the large molecule size of immunotoxins and their resulting long half-life.

Thus, improved immunotherapeutic agents that will selectively eliminate leukemic blasts without damaging pluripotent lympho-hematopoietic progenitor cells are needed.

Summary of the Invention

The present invention provides a recombinant polypeptide cytotoxin which comprises a cytokine or cytokine subunit, which binds to a receptor expressed on a mammalian cell, fused to a polypeptide cytotoxin. The
cytokines or cytokine subunits suitable for use in the present invention are preferably those whose receptors are those which do not bind to a receptor expressed on normal pluripotent bone marrow progenitor cells. Preferably, the cytokine will be granulocyte macrophage colony stimulating factor (GMCSF), IL-3 or subunits thereof. Most preferably, the cytokine will be GMCSF or active subunits thereof.

The polypeptide cytotoxin portion of the recombinant polypeptide cytotoxin preferably does not contain a binding region for mammalian cells. More preferably, the cytokine or cytokine subunit is fused to the polypeptide cytotoxin so that the cytokine or cytokine subunit replaces the cell-binding portion of the polypeptide cytotoxin. Additionally, the polypeptide cytotoxin portion of the present recombinant polypeptide cytotoxin preferably acts to inhibit the protein synthesis of the target cell, thus killing the cell by apoptosis. More preferably, the polypeptide cytotoxin will be a bacterial toxin or a plant toxin. Most preferably, the polypeptide cytotoxin will be derived from diphtheria toxin (DT). The recombinant polypeptide cytotoxins resulting from the fusion of the catalytic and translocation domains of DT (DT_{ct}) and the preferred cytokine can be designated DT_{ct}-GMCSF. In this embodiment of the invention, it is preferred that the receptor be GMCSF-R.

In this embodiment of the present invention, the mammalian cell will preferably be a cancer cell. Preferably, if the mammalian cell is a cancer cell, it is a leukemia cell or a solid tumor cell, such as a multi-drug resistant or radiation resistant cancer cell.

The present invention also provides a recombinant polypeptide cytotoxin which comprises a single chain antibody subunit fused to a polypeptide cytotoxin. Preferably, the antibody or antibody subunit is capable of binding to a CD19 receptor expressed on a mammalian B-lineage cell and does not bind to a receptor expressed on normal pluripotent bone marrow progenitor cells. More preferably, the antibody subunit is the Fab fragment of B43. It is preferred that the polypeptide cytotoxin of the present invention inhibits protein synthesis of the target cell to the extent that the cell is killed by
apoptosis. More preferably, the polypeptide cytotoxin is a bacterial or plant toxin. Most preferably, the polypeptide toxin is a diphtheria toxin subunit which does not contain the native binding domain, pokeweed antiviral protein (PAP) or an active subunit thereof. The recombinant polypeptide cytotoxins resulting from the fusion of DT<sub>ct</sub> and the preferred antibody can be designated DT<sub>ct</sub>-B43, while the recombinant polypeptide cytotoxins resulting from the fusion of PAP and the preferred antibody can be designated B43-PAP.

In this embodiment of the present invention, the mammalian cell will preferably be a cancer cell. Preferably, the mammalian cell is a leukemia cell or a solid tumor cell, such as a multi-drug resistant or radiation resistant cancer cell.

Also provided in the present invention is a method of suppressing the proliferation of a population of mammalian cells which overexpress the GMCSF receptor. The method comprises reacting the proliferating mammalian cells with a recombinant polypeptide cytotoxin comprising GMCSF or a subunit thereof which binds to said receptor, fused to a polypeptide cytotoxin. Preferably, the native binding domain of the polypeptide cytotoxin is absent and more preferably, GMCSF is fused to the polypeptide cytotoxin so that GMCSF replaces the cell-binding portion of the toxin. Additionally, the polypeptide cytotoxin portion of the present recombinant polypeptide cytotoxin preferably acts to inhibit the protein synthesis of the target cell, thus killing the cell by apoptosis. Furthermore, it is preferred that the polypeptide cytotoxin is a plant toxin or a bacterial toxin. More preferably, the polypeptide cytotoxin will be derived from diphtheria toxin.

It is further preferred, in this embodiment of the invention, that the mammalian cells are cancer cells. More preferably, the mammalian cells are leukemia cells, solid tumor cells or human macrophages. Furthermore, it is preferred that the mammalian cells are multi-drug resistant or radiation resistant. If the mammalian cells are human macrophages, it is further preferred that the proliferation of the human macrophages is due to contact with foreign tissue antigens in the context of organ transplantation.
The present invention also provides a therapeutic method for the
treatment of target cancers. The method comprises parenterally administering
to a patient who is afflicted with a target cancer an effective amount of a
pharmaceutical composition comprising a recombinant polypeptide cytotoxin;
which recombinant polypeptide cytotoxin comprises a single chain antibody
subunit fused to a polypeptide cytotoxin, in combination with a
pharmacologically acceptable carrier. Preferably, the single chain antibody
subunit will be the Fv fragment of B43. It is further preferred that the
polypeptide cytotoxin be either a diptheria toxin subunit that does not contain
the native binding domain or pokeweed antiviral protein. As used herein, the
phrase "target cancer" refers to diseases associated with the proliferation of
mammalian cells expressing the antigen recognized by B43, i.e., the CD19
antigen. Such target cancers include, but are not limited to, B-lineage acute
lymphoblastic leukemia, chronic lymphocytic leukemia, B-lineage lymphoma,
blast crisis of chronic myelosytic leukemia, hairy cell leukemia, AIDS
lymphoma, EBV-lymphoma.

Further provided is a therapeutic method for treating target cancers
which comprises parenterally administering to a patient who is afflicted with a
target cancer an effective amount of a pharmaceutical composition comprising a
recombinant polypeptide cytotoxin; which recombinant polypeptide cytotoxin
comprises a cytokine or cytokine subunit fused to a polypeptide cytotoxin, in
combination with a pharmacologically acceptable carrier. Preferably, the
cytokine will be GMCSF. It is further preferred that the polypeptide cytotoxin
be a diptheria toxin subunit that does not contain the native binding domain.
As used herein, the phrase "target cancer" refers to diseases associated with the
proliferation of mammalian cells expressing the receptor recognized by
GMCSF, i.e., GMCSF-R. Such target cancers include, but are not limited to,
acute or chronic myelogenous leukemia, mixed lineage leukemia, breast cancer,
colon cancer, prostate cancer, lung cancer and T-cell leukemia.
**Brief Description of the Figures**

Figure 1(A) is a photograph depicting the structure of the recombinant DT<sub>ct</sub>-GMCSF fusion toxin and comparison to diphtheria toxin.

Figure 1(B) is a depiction of the recombinant growth factor - toxin fusion expression vector pET11d-DT<sub>ct</sub>-GMCSF.

Figure 1(C) depicts the purification of recombinant DT<sub>ct</sub>-GMCSF. The expression and sequential anti-DT immunoaffinity and mono-Q HPLC purification of DT<sub>ct</sub>-GMCSF from IPTG-induced 1 liter cultures of *E. coli* were analyzed by SDS-PAGE, and anti-DT and anti-GMCSF immunoblots.

Figure 2(A) depicts the results of experiments conducted to evaluate specific DT<sub>ct</sub>-GMCSF protein synthesis inhibition and cytotoxicity to leukemia cells mediated through GMCSF-R binding. Protein synthesis inhibition (PSI) (filled circles) and cytotoxicity (MTT assay) (open triangles) to GMCSF-R bearing cell human leukemia line TF-1, and cytotoxicity against GMCSF-R bearing human leukemia cell lines HL-60 (filled squares), THP-1 (open circles), and MV4-11 (inverted open triangles). Controls included GMCSF-R negative human erythroleukemia cell line K562 (open squares) and murine leukemia cell line NFS60 (filled triangles).

Figure 2(B) illustrates the blocking effects of excess native recombinant GMCSF on the cytotoxicity of DT<sub>ct</sub>-GMCSF against HL60 cells.

Figure 3(A) depicts the cytotoxicity of DT<sub>ct</sub>-GMCSF to HL60 and multidrug-resistant HL60/VCR and HL60/ADR cell lines, expressing P-glycoprotein and mpr, respectively.

Figure 3(B) illustrates the resistance of HL60/VCR and HL60/ADR cells to daunorubicin.

Figure 3(C) illustrates the resistance of HL60/VCR and HL60/ADR cells to vincristine.

Figure 4 is a depiction of the morphological features of DTct-GMCSF treated leukemia cells undergoing apoptosis. Specifically, figure 4(A) depicts untreated control cells. Figure 4(B) illustrates that apoptosis is not induced in GMCSF-R positive leukemia cells by treatment with native recombinant
GMCSF. Figures 4(C) and 4(D) depict the morphologic signs of extensive apoptotic damage, including pronounced shrinkage, nuclear chromatin condensation, segmentation of the nucleus, and plasma membrane blebbing within 4 hours of exposure to the fusion toxin. Figures (E) and (F) show the damage caused by treatment with DT<sub>ct</sub>-GMCSF after 24 hours.

Figure 5(A) illustrates that DNA from DT<sub>ct</sub>-GMCSF treated HL60 cells show a ladder-like fragmentation pattern and a dose-dependent decrease in the amount of intact DNA, consistent with apoptosis.

Figure 5(B) illustrates that native recombinant GMCSF did not cause DNA fragmentation indicative of apoptosis.

Figure 5(C) shows that DT<sub>ct</sub>-GMCSF did not cause DNA fragmentation in GMCSF-R negative NALM-6 B-lineage lymphoid leukemia cells.

Figure 5(D) depicts that DT<sub>ct</sub>-GMCSF effected apoptotic destruction of radiation-resistant RS4;11 cells in a GMCSF-R specific fashion.

Figure 6(A) is illustrative of the effects of DT<sub>ct</sub>-GMCSF on the in vitro clonogenic growth of normal bone marrow progenitor cells vs. myeloid leukemia cells.

Figure 6(B) is a depiction of the morphological features of CFU-GEMM mixed lineage cells in cultures of DT<sub>ct</sub>-GMCSF treated bone marrow cells.

Figure 7 shows that DT<sub>ct</sub>-GMCSF induces apoptosis in primary leukemia cells from therapy-refractory AML patients. Specifically, figure 7(A) depicts the analysis of Cases 1, 2, and 3. Figure 7(B) depicts the analysis of Case numbers 4, 5 and 6.

Figure 8 is a depiction of leukemic progenitor cell-derived AML blast colony formation in in vitro cultures of GMCSF (1000 ng/mL)-treated (Figure 8(A)) vs. DT<sub>ct</sub>-GMCSF (1000 ng/mL)-treated (Figure 8(B)) primary leukemic cells from a therapy-refractory AML patient.

Figure 9 illustrates the tumor free survival of sublethally irradiated SCID mice injected with 5 x 10<sup>6</sup> HL60 cells intravenously and subsequently treated
with either PBS (N=19) or DT\textsubscript{ct}-GMCSF (5 mcg/mouse, N=5; 10 mcg/mouse, N=5).

Figure 10 illustrates the effects of DT\textsubscript{ct}-GMCSF treatment on complete blood count of a cynomologus monkey. This monkey received daily intravenous injections of 50 mcg/kg DT\textsubscript{ct}-GMCSF for a total of 5 consecutive days.

Detailed Description of the Invention

Recombinant Polypeptide Cytotoxins

Recombinant polypeptide cytotoxins are hybrid cytotoxic proteins made by recombinant DNA technology that are designed to selectively kill cancer cells. Recombinant polypeptide cytotoxins are synthesized by the fusion of a cell-targeting moiety that binds to a receptor on a mammalian to a polypeptide cytotoxin. The toxic moiety is a portion of a bacterial or plant toxin. The activity of these recombinant polypeptide cytotoxins depends not only on the toxin utilized, but also on efficient binding of antibody to antigen, endocytosis, and intracellular release of functional ribosome inactivating proteins. Since it is established that many cancer cells overproduce cytokine receptors, the targets for this type of therapy can be growth factor receptors, differentiation antigens, or other less characterized cell surface antigens. Thus, effective cell-targeting moieties include, but are not limited to, cytokines, cytokine subunits, antibodies or antibody subunits.

A. Cell Targeting Moieties and their Targets

1. Cytokines

   a. Granulocyte-Macrophage Colony-Stimulating Factor (GMCSF)

   Native GMCSF is a glycosylated protein with an amino acid length of 127 residues and a molecular weight of 14-28 kd. The gene that encodes GMCSF in humans is found on the long arm of chromosome 5, linked in tandem to the IL-3 gene, and mapping closely to the genes for other hematopoietic cytokines (including IL-4 and IL-5) and their receptors. Cannistern et al., J. Biol. Chem., 265, 12656 (1990); Van Leeuwen et al., Blood, 73,
1142 (1989). The native and recombinant forms of GMCSF stimulate the proliferation, differentiation and function of myeloid lineage progenitor cells, and enhance the functional activation of granulocytes, monocytes, and macrophages. Gasson, Blood, 77, 1131 (1991); Seif et al., Science, 230, 872 (1985). Furthermore, a defective regulation of expression of the genes for individual cytokines or their receptors (e.g., GMCSF and GMCSF-R), which causes a pathological autocrine or paracrine stimulation of leukemic cell growth, has been implicated in the leukemogenesis of AML. Rogers et al., Exp. Hematol., 22, 593 (1994). It has been reported that leukemic cells from approximately two-thirds of patients with AML show an autonomous growth pattern because of autocrine GMCSF production and secretion. Rogers et al., cited supra; Young and Griffin, Blood, 68, 1178 (1986).

The biological effects of GMCSF are species-specific and mediated through the activation of a specific receptor. The heterodimeric high affinity GMCSF-R is composed of an α-chain specific for GMCSF and a β-chain that can also associate with the interleukin 3 and interleukin 5 receptor α-chains. Kastelein et al., Oncogene, 8, 231 (1993). Reflecting a common molecular theme among many cytokine receptors, the functional high affinity GMCSF receptor shares a common β subunit with the IL-3 and IL-5 receptors, and explaining the partial overlap in biological effects of GMCSF and IL-3. The high affinity GMCSF-R is expressed at high levels on myeloid leukemia cells and may provide an appropriate target for biotherapy of AML, since it is not expressed on the surface of pluripotent lymphohematopoietic stem cell populations. Higashigawa et al., Leuk. Res., 16, 1049 (1992).

b. Interleukin 3 (IL-3)

IL-3, also known as multi-colony-stimulating factor (multi-CSF), is a glycosylated protein with an amino acid length of 133 amino acid residues and native molecular weight of 15 to 30 kDa in humans. Yang et al., Cell, 47, 3 (1986); Lee et al., Nature, 289, 407 (1981). Its gene is located on the 5q23-13 region on long arm of chromosome 5, in relatively close proximity to the GMCSF gene, and linked to other cytokine genes including IL-4 and IL-5.
Human IL-3 is active in primates, however, it demonstrates species specificity in that it is not active in rodents. The biological effects of IL-3 in humans are mediated through a high affinity receptor that is composed of a β chain that is shared with the GMCSF and IL-5 receptors, and an α chain that is apparently specific for IL-3. Although the α chain alone may bind IL-3 with relatively low affinity, the β chain alone does not bind the cytokine, and both the α and β chain together are required for high affinity binding and signal transduction. IL-3 stimulates proliferation in progenitors at a somewhat earlier stage than GMCSF, but also does not stimulate the pluripotent primitive hematopoietic stem cell. IL-3 also stimulates colony formation and differentiation in committed progenitors in granulocytic, macrophage, mast cell, megakaryocytic and erythroid lineages.

2. Antibodies

Monoclonal antibodies (MoAbs) are produced by the fusion of spleen lymphocytes with malignant cells (myelomas) of bone marrow primary tumors. Milstein, Sci. Am., 243, 66 (1980). The procedure yields a hybrid cell line, arising from a single fused cell hybrid, or clone, which possesses characteristics of both the lymphocytes and myeloma cell lines. Like the lymphocytes (taken from animals primed with sheep red blood cells as antigens), the fused hybrids or hybridomas secrete antibodies (immunoglobulins) reactive with the antigen. Moreover, like the myeloma cell lines, the hybrid cell lines are immortal. Specifically, whereas antisera derived from vaccinated animals are variable mixtures of antibodies which cannot be identically reproduced, the single-type of immunoglobulin secreted by a hybridoma is specific to one and only one determinant on the antigen, a complex molecule having a multiplicity of antigenic molecular substructures, or determinants (epitopes). Hence, monoclonal antibodies raised against a single antigen may be distinct from each other depending on the determinant that induced their formation. However, all of the antibodies produced by a given clone are identical. Furthermore, hybridoma cell lines can be reproduced indefinitely, are easily propagated in
vitro and in vivo, and yield monoclonal antibodies in extremely high concentration.

a. B43

B43 is a murine IgG1, κ monoclonal antibody (MoAb) recognizing a 95 kDa target B lineage restricted phosphoglycoprotein, which is identified as the CD19 antigen according to the World Health Organization (WHO) established CD (cluster of differentiation) nomenclature. The chemical, immunological and biological features of B43 MoAb have been described in detail in previously published reports. Uckun et al., Blood, 71, 13 (1988).

CD19 antigen is a B-lineage specific surface receptor which is expressed on malignant cells from 85% of patients with acute lymphoblastic leukemia (ALL). Uckun et al., Blood, 71, 13 (1988). CD19 is found on the surface of each B-lineage lymphoma cell and B-lineage cell at a high density (> 1,000,000 molecules/cell and > 50,000 molecules/cell, respectively) but is absent from the parenchymal cells of life-maintaining nonhematopoietic organs, as well as from blood related myeloid and erythroid cells, T-cells and bone marrow stem cells, reducing the opportunity for nonspecific toxicity when anti-CD19 antibodies are used in biotherapy. Uckun et al., J. Exp. Med., 163, 347 (1986). This B-lineage specific antigen shows a high affinity for the B43 (anti-CD19) monoclonal antibody (Ka > 10^8 M^-1), undergoes antibody induced internalization upon binding of B43 and is not shed from the cell surface. Uckun et al., J. Exp. Med., 163, 347 (1986). CD19+ acute lymphoblastic leukemias are believed to originate from putative developmental lesions in normal B-cell precursor clones during early phases of ontogeny and are therefore classified as B-lineage leukemia F.M. Uckun, Blood, 76, 1908 (1990).

B. Polypeptide Cytotoxins

Delivery of a highly potent cytotoxic molecule to leukemia and lymphoma cells while sparing normal cells is a major goal for the development of new anticancer therapies. To be optimally effective, such an approach requires that internalization of relatively small numbers of toxin molecules be lethal to target cells, as there are limited receptor sites on the cell surface. The
polypeptide cytotoxins produced by certain bacteria and plants that inactivate cellular protein synthesis meet this criteria as, unlike most chemotherapeutic agents which act in a stoichiometric manner, they are catalytic in their lethal activity.

Two classes of polypeptide cytotoxins that inactivate protein synthesis have been widely employed in the construction of recombinant polypeptide cytotoxins and related immunotoxins. Diphtheria toxin (DT) and *Pseudomonas aeruginosa* exotoxin A represent one class of these toxins, and kill cells by catalyzing the ADP-ribosylation and inactivation of elongation factor 2, an essential cofactor in protein synthesis.

Lethally inhibiting protein synthesis in a complementary manner, members of the other class of toxins covalently modify the ribosome such that it can no longer productively interact with elongation factor 2. This latter family of toxins includes pokeweed antiviral protein (PAP), ricin, abrin, gelonin, saporin, and alpha-sarcin. The ribosome inactivating proteins derived from plants consist of either two chains, including a binding chain and catalytic chain (e.g. ricin), or a single catalytic chain alone (e.g. PAP or saporin).

1. **PAP**

There are three subtypes of pokeweed antiviral protein (PAP) the expression of which are dependent upon the season. PAP is found in spring leaves, PAP II is found in late summer leaves, and PAP-S is found in seeds. Irvin, *Pharmacol. Ther.*, 21, 371 (1983). Small differences exist in their sizes (all are approximately 29,000 MW) and there are only small differences, if any, between their ability to inhibit ribosomes catalytically. Houston et al., "Immunotoxins made with Toxins and Hemitoxins other than Ricin", in *Immunological Antibody Conjugates in Radioimaging and Therapy of Cancer*, C.W. Vogel, ed., New York, Oxford University Press, P. 71 (1987).

PAP is a member of the hemitoxin group of toxins and thus inactivates ribosomes by the specific removal of a single adenine from the conserved loop sequence found near the 3' terminus of all larger rRNAs. Irvin et al., *Pharmacology and Therapeutics*, 55, 279, (1992). This specific depurination
greatly reduces the capability of elongation factors to interact with ribosomes and results in an irreversible shut-down of protein synthesis. Irvin et al., cited supra. Furthermore, PAP is one of the most active ribosomal inactivating proteins. In a comparison of cytotoxicity of anti-mouse IgG immunotoxins gelonin, ricin A chain, momordin, dianthin 32, saporin, and PAP, the PAP constructs were among the most potent immunotoxins tested. Irvin et al., cited supra. Bolognesi et al., “A comparison of anti-lymphocyte immunotoxins containing different ribosome-inactivating proteins and antibodies”, Clin. Exp. Immunol., 89, 341 (1992).

2. Diphtheria toxin

Diphtheria toxin (DT) is one of the most toxic substances found in nature, and human cells are indiscriminantly killed by a single molecule of the toxin entering the cytoplasm. Yamaizumi et al., Cell, 15, 245 (1978); Perentesis et al., Biofactors, 3, 173 (1992). The toxin is a 535 amino acid, single chain polypeptide with a deduced molecular weight of 58,342 daltons and an apparent molecular weight on SDS-polyacrylamide gels of 62,000 - 63,000 daltons. DT kills cells by catalyzing ADP-ribosylation and consequent inactivation of elongation factor 2 (EF-2), an essential cofactor in protein synthesis, at a unique post-translationally modified histidine residue, diphthamide. Id. DT-modified EF-2 is unable to interact with ribosomes, leading to an irreversible inhibition of protein synthesis and cell death. Id. Molecular genetic, biochemical, and X-ray crystallographic analyses have revealed that DT possesses functionally distinct structural domains corresponding to (a) an ADP-ribosyltransferase catalytic activity, (b) a membrane translocation activity, and (c) a unique receptor binding moiety. Gill et al., J. Biol. Chem., 246, 1492 (1971).

The other known enzyme that kills eukaryotic cells by inactivating protein synthesis is Pseudomonas aeruginosa exotoxin A. Functionally, it is very similar to diphtheria, since it has an enzymatic domain and a binding/membrane translocation domain. However, diphtheria toxin and Pseudomonas exotoxin A show little immunological cross-reactivity and their
DNA and amino acid sequences are not homologous. Sadoff et al., Infect. Immun., 37, 250 (1982).

C. Production and Purification of Recombinant Polypeptide Cytotoxins

Recent refinement of the DT crystallographic structure has revealed that amino acid residues 380-386, located in a small loop separating the carboxyl terminal receptor binding domain (domain r) from the rest of the toxin (domain ct), allow the entire 15 kDa binding domain to flexibly rotate as a unit by 180°, with atomic movement of up to 65 Å. Bennett et al., Proc. Natl. Acad. Sci. USA, 91, 3127 (1994). Rotation of the binding domain permits DT dimerization through noncovalent interactions of the binding domain of one DT molecule with the catalytic and translocation domains of a second DT molecule, and vice versa. Important for genetic engineering considerations, the native binding domain of DT bears rudimentary homology to folds of immunoglobulin molecule binding domains.

The exploitation of the identification of this distinct flexible molecular hinge separating the native binding domain of DT (DT_{r}) from its catalytic and translocation domains (DT_{ct}) has been used in the present invention to design and produce novel recombinant polypeptide cytotoxins, directed against high affinity receptors which are overexpressed on such cancer cells as myeloid leukemia and acute lymphoblastic leukemia. In the construction of this recombinant polypeptide cytotoxin, the portion of the DT gene encoding amino acid residues 1-385 including both the lethal catalytic ADP-riboosyltransferase and the transmembrane passage domains was preserved. This portion of the DT gene (i.e., DT_{ct}) was fused either to the gene that encodes the mature form of a cytokine suitable for use in the present invention or a novel genetically engineered B43 antibody Fv fragment at the site of the flexible molecular hinge, effectively deleting the native DT binding domain (DT_{r}). In addition, a synthetic DNA sequence encoding a short Ser-(Gly)_{4}-Ser-Met intervening linker was inserted at the hinge site separating the DT and cytokine or B43 scFv moieties to insure that the binding domains would be available for participation in high affinity receptor binding (DT_{ct}-GMCSF - Figure 1(A)). This rational
drug design of recombinant polypeptide cytotoxins was intended to preserve essential structure-function relationships identified in crystallographic analyses of both the DT and cytokine or antibody molecules. Rambaldi et al., *Blood*, 81, 1376 (1993).

The pET11d expression vector (Novagen, Inc.; 597 Science Drive, Madison, WI 53711) employed for the production of recombinant polypeptide cytotoxins in *E. coli* contains a hybrid bacteriophage T7 promoter with a 3′ lac operator sequence fusion and an internal copy of lacI to suppress basal expression, an efficient Shine-Dalgarno sequence for translational efficiency, and an NcoI cloning site for the insertion of recombinant scFv, dsFv, and toxin gene fusions. See Figure 1(B) for a depiction of pET11d-DT<sub>T</sub>CT-GMCSF. The gene encoding the bacteriophage T7 polymerase gene is incorporated by lysogeny into the genome of the *E. coli* expression host, HMS174(de3)plysS, and is under the control of the lac UV5 promoter. The pLysS gene in HMS174(de3)plysS produces a low amount of the T7 lysozyme, a natural inhibitor of T7 RNA polymerase, to provide additional stringency of gene expression regulation. Expression of the recombinant polypeptide cytotoxins from within pET11d expression vectors is induced by the addition of isopropylthiogalactoside (IPTG) to the media containing the *E. coli* expression host.

The recombinant polypeptide cytotoxins are individually expressed in HMS174(de3)plysS and the soluble product is recovered from cells disrupted by freeze-thaw cycles and sonication. The soluble fraction containing the recombinant polypeptide cytotoxin is subsequently purified through sequential filtration, anti-diphtheria toxin immunoaffinity chromatography, filtration and dialysis, anion exchange high performance liquid chromatography, additional filtration endotoxin removal resins, and final filtration and dialysis. Insoluble product can be rendered to a soluble form for purification by dissolution in 7M guanidine HCl with a slow renaturation under controlled conditions to a physiological buffer such as phosphate buffered saline.
D. Apoptosis

Apoptosis, also known as programmed cell death, is a common mode of eukaryotic cell death, with distinct ultrastructural features and a ladder-like DNA fragmentation pattern due to endonuclease-mediated cleavage of DNA into oligonucleosome-length fragment. Uckun et al., Proc. Natl. Acad. Sci. USA, 89, 9005 (1992). Ionizing radiation, as well as several chemotherapeutic drugs with diverse molecular targets, such as prednisone, cisplatin, methotrexate, L-asparaginase, etoposide, 5-fluorouracil, cyclophosphamide have been reported to induce apoptotic cell death in human cells. Id. Failure of normal apoptosis pathways or resistance to chemotherapy-induced apoptosis may be important mechanisms in leukemogenesis and the biology of high risk chemotherapy refractory disease, respectively.

E. Modes of Administration of the Recombinant Polypeptide Cytotoxins

The recombinant polypeptide cytotoxins of the present invention can be formulated as pharmaceutical compositions and administered to a mammalian host, such as a human patient, in a variety of forms adapted to the chosen parenteral route of administration, i.e., by intravenous, intramuscular or subcutaneous routes.

1. Dosage Forms

It is preferred that the recombinant polypeptide cytotoxins of the present invention be parenterally administered, i.e., intravenously, or subcutaneously by infusion or injection. Solutions or suspensions of the recombinant polypeptide cytotoxins can be prepared in water, or isotonic saline, such as PBS, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, DMA, vegetable oils, triacetin, and mixtures thereof. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms. Additionally, more specific delivery of the recombinant polypeptide cytotoxins to the lungs may be accomplished via aerosol delivery systems. The pharmaceutical dosage form suitable for aerosol delivery can include adipot formulations such as a liposome of suitable size.
The pharmaceutical dosage form suitable for injection or infusion use can include sterile aqueous solutions or dispersions or sterile powders comprising the recombinant polypeptide cytotoxin which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions. In all cases, the ultimate dosage form must be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, lipids (for example, dimyristoyl phosphatidyl choline) and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersion or by the use of nontoxic surfactants. The prevention of the action of microorganisms can be accomplished by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be desirable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the inclusion in the compositions of agents delaying absorption, for example, aluminum monostearate hydrogels and gelatin.

Sterile injectable or infusible solutions are prepared by incorporating the recombinant polypeptide cytotoxin in the required amount in the appropriate solvent with various of the other ingredients enumerated above, and as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable or infusible solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

2. **Dosages**

The dosage of the recombinant polypeptide cytotoxins in said composition can be varied widely, in accord with the size, age and condition of
the patient and the target cancer. Based on animal data, it is expected that the dosage can be varied between 0.025 mg/kg/day and 1 mg/kg/day, administered over a period of about 3 to 5 days.

The invention will be further described by reference to the following detailed examples.

**Example 1. Construction and Purification of the Recombinant Polypeptide Cytotoxins**

**A. DT$_{ct}$-GMCSF**

The recombinant polypeptide cytotoxin expression vector pET11d-DT$_{ct}$-GMCSF was constructed in three major steps as depicted in Figure 1. An 392 bp NcoI - BamHI DNA fragment containing the coding sequence of mature hGMCSF was cloned between the Nco I and Bam HI sites of plasmid pET11d downstream of the T7 promoter to produce pET11d-GMCSF. Polymerase chain reaction (PCR) mutagenesis of the diphtheria toxin gene was employed to obtain an Ncol gene cassette that encoded 385 amino terminal residues of diphtheria toxin including the entire fragment A ADP-ribosyltransferase catalytic domain and the contiguous proximal portion of fragment B that is associated with toxin translocation across cellular membranes. PCR was employed for mutagenesis of the diphtheria toxin gene to delete the coding region for the native toxin binding domain, and provide coding sequences for a translation initiation ATG codon, a seven residue linker segment for fusion with the GMCSF gene, and convenient flanking NcoI restriction enzyme sites for cloning. PCR mutagenesis primers included a 5’ primer:

$(5’$-GCCATGGGCGCTGATGATTTGTTGATTC-3’$)$ introducing an Ncol restriction enzyme site and ATG codon, and a 3’ primer $(5’$-GCCATGGAGCCACCTCCACCCGATTTATGCCCGGAGAATACGC-3’$)$ incorporating sequences encoding a linker domain for steric spacing of the GMCSF gene and an Ncol restriction enzyme site. Expression plasmid pET11d-DT$_{ct}$-GMCSF was constructed by the cloning of the intact DT Nco I gene cassette into the Nco I site of pET11d-GMCSF (Figure 1(B)). Cloning strategies
and other genetic manipulations were positioned to assure maintenance of the translational reading frame, and fidelity of PCR amplification and genetic constructions were confirmed by DNA sequencing. Oligonucleotide primers were synthesized with an Applied Biosystems 394 DNA synthesizer at the University of Minnesota Microchemical Facility. A synthetic cDNA encoding human GMCSF using *E. coli* codon preferences was obtained from R & D Systems (Minneapolis, MN).

The plasmid containing the diphtheria toxin gene has been described previously. [J. Perentesis et al., *Proc. Natl. Acad. Sci. USA*, 85, 8386 (1988)]

Plasmid DNAs were prepared by either the alkaline lysis method with purification on cesium chloride/ethidium bromide gradients, or by use of the Wizard DNA purification resin (Promega, Madison, WI). DNA fragments amplified by PCR were initially cloned into the pT7Blue vector as directed by the manufacturer (Novagen), with DNA sequencing confirmation by the dideoxy method of Sanger using CircumVent thermal cycling reagents (New England Biolabs, Beverly, MA). Restriction endonucleases, Taq DNA polymerase, and T4 DNA ligase were procured from BRL-Life Technologies (Gaithersburg, MD), Promega, New England Biolabs, or Perkin Elmer (Norwalk, CT), and used according to the specifications directed by the manufacturer.


SDS-polyacrylamide gel analysis of the uninduced and induced whole cell extract, immunoaffinity column eluate, and Mono Q chromatography column fractions revealed production and >95% purity of a monomeric protein with a molecular mass of about 58 kDa, the expected molecular mass of DT<sub>CT</sub>-GMCSF as deduced from its nucleic acid sequence (Figure 1(C)). The integrity of expression of both the diphtheria toxin and GMCSF moieties of
DT_{ct}-GMCSF was confirmed in immunoblot analysis employing antisera to diphtheria toxin or GMCSF (Figure 1(C)). In vitro analysis of the catalytic activity of DT_{ct}-GMCSF (see Example 3) demonstrated the characteristic lethal enzymatic activity of diphtheria toxin, the ability to catalyze the ADP-ribosylation of translation factor EF-2.

B. DT_{ct}-B43

Gene cassettes encoding a B43 scFv with either a VLVH or a VHVL orientation were constructed, since one cannot predict a priori which orientation would recreate the native B43 binding site with the greatest affinity. In addition, each of the VLVH or VHVL B43 scFv gene cassettes was constructed in two versions, with two alternative linkers to connect the VL and VH cDNAs. The use of these two alternate linkers also permits identification of the B43 scFv versions that recreate the greatest affinity recombinant B43 binding when the scFv is fused with diphtheria toxin or other molecules.

The anti-human CD19 monoclonal antibody B43 (murine IgG, kappa) has been previously described by Uckun et al., Blood, 71, 13 (1988). Random oligonucleotide priming of messenger RNA isolated from the B43 production hybridoma was used to produce cDNA with reverse transcriptase. Specific B43 VH and VL cDNA is produced by PCR using primers based on the B43 VH and VL sequences and incorporating novel additional restriction endonuclease sequences at the 5’ and 3’ termini of the antibody gene sequences. The primer sequences include:

PVH5’-1: 5’-GCC-ATG-GTT-CAG-CTG-CAG-CAG-TCT-GGG-GCT-GAG-C-3’

PVH3’-1: 5’-CCA-TGG-CTA-TGA-GGA-GAC-GGA-GAC-TGA-GGT-TCC-TTG-3’

PVL5’-1: 5’-GCC-ATG-GAC-ATT-GTG-CTG-ACC-CAA-TCT-CCA-GCT-TCC-3’

PVL3’-1: 5’-CCA-TGG-CTA-TTT-GAT-TTC-CAG-CTT-GGT-GCC-TCC-ACC-GAA-CG-3’
The restriction endonuclease cleavage sites that were incorporated into the individual B43 \( V_H \) and \( V_L \) cDNA fragments are underscored. B43 \( V_H \) and \( V_L \) cDNAs were individually cloned into standard TA vectors (Invitrogen Corp., San Diego CA) for the determination of DNA sequence by thermal cycle sequencing technology employing dideoxy chain termination methodology.

Construction of the B43 \( V_L V_H \) and \( V_H V_L \) scFv gene cassettes was accomplished by genetic fusion of the cDNA sequences for the respective \( V_L V_H \) and \( V_H V_L \) scFv gene segments with the introduction of novel linking sequences. For each B43 \( V_L V_H \) or \( V_H V_L \) scFv gene cassette, two different genetic fusion were constructed, each employing one of two different linkers to connect the \( V_L \) and \( V_H \) cDNAs. The unique version “A” linker encodes “Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Thr-Gly-Gly-Gly-Ser”, and the unique version “B” linker encodes “Gly-Ser-Thr-Ser-Gly-Ser-Gly-Lys-Ser-Ser-Glu-Gly-Lys-Gly”.

PCR mutagenesis was employed to produce unique \( V_L \) and \( V_H \) cDNAs that are thus individually modified to include the addition of unique sequences to encode linkers between the \( V_L \) and \( V_H \) cDNAs. Overlapping PCR was then employed to then produce B43 \( V_L V_H \) and \( V_H V_L \) scFv cassettes.

The detail of the primers for overlapping PCR is as follows:

\[
PVL5'-1LA: \; 5'-TCC-GGA-GGA-GGC-GGT-ACC-GGT-GGT-GGC-GGT-AGC-GAC-ATT-GTG-CTG-ACC-CAA-TC-3'
\]

\[
PVL5'-1LB: \; 5'-CT-GGT-TCC-GGA-AAA-TCT-TCT-GAA-GGT-AAA-GGT-GAC-ATT-GTG-CTG-ACC-CAA-TC-3'
\]

\[
\]

\[
PVH3'-1LB: \; 5'-C-AGA-AGA-TTT-TCC-GGA-ACC-AGA-GGT-AGA-ACC-TGA-GGA-GAC-GGA-GAC-TG-3'
\]
The predicted encoded sequence of the “A” and “B” versions of the linkers in the B43 VLVH and VHVL scFv cassettes is as follows:

**LINKER A:**

```
CCG-CCT-CCT-AGG-CCT-CCT-CCG-CCA-TGG-CCA-CCA-CCG-CCA-TCG
GLY-GLY-GLY-SER-GLY-GLY-GLY-GLY-GLY-THR-GLY-GLY-GLY-GLY-SER
```

**LINKER B:**

```
GGT-TCT-ACC-TCT-GGT-TCC-GGA-AAA-TCT-TCT-GAA-GGT-AAA-GGT
CCA-AGA-TGG-AGA-CCA-AGG-CCT-TTT-AGA-AGA-CTT-CCA-TTT-CCA
GLY-SER-THR-SER-GLY-SER-GLY-LYS-SER-SER-GLU-GLY-LYS-GLY
```

The B43 VLVHLA, VLHVHB, VHVLLA and VHVLBB scFv cassettes were each individually cloned into standard TA vectors for the determination of DNA sequence by thermal cycle sequencing technology employing dideoxy chain termination methodology.

The recombinant polypeptide cytotoxin expression vectors pET11d-DTct-B43 VLVHLA, VLHVHB, VHVLLA and VHVLBB were each constructed in three major steps. Briefly, an NcoI DNA fragment containing the coding sequence of each B43 scFv cassette is cloned into the Nco I site of plasmid pET11d downstream of the T7 promoter to produce pET11d-B43 VLVHLA, VLHVHB, VHVLLA or VHVLBB. PCR mutagenesis of the diphtheria toxin gene was employed to obtain a NcoI gene cassette that encoded 385 amino terminal residues of diphtheria toxin including the entire fragment A ADP-ribosyltransferase catalytic domain and the contiguous proximal portion of fragment B that is associated with toxin translocation across cellular membranes. PCR was employed for mutagenesis of the diphtheria toxin gene to delete the coding region for the native toxin binding domain, and to provide coding sequences for a translation initiation ATG codon, a seven residue linker segment for fusion with the B43 gene, and convenient flanking NcoI restriction sites for cloning into the B43 expression vectors.
enzyme sites for cloning. PCR mutagenesis primers included a 5' primer (5'-GGCATGGGCGCTGATGATGTGTTGATT-3') introducing an NcoI restriction enzyme site and ATG codon, and a 3' primer (5'GGATATTCTCCGGCGCATAAAATCGGTTGAGGTTGCGATCACG-3') incorporating sequences encoding a linker domain for steric spacing of the scFv cassette and an NcoI restriction enzyme site. This DT<sub>ct</sub> gene cassette thus included: (i) the addition of an ATG methionine translation initiation codon immediately 5' of the initial GGC glycine codon of mature diphtheria toxin, (ii) a short 3' linker sequence encoding seven amino acid [Ser-(Gly)4-Ser-Met] residues downstream of diphtheria toxin lysine residue 385, and (iii) flanking Nco I restriction endonuclease sites. Each DT<sub>ct</sub>-B43 VLVHLA, VLVHLB, VHVLAA and VHVLBB expression plasmid was constructed by the cloning of the intact DT Nco I gene cassette into the Nco I site of the respective pET11d-B43. Cloning strategies and other genetic manipulations were positioned to assure maintenance of the translational reading frame, and fidelity of PCR amplification and genetic constructions were confirmed by DNA sequencing. Oligonucleotide primers were synthesized with an Applied Biosystems 394 DNA synthesizer at the University of Minnesota Microchemical Facility.

**Example 2. Expression of Recombinant Polypeptide Cytotoxins in E. coli and Subsequent Purification**

**A. DT<sub>ct</sub>-GMCSF**

High efficiency expression of DT<sub>ct</sub>-GMCSF was achieved in *E. coli* followed by serial purification through anti-diphtheria toxin affinity chromatography, anion exchange chromatography and extensive dialysis. All manipulations of *E. coli* bearing intact recombinant fusion toxin were performed under Biosafety Level 3 (BL3) containment practices. *E. coli* HMS174(de3)plysS transformed with pET11dDT-GMCSF was grown at 37°C in LB medium with carbenicillin (50 μg/ml) to an absorbance (Å600) of 0.4-0.6. Expression of the fusion gene was induced by the addition of isopropyl-B-D-
thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The bacterial cells were collected by centrifugation after one hour of induction, and resuspended in 50 mM potassium phosphate, 10 mM EDTA, 750 mM NaCl, 0.1% Tween 20, pH 8.0. Lysis of the cells was achieved by freezing in a dry ice/ethanol bath followed by thawing and sonication. The soluble extract was filtered through a 0.2 μm filter (Millipore, Bedford, MA) and applied to a 4 ml anti-diphtheria toxin immunoaffinity column.

The immunoaffinity column was prepared with Affinica Antibody orientation Kit (Schleicher and Schuell, Keene, NH) using equine diphtheria antitoxin. Bound proteins were subsequently eluted from the immunoaffinity column with 4 M guanidine hydrochloride, 100 mM potassium phosphate, 0.1% Tween 20, pH 7.2. Eluate fractions containing the fusion toxin were dialyzed exhaustively in 20 mM Tris, 150 mM NaCl, pH 7.5 with 5 buffer changes over 18 hrs using 12kD exclusion dialysis tubing. The dialyzed fusion toxin was applied onto a Mono Q HR 5/5 chromatography column (Pharmacia, Piscataway, NJ) and eluted at a flow rate of 1 ml/min over 30 min with a linear gradient of NaCl (150-500mM in 20mM Tris, pH 7.5). The purified fusion toxin was concentrated 10-20 fold in a 30 kD exclusion filter (Amicon) followed by exhaustive dialysis in 2 L PBS with 5 buffer changes over 18 hrs. Protein concentration was determined by Bio-Rad Protein Assay. Over 100 μg of purified DT-GMCSF protein was consistently obtained from 1 liter pilot cultures.

B. DT<sub>ct</sub>-B43

*E. coli* HMS174(de3)plysS transformed with pET11dDT<sub>ct</sub>-B43 will be grown at 37°C in LB medium with carbenicillin (50 μg/ml) to an absorbance (A600) of 0.4-0.6. Expression of the fusion gene will be induced by the addition of isopropyl-B-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The bacterial cells will be collected by centrifugation after one hour of induction, and resuspended in 50 mM potassium phosphate, 10 mM EDTA, 750 mM NaCl, 0.1% Tween 20, pH 8.0. Lysis of the cells will be achieved by freezing in a dry ice/ethanol bath followed by thawing and sonication.
The soluble extract will be filtered through a 0.2 μm filter (Millipore, Bedford, MA) and applied to a 4 ml anti-diphtheria toxin immunoaffinity column. The immunoaffinity column will be prepared with Affinica Antibody orientation Kit (Schleicher and Schuell, Keene, NH) using equine diphtheria antitoxin. Bound proteins will be subsequently eluted from the immunoaffinity column with 4 M guanidine hydrochloride, 100 mM potassium phosphate, 0.1% Tween 20, pH 7.2. Eluate fractions containing the fusion toxin will be dialyzed in 20 mM Tris, 150 mM NaCl, pH 7.5 with 5 buffer changes over 18 hrs using 12kD exclusion dialysis tubing. The dialyzed fusion toxin will be applied onto a Mono Q HR 5/5 chromatography column (Pharmacia, Piscataway, NJ) and eluted at a flow rate of 1 ml/min over 30 min with a linear gradient of NaCl (150-500mM in 20mM Tris, pH 7.5). The purified fusion toxin will be concentrated 10-20 fold in a 30 kD exclusion filter (Amicon) followed by exhaustive dialysis in 2 L PBS with 5 buffer changes over 18 hrs. Protein concentration will be determined by Bio-Rad Protein Assay.

**Example 3. Biochemical Characterization of Recombinant Polypeptide**

**Cytotoxins**

For biochemical characterization of the recombinant polypeptide cytotoxins, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses using equine diphtheria antitoxin (anti-DT; Connaught) antibodies will be performed by standard methods using 10-15% gels in a Mini-Protean II gel apparatus (Bio-Rad). Diphtheria toxin and human GMCSF standards will be obtained from Connaught Laboratories and R & D Systems (Minneapolis, MN), respectively. Primary antibodies will be used at a dilution of 1:5000. Secondary antibodies, goat anti-horse (Bethyl laboratories) and rabbit anti-goat (Calbiochem), covalently linked to horseradish peroxidase, will be used at a 1:10,000 dilution.

The ADP-ribosyltransferase catalytic activity of the recombinant polypeptide cytotoxins will be determined by measuring incorporation into purified *Saccharomyces cerevisiae* EF-2 or into the EF-2 of reticulocyte lysates.
Approximately 300 ng of recombinant polypeptide cytotoxin or 200 ng of nicked diphtheria toxin will be incubated at 37°C with 10 μg of EF-2 in a reaction mixture containing 20 mM Tris-HCl (pH 7.5), 50 mM dithiothreitol, 1 mM EDTA, and 2.5 μM [adenylate-32P]NAD+ (=1 x 10^6 cpm) and analyzed for ADP-ribose incorporation as described by Chen et al., *Mol. Cell Biol.*, 5, 3357 (1985).

**Example 4. Cytotoxic Activity of Recombinant Polypeptide Cytotoxins against Human Leukemia Cells**

The GMCSF-R specific cytotoxic activity of DTc- GMCSF against myeloid leukemia cells was examined in protein synthesis inhibition assays, a tetrazolium bromide (MTT) assay of cell viability, and clonogenic assays.

**A. Myeloid leukemia cells**

TF1, a GMCSF-dependent human early myeloid/erythroleukemia cell line, was provided by Dr. J. Winkleman (University of Cincinnati, Cincinnati, OH). Other GMCSF-R bearing human leukemia cell lines included HL60, a human acute promyelocytic leukemia cell line, THP-1, a human monocytic leukemia cell line, and MV4-11, a mixed lineage leukemia cell line. These cell lines, as well as the GMCSF-R negative control cell line K562, were obtained from the American Type Culture Collection (Rockville, MD). The murine leukemia cell line NFS-60 was the gift of Dr. J. Ihle (St. Jude Children’s Research Hospital, Memphis, TN). TF-1 cells were maintained in RPMI, 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 5 ng/ml human GMCSF. NFS-60 cells were maintained in RPMI, 10% FBS, 50 u/ml penicillin, 50 μg/ml streptomycin, and 0.5 ng/ml human GMCSF. HL-60 and MV4-11 cells were maintained in IMDM, 10% FBS and 50 U/ml penicillin, and 50 μg/ml streptomycin. THP-1 and K562 cells were maintained in RPMI, 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 5x10^-5 M 2-mercaptoethanol (for THP-1 cells only). RS4;11 is a highly radiation-resistant mixed lineage acute leukemia cell line expressing high levels of Bcl-2 protein and NALM-6 is a pre-B leukemia cell line. Primary leukemic cells were obtained from the previously
cryopreserved AML bone marrow samples of therapy-refractory patients stored in the liquid nitrogen tanks of the Children's Cancer Group Cell Bank at the University of Minnesota.

B. Protein synthesis inhibition assays

For protein synthesis inhibition assays, 1 x 10^5 cells per well were seeded into 96 well V-bottom plates containing leucine-free Roswell Park Memorial Institute (RPMI) medium prior to the addition of DT or DT_{ct}-GMCSF and incubated at 37°C for 48 hours. MEM +H-leucine (L-[4,5,3H]) (DuPont-NEN) was added to a final concentration of 1 μCi/well with a four hour pulse incubation. Cells were lysed with 4M KOH and the insoluble protein was precipitated with 40% trichloroacetic acid. A cell harvester (PHD., Cambridge Technology Inc.) was used to collect the insoluble protein on glass fiber filters, and radioisotope incorporation into protein was measured in a Beckman LS7000 scintillation counter.

C. Tetrazolium bromide (MTT) assay of cell viability

For MTT assays, cells were seeded into 96-well plates at a final concentration of 5 x 10^3 cells/well for TF-1, and 5-9 x 10^4 cells/well for HL-60, K562, NFS-60, MV4-11 and THP-1, and incubated at 37°C in a humidified incubator under a 5% CO_2 atmosphere for 16-24 hours. DT or the DT_{ct}-GMCSF fusion toxin was added to each well and the incubation was continued for an additional 72-96 hours. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to a final concentration of 0.5 mg/ml with 4 hours of further incubation at 37°C. The dye was solubilized with 50% Isobutanol/10% SDS and cell viability was determined by measure of absorption (A595) using a Bio-Rad Elisa Reader. The MTT assay has been found to be "more reproducible and reliable than ^3H-thymidine incorporation or cell enumeration, although equivalent results are obtained". Kanakura et al., Blood, 76, 706 (1990).
D. Clonogenic assays

A serial dilution clonogenic assay system was used to evaluate the anti-leukemic efficacy of DT<sub>CT</sub>-GMCSF against human leukemia cell lines, as previously described by Uckun et al., *J. Immunol.*, 134, 2010 (1985). The sensitivity of primary AML cells to DT<sub>CT</sub>-GMCSF was examined in a leukemic progenitor cell assay system as described by Uckun et al., *J. Exp. Med.*, 163, 347 (1986). Colony assays of normal bone marrow progenitor cells CFU-GEMM, CFU-GM, and BFU-E were conducted as previously described by Uckun et al., *J. Immunol.*, 134, 3504 (1985).

E. Results

DT<sub>CT</sub>-GMCSF effectively inhibited protein synthesis in the GMCSF-dependent human myeloid leukemia cell line TF-1 with an IC<sub>50</sub> of approximately 1 ng/ml (=17 pM). DT<sub>CT</sub>-GMCSF was cytotoxic to TF-1 cells in an MTT cell viability assay with a similar IC<sub>50</sub> value (Figure 2(A)). In addition, DT<sub>CT</sub>-GMCSF was selectively cytotoxic to the high affinity GMCSF-R bearing human myeloid leukemia cell lines HL60 and THP-1, as well as the mixed lineage leukemia cell line MV4-11 with IC<sub>50</sub> values ranging from 1 ng/ml (= 17 pM) (HL60) to 40 ng/ml (= 680 pM) (THP-1 and MV4-11) (Figure 2(A)). In contrast to DT<sub>CT</sub>-GMCSF, a control fusion toxin, DT<sub>CT</sub>-interleukin 3 (DT<sub>CT</sub>-IL3), did not kill GMCSF-R positive HL60 cells which do not display the appropriate receptor for IL-3 (Figure 2(B)), indicating that the DT domain did not possess nonspecific toxicity. Human erythroleukemia cell line K562 and murine leukemia cell line NFS60, neither of which express the high affinity GMCSF-R, were not killed by DT<sub>CT</sub>-GMCSF even at 1000 ng/mL concentration (= 17 nM), supporting the notion that the cytotoxicity of DT<sub>CT</sub>-GMCSF was mediated through specific binding to the high affinity GMCSF-R. Further, the addition of excess native GMCSF abolished the cytotoxicity of DT<sub>CT</sub>-GMCSF to myeloid leukemia cells, presumably by competition for binding to the GMCSF-R (Figure 2(B)). Control studies indicated that GMCSF alone did not stimulate the
proliferation or differentiation of HL60 cells in the DT<sub>ct</sub>-GMCSF treatment groups. Taken together, these results provide evidence that recombinant DT<sub>ct</sub>-GMCSF is selectively cytotoxic to GMCSF-R bearing human leukemia cells and that cytotoxicity depends on high affinity GMCSF-R binding and internalization.

**Example 5. Cytotoxic Activity of Recombinant Polypeptide Cytotoxins against Human Leukemia Cells Possessing the MDR Phenotype**

The main cause of the failure of contemporary treatment regimens for AML is the emergence of clinical multidrug resistance (MDR) and/or radiation resistance. Sato et al., *Brit. J. Hematol.*, 75, 340 (1990). MDR has been associated with increased expression of a 170 kDa membrane glycoprotein (P-glycoprotein), as well as a recently described 190 kDa membrane glycoprotein (mrp, multidrug-resistance associated protein), that are both members of a superfamily of ATP-dependent transport and drug efflux proteins. Higgins et al., *Annu. Rev. Cell Biol.*, 8, 67 (1992). Overexpression of these proteins results in decreased intracellular accumulation of a wide range of unrelated lipophilic anticancer drugs in cancer cells, and in the case of P-glycoprotein, has been associated with treatment failures of AML patients. Arecci, *Blood*, 81, 2215 (1993). In this experiment, the ability of DT<sub>ct</sub>-GMCSF to kill multidrug resistant subclones of HL60 cells, including HL60/VCR cells which express a P-glycoprotein associated MDR phenotype and HL60/ADR cells which express a mrp-associated MDR phenotype, was examined.

In assays of cellular viability, DT<sub>ct</sub>-GMCSF was cytotoxic to both HL60/VCR and HL60/ADR cells (Figure 3A). Consistent with previous reports, both HL60/VCR and HL60/ADR cell lines exhibited significant resistance to cytotoxicity by daunorubicin (Figure 3B) or vincristine (Figure 3C). McGrath et al., *Biochem. Biophys. Res. Commun.*, 145, 1171 (1987); Marsh et al., *Cancer Res.*, 46, 4053 (1986). Thus, overexpression of P-glycoprotein or mrp does not appear to cause a significant decrease in
DT_{ct}-GMCSF accumulation or cytotoxicity in GMCSF-R positive human myeloid leukemia cells.

Example 6. Ability of Recombinant Polypeptide Cytotoxins to cause Apoptotic Cell Death in Human Leukemic Cell Lines

Inhibitors of protein synthesis, including diphtheria toxin and cycloheximide, have been demonstrated to activate apoptotic cell death programs in a variety of leukemia cell lines. Kochi et al., *Exp. Cell Res.*, 208, 296 (1993). Therefore, this experiment was designed to determine whether the inhibition of protein synthesis effected by the DT_{ct}-GMCSF-specific ADP-ribosylation of the diphthamide site of EF-2 could subsequently trigger apoptosis in GMCSF-R positive human leukemia cells. Since the induction of apoptosis by some anti-cancer agents has been reported to be p53-dependent, and Bcl-2 protein is able to protect cells against p53-dependent as well as p53-independent apoptosis, (a) p53-deficient HL-60 cells and (b) p53-positive radiation-resistant mixed lineage RS4;11 cells expressing very high levels of Bcl-2 were used as targets. Uckun et al., cited *supra*.

DT_{ct}-GMCSF caused apoptosis of HL60 cells and RS4;11 cells in a concentration- and time-dependent fashion. A 4 hour or 24 hour exposure to 0.1 ng/mL or 1.0 ng/mL DT_{ct}-GMCSF did not induce apoptosis, while 10 ng/mL DT_{ct}-GMCSF induced apoptosis after 24 hours and 100 ng/mL or 1000 ng/mL DT_{ct}-GMCSF induced apoptosis after 4 hours. A 24 hour exposure to 100 or 1000 ng/mL DT_{ct}-GMCSF was more effective than a 4-hour exposure. DT_{ct}-GMCSF (100 ng/mL) treated cells showed distinctive morphologic signs of extensive apoptotic damage by light microscopy, including pronounced shrinkage, nuclear chromatin condensation, segmentation of the nucleus, and plasma membrane blebbing within 4 hours of exposure to the fusion toxin (Figures 4(C) & 4(D)). After 24 hours of treatment with DT_{ct}-GMCSF, very few cells remained detectable and virtually all of these cells showed signs of extensive damage consistent with apoptosis (Figures 4(E)
Some of the DT<sub>ct</sub>-GMCSF treated cells showed distinctive features of necrosis (NC), including swelling of the nucleus and cytoplasm and loss of nuclear and cytoplasmic basophilia (Figure 4(E)). By comparison, GMCSF alone did not induce apoptosis. (See Figures 4(A)[Control] and 4(B)[GMCSF treated cells]).

On agarose gels, DNA from DT<sub>ct</sub>-GMCSF treated HL60 cells showed a ladder-like fragmentation pattern and a dose-dependent decrease in the amount of intact DNA, consistent with apoptosis (Figure 5(A)). To detect apoptotic changes, cells were harvested 24 hours after continuous exposure to the DT<sub>ct</sub>-GMCSF fusion toxin, and DNA from SDS lysates according to the method previously described by Uckun et al., *Proc. Natl. Acad. Sci. USA*, 89, 9005 (1992) or Triton-X-100 lysates according to the method previously described by Cohen et al., *J. Immunol.*, 132, 38 (1984) was prepared for analysis of fragmentation. DNA was separated by electrophoresis through a 1% agarose gel, and the DNA fragments were visualized by UV light after staining with ethidium bromide. Controls included DNA from PBS treated cells cultured for 24 hours, DNA from cells treated with 1,000 ng/mL GMCSF for 24 hours, DNA from cells irradiated with 2 Gy γ-rays 24 hours prior to harvest, and DNA from cells preincubated for 2 hours with 3,000 ng/mL native recombinant GMCSF prior to treatment with 100 ng/mL DT<sub>ct</sub>-GMCSF for 24 hours.

The DT<sub>ct</sub>-GMCSF induced DNA fragmentation and apoptotic alterations in morphology in HL60 cells were not associated with evidence of terminal maturation, nor were they associated with growth factor deprivation, since these cells are GMCSF independent. Many agents which induce apoptosis are stimulators of oxidative metabolism and invoke oxidative stress as a common mediator of apoptosis. Uckun et al., cited supra. DT<sub>ct</sub>-GMCSF induced apoptosis of HL60 cells was not caused by an oxidative stress due to formation of reactive oxygen intermediates because it was not prevented by the radical scavenger N-acetylcysteine (NAC) at 100 nM-1 μM concentrations.

Furthermore, DT<sub>ct</sub>-GMCSF induced apoptosis of HL60 cells was not triggered by a decrease of their anti-apoptotic Bcl-2 oncoprotein levels. DT<sub>ct</sub>-GMCSF-
induced DNA fragmentation was most likely a consequence of the DT catalytic domain-induced inhibition of protein synthesis, since native recombinant GMCSF did not cause DNA fragmentation (Figure 5(B)), and a variety of biochemical and genetic studies have demonstrated that the DT catalytic domain does not possess direct deoxyribonuclease activity. Wilson et al., *Science*, 250, 834 (1990).

$D_T$-GMCSF induced apoptosis was mediated by the GMCSF-R specific binding of the fusion toxin to leukemia cells since prior incubation with excess unmodified native recombinant GMCSF prevented $D_T$-GMCSF-associated DNA fragmentation in HL60 cells (Figure 5(B)) and $D_T$-GMCSF did not cause DNA fragmentation in GMCSF-R negative NALM-6 B-lineage lymphoid leukemia cells (Figure 5(C)). Thus, $D_T$-GMCSF resulted in apoptosis of GMCSF-R positive human myeloid leukemia cells and this cytotoxicity is p53-independent. Similarly, $D_T$-GMCSF effected apoptotic destruction of radiation-resistant RS4;11 cells in a GMCSF-R specific fashion (Figure 5(D)), providing evidence that high expression levels of Bcl-2 oncoprotein associated with radiation resistance do not render GMCSF-R positive cells resistant to the potent cytotoxicity of $D_T$-GMCSF fusion toxin.

**Example 7. Anti-Leukemic Efficacy of Recombinant Polypeptide Cytotoxins against Human Leukemia Cells as Determined by an In Vitro Limiting Dilution Clonogenic Assay System**

The activity of a new agent against the bulk population of leukemia cells does not always predict its activity against the clonogenic self-renewing subpopulations of leukemia cells. Therefore, a highly sensitive *in vitro* limiting dilution clonogenic assay system was next used to determine the anti-leukemic efficacy of $D_T$-GMCSF against the clonogenic fraction of an HL60 cell line. A serial dilution clonogenic assay system was used to evaluate the anti-leukemic efficacy of $D_T$-GMCSF against human leukemia cell lines, as previously described by Uckun et al., *J. Immunol.*, 134, 2010 (1985). The sensitivity of primary AML cells to $D_T$-GMCSF was examined in a leukemic progenitor cell
assay system as previously described by Myers et al., Transplantation, 46, 240 (1988). Colony assays of normal bone marrow progenitor cells CFU-GEMM, CFU-GM, and BFU-E have been previously described by Uckun et al., J. Immunol., 134, 3504 (1985).

$\text{DT}_{ct}$-GMCSF killed clonogenic HL60 cells in a dose-dependent fashion with $>99.99\%$ destruction at 100 ng/mL or 1000 ng/mL (Figure 6(A)). Thus, the clonogenic subpopulation of HL60 cells were not spared from the cytotoxic effects of $\text{DT}_{ct}$-GMCSF.

Example 8. Lack of Toxicity of Recombinant Polypeptide Cytotoxins against Normal Pluripotent or Erythroid Bone Marrow Progenitor Cells

Contemporary intensive chemotheraphy regimens for AML are ultimately ineffective for most patients because they are toxic to normal bone marrow progenitors. The effects of $\text{DT}_{ct}$-GMCSF were examined on in vitro hematopoietic colony formation by normal bone marrow progenitor cells. While $\text{DT}_{ct}$-GMCSF inhibited granulocyte-macrophage colony formation by committed myeloid progenitor cells (i.e., CFU-GM), it did not affect erythroid burst formation by committed erythroid progenitor cells (i.e., BFU-E), or mixed granulocyte-erythroid-macrophage-megakaryocyte colony formation by pluripotent multilineage progenitor cells (i.e., CFU-GEMM) (Figure 6(A)). CFU-GEMM-derived mixed lineage colonies in cultures of $\text{DT}_{ct}$-GMCSF treated bone marrow cells were morphologically very similar to mixed lineage colonies in cultures of untreated bone marrow cells and they had a normal size and cellular composition (Figure 6(B)). Thus, $\text{DT}_{ct}$-GMCSF does not adversely affect the in vitro differentiation capacity of this pluripotent bone marrow progenitor cell population.
Example 9. Ability of Recombinant Polypeptide Cytotoxins to Induce Apoptosis in Primary Leukemic Cells from Patients with Chemotherapy-Refractory Relapsed Leukemia

The ability of DTct-GMCSF to induce apoptosis in primary leukemic cells from 6 patients with chemotherapy-refractory relapsed AML was also examined. DTct-GMCSF induced apoptotic DNA fragmentation in leukemic cells from 4 of these 6 patients (Figures 7(A) and 7(B)). In one case (Case #1), DTct-GMCSF was as effective as 2 Gy γ-rays in inducing apoptosis. In 3 additional cases (Case #s 3, 5, 6), DTct-GMCSF induced DNA fragmentation whereas 2 Gy γ-rays did not. Thus, DTct-GMCSF caused apoptosis of primary leukemic cells from a significant portion of relapsed AML patients regardless of their radiation sensitivity. Similarly, DTct-GMCSF caused apoptosis of HL60/VCR cells which express a P-glycoprotein associated MDR phenotype and HL60/ADR cells which express a Mrp-associated MDR phenotype.

Example 10. Anti-Leukemic Activity of Recombinant Polypeptide Cytotoxins against Clonogenic Leukemic Cells taken directly from Leukemia Patients

Leukemic progenitor cell assays were used to examine the anti-leukemic activity of DTct-GMCSF against primary leukemic cells from 7 therapy-refractory AML patients. Specifically, a serial dilution clonogenic assay system was used to evaluate the anti-leukemic efficacy of DTct-GMCSF against human leukemia cell lines, as previously described by Uckun et al., J. Immunol., 134, 2010 (1985). The sensitivity of primary AML cells to DTct-GMCSF was examined in a leukemic progenitor cell assay system as described by Myers et al., Transplantation, 46, 240 (1988). Colony assays of normal bone marrow progenitor cells CFU-GEMM, CFU-GM, and BFU-E were conducted as previously described by Uckun et al., J. Immunol., 134, 3504 (1985). As shown in Table I, below, DTct-GMCSF killed 92-99.9% of leukemic progenitor cells from 5 of the 7 cases studied. The observed inhibition of blast colony formation was due to destruction of leukemic progenitor cells rather than an impairment
in their ability to proliferate in methylcellulose cultures because cultures of 
DT<sub>c</sub>T-GMCSF treated AML blasts did not contain microclusters (Figure 8). 
These results provide direct evidence that DT<sub>c</sub>T-GMCSF can kill clonogenic 
leukemia cells from a substantial proportion of AML patients who have failed 
conventional chemotherapy. Similarly, DT<sub>c</sub>T-GMCSF effectively killed 
clonogenic cells of RS4;11 and HL60 cell lines that were used as positive 
controls (Table I).
Table 1
Effects of DT<sub>c</sub>-GMCSF on primary leukemic progenitor cells from therapy refractory AML patients

<table>
<thead>
<tr>
<th>Leukemic Progenitors</th>
<th>Control</th>
<th>DAB-GMCSF (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>AML Case 1</td>
<td>1496 (-)</td>
<td>0 (&gt;99.9)</td>
</tr>
<tr>
<td>AML Case 2</td>
<td>2204 (-)</td>
<td>1857 (15.7)</td>
</tr>
<tr>
<td>AML Case 3</td>
<td>90 (-)</td>
<td>0 (&gt;98.8)</td>
</tr>
<tr>
<td>AML Case 4</td>
<td>642 (-)</td>
<td>512 (20.2)</td>
</tr>
<tr>
<td>AML Case 5</td>
<td>19 (-)</td>
<td>1 (94.7)</td>
</tr>
<tr>
<td>AML Case 6</td>
<td>13 (-)</td>
<td>0 (&gt;92.3)</td>
</tr>
<tr>
<td>AML Case 7</td>
<td>41 (-)</td>
<td>0 (&gt;97.6)</td>
</tr>
<tr>
<td>AML HL60</td>
<td>785 (-)</td>
<td>4 (99.5)</td>
</tr>
<tr>
<td>t(4;11) ALL RS4;11</td>
<td>3923 (-)</td>
<td>785 (80)</td>
</tr>
</tbody>
</table>
Example 11. Pharmacokinetic Features of Recombinant Polypeptide

Cytotoxins

Radiolabeled DT\textsubscript{Ct}-GMCSF was used to examine the pharmacokinetic features of DT\textsubscript{Ct}-GMCSF in a SCID mouse model which was found to be highly predictive for the clinical pharmacology results obtained with immunotoxins. The radiolabeling of the recombinant polypeptide cytotoxins was performed as described by Uckun et al., *Science*, **267**, 886 (1995). A two compartment model was implemented in the ADAPT-II program was used in order to determine its volume of distribution and plasma clearance as described by Uckun et al., cited supra. The volume of distribution of DT\textsubscript{Ct}-GMCSF was 11.1 mL and its plasma clearance was 0.67 mL/hour with an alpha half-life of 0.82 hours, a beta half-life of 20.74 hours, and a plasma area under curve (AUC) of 1.13 \( \mu \)g.hour/mL.

These drug disposition results provide direct evidence that a daily administration schedule of DT\textsubscript{Ct}-GMCSF will not cause potentially dangerous drug accumulation.

Parenchymal accumulation of DT\textsubscript{Ct}-GMCSF was relatively low in brain, liver, muscle, and higher in skin, kidney, spleen, and lungs. Parenchymal concentrations were close to total tissue concentrations, which is explained by the good capillary permeability of recombinant polypeptide cytotoxins as a result of their low molecular weight (59kDa). Coefficients of the physiological model employed in these analyses are shown in Table II, below. These results provide direct evidence that DT\textsubscript{Ct}-GMCSF will effectively penetrate all organs infiltrated by human leukemia cells, including spleen, kidney, skin and lungs.
### Table II
Results of Physiological Modelling of DT_GMCSF

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Degradation Rate, 1/h</th>
<th>Transcapillary Transport Rate, 1/h</th>
<th>Liver Elimination Rate, 1/h</th>
<th>Lymph Flow Elimination Rate, 1/h</th>
<th>Endocytosis Rate, 1/h</th>
<th>Cell Excretion Rate, 1/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>0.31</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>0.15</td>
<td>0.021</td>
</tr>
<tr>
<td>Brain</td>
<td>N.A.</td>
<td>1.03</td>
<td>N.A.</td>
<td>0.011</td>
<td>0.70</td>
<td>0.021</td>
</tr>
<tr>
<td>Heart</td>
<td>N.A.</td>
<td>1.9</td>
<td>N.A.</td>
<td>0.89</td>
<td>0.19</td>
<td>0.021</td>
</tr>
<tr>
<td>Lungs</td>
<td>N.A.</td>
<td>0.6</td>
<td>N.A.</td>
<td>0.0007</td>
<td>0.042</td>
<td>0.021</td>
</tr>
<tr>
<td>Spleen</td>
<td>N.A.</td>
<td>5.8</td>
<td>N.A.</td>
<td>0.13</td>
<td>0.073</td>
<td>0.021</td>
</tr>
<tr>
<td>Kidney</td>
<td>N.A.</td>
<td>7</td>
<td>N.A.</td>
<td>0.50</td>
<td>0.025</td>
<td>0.021</td>
</tr>
<tr>
<td>Skin</td>
<td>N.A.</td>
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<td>N.A.</td>
<td>0.44</td>
<td>0.009</td>
<td>0.021</td>
</tr>
<tr>
<td>Muscle</td>
<td>N.A.</td>
<td>15.3</td>
<td>N.A.</td>
<td>0.83</td>
<td>0.12</td>
<td>0.021</td>
</tr>
<tr>
<td>Liver</td>
<td>N.A.</td>
<td>0.35</td>
<td>0.02</td>
<td>0.0001</td>
<td>0.065</td>
<td>0.021</td>
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</tbody>
</table>

Rest of body apparent volume = 6.53 ml

NA = Not Applicable
Example 12. In Vivo Anti-Leukemic Activity of Recombinant Polypeptide Cytotoxins in SCID Mouse Xenograft Models of Human Leukemia

The in vivo anti-leukemic efficacy of DT<sub>CT</sub>-GMCSF against human leukemia cells was evaluated in two different SCID mouse xenograft models of human leukemia. The first model system is based upon the ability of HL60 cells to cause disseminated and fatal AML in sublethally irradiated SCID mice. The second model system is based on the ability of RS4;11 cells to cause disseminated and fatal human t(4;11) mixed lineage leukemia in SCID mice. In both model systems, control mice treated with phosphate buffered saline (PBS) died of disseminated human leukemia within 45 days. See Figure 9 for the results in the HL60 model system. By comparison, one hundred percent of SCID mice treated with 5 μg or 10 μg DT<sub>CT</sub>-GMCSF administered parenterally by intraperitoneal injections remained alive free of leukemia for more than 75 days. Most importantly, this outcome was achieved without any significant toxicity to the mice. These results provide direct evidence that DT<sub>CT</sub>-GMCSF is an active anti-leukemic agent against human leukemia in vivo.

Example 13. In Vivo Activity and Toxicity of Recombinant Polypeptide Cytotoxins in Cynomolgous Monkeys

The activity and toxicity of DT<sub>CT</sub>-GMCSF was next evaluated in cynomolgous monkeys. Monkeys received intravenous infusions of 50 μg/kg/day of DT<sub>CT</sub>-GMCSF for a total of 5 consecutive treatment days. DT<sub>CT</sub>-GMCSF caused marked and unexpectedly rapid neutropenia and myeloid hypoplasia within 5 days which was severe but transient, consistent with sparing of pluripotent bone marrow progenitor cells. By day 10, monkeys had normal counts consistent with full hematologic recovery. See Figure 10. Transient diarrhea was observed but did not cause significant clinical problems. The blood chemistry indicated that DT<sub>CT</sub>-GMCSF cause mild capillary leak as reflected by a decrease in serum albumin levels (ALB) and mild liver inflammation as reflected by a mild elevation of the liver enzyme ALT. No other abnormalities were noted in the blood chemistry. See Table III, below.
Table III

Blood Chemistry Profile of DT<sub>cr</sub>-GMCSF Treated Monkey 52H

<table>
<thead>
<tr>
<th>Day</th>
<th>Na</th>
<th>K</th>
<th>Cl</th>
<th>HCO3</th>
<th>Gluc</th>
<th>BUN</th>
<th>Creat</th>
<th>Ca</th>
<th>Bili</th>
<th>ALT</th>
<th>Alb</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>148</td>
<td>4.0</td>
<td>111</td>
<td>50</td>
<td>17</td>
<td>0.8</td>
<td>10.3</td>
<td>0.3</td>
<td>96</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
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<td>146</td>
<td>3.8</td>
<td>108</td>
<td>27</td>
<td>30</td>
<td>19</td>
<td>0.6</td>
<td>10.1</td>
<td>0.2</td>
<td>176</td>
<td>3.6</td>
</tr>
<tr>
<td>2</td>
<td>152</td>
<td>3.6</td>
<td>105</td>
<td>18</td>
<td>42</td>
<td>15</td>
<td>0.9</td>
<td>10.1</td>
<td>0.3</td>
<td>335</td>
<td>4.0</td>
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<tr>
<td>3</td>
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<tr>
<td>4</td>
<td>144</td>
<td>3.6</td>
<td>106</td>
<td>24</td>
<td>67</td>
<td>17</td>
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<td>0.3</td>
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<tr>
<td>5</td>
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<td>4.3</td>
<td>109</td>
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<td>66</td>
<td>17</td>
<td>0.8</td>
<td>9.3</td>
<td>0.3</td>
<td>193</td>
<td>2.8</td>
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<tr>
<td>8</td>
<td>147</td>
<td>3.6</td>
<td>107</td>
<td>27</td>
<td>66</td>
<td>16</td>
<td>0.8</td>
<td>8.5</td>
<td>0.1</td>
<td>103</td>
<td>3.0</td>
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<td>4.3</td>
<td>108</td>
<td>27</td>
<td>43</td>
<td>5</td>
<td>0.6</td>
<td>8.7</td>
<td>0.3</td>
<td>34</td>
<td>ND</td>
</tr>
</tbody>
</table>

Na = sodium
K = potassium
Cl = chlorine
HCO3 = bicarbonate
Gluc = glucose

BUN =
Creat = creatinine
Ca = calcium
Bili = bilirubin
Alb = albumin
There was no toxicity to the lungs, heart, kidneys, or nervous system. DT<sub>ct</sub>-GMCSF caused moderate thrombocytopenia providing unprecedented evidence that GMCSF receptors are expressed on some of the megakaryocytic precursors. Thus, human patients are likely to require platelet transfusions during and for a limited period after treatment with DT<sub>ct</sub>-GMCSF. These results provide direct evidence that DT<sub>ct</sub>-GMCSF is active against primate myeloid cells without affecting pluripotent bone marrow progenitor cells or causing significant toxicity to non-hematopoietic organs.

The above data demonstrates that DT<sub>ct</sub>-GMCSF is a potent anti-neoplastic agent against GMCSF-R bearing leukemia cells. DT<sub>ct</sub>-GMCSF induces apoptosis in leukemic cells deficient in p53 expression, leukemic cells expressing high levels of Bcl-2, multi-drug-resistant leukemia cell lines, and primary leukemic cells from AML patients who have failed intensive multiagent chemotherapy. DT<sub>ct</sub>-GMCSF killed virtually 100% of primary leukemic progenitor cells from therapy-refractory AML patients under conditions that did not adversely affect the proliferative capacity or differentiation of pluripotent normal hematopoietic progenitor cells. DT<sub>ct</sub>-GMCSF is effective against human leukemia in in vivo SCID mouse models. Furthermore, DT<sub>ct</sub>-GMCSF kills GMCSF-R bearing myeloid cells in cynomologous monkeys. Thus, the recombinant polypeptide cytotoxins described herein show great potential for the treatment of target human diseases.
WHAT IS CLAIMED IS:

1. A recombinant polypeptide cytotoxin comprising GMCSF, IL3 or an active subunit thereof which binds to a receptor expressed on a mammalian cell, wherein said GMCSF, IL3 or an active subunit thereof is fused to a polypeptide cytotoxin which inhibits protein synthesis by said cell so that the cell is killed by apoptosis, wherein said polypeptide cytotoxin does not comprise a binding region for mammalian cells, and wherein the GMCSF, IL3 or an active subunit thereof does not bind to a receptor expressed on normal pluripotent bone marrow progenitor cells.

2. The recombinant polypeptide cytotoxin of claim 1 wherein the GMCSF, IL3 or an active subunit thereof is linked to the polypeptide cytotoxin so that the GMCSF, IL3 or an active subunit thereof replaces the cell-binding region of the polypeptide cytotoxin.

3. The recombinant polypeptide cytotoxin of claim 1 wherein the receptor is GMCSF-R.

4. A recombinant polypeptide cytotoxin comprising a single chain antibody subunit which binds to a CD19 receptor expressed on a mammalian B-lineage cell, wherein said antibody subunit is fused to a polypeptide cytotoxin which inhibits protein synthesis by said cell so that the cell is killed by apoptosis and which polypeptide cytotoxin does not comprise a native binding domain, and wherein the antibody subunit does not bind to a receptor expressed on normal pluripotent bone marrow progenitor cells.

5. The recombinant polypeptide cytotoxin of claims 1 or 4 wherein the polypeptide cytotoxin is a bacterial or plant toxin.
6. The recombinant polypeptide cytotoxin of claim 5 wherein the polypeptide cytotoxin is derived from diphtheria toxin.

7. The recombinant polypeptide cytotoxin of claim 5 wherein the polypeptide cytotoxin is pokeweed antiviral protein or an active subunit thereof.

8. The recombinant polypeptide cytotoxin of claim 4 wherein the antibody subunit is the Fv fragment of B43.

9. The recombinant polypeptide cytotoxin of claims 1 or 4 wherein the mammalian cell is a cancer cell.

10. The recombinant polypeptide cytotoxin of claim 9 wherein the cancer cell is a leukemia cell.

11. The recombinant polypeptide cytotoxin of claim 10 wherein the cancer cell is a B-lineage acute lymphoblastic leukemia cell, a chronic lymphocytic leukemia cell, a B-lineage lymphoma cell, a blast crisis of chronic myelosytic leukemia cell, a hairy cell leukemia cell, an acute or chronic myelogenous leukemia cell, a mixed lineage leukemia cell or a T-cell leukemia cell.

12. The recombinant polypeptide cytotoxin of claim 9 wherein the cancer cell is a lymphoma cell.

13. The recombinant polypeptide cytotoxin of claim 12 wherein the cancer cell is an AIDS lymphoma cell, an EBV-lymphoma cell, a breast cancer cell, a colon cancer cell, a prostate cancer cell, or a lung cancer cell.

14. The recombinant polypeptide cytotoxin of claim 9 wherein the cancer cell is a multi-drug or radiation resistant cancer cell.
15. A method comprising suppressing the proliferation of a population of mammalian cells which express the GMCSF receptor by reacting said cells with a recombinant toxin comprising GMCSF or a subunit thereof which binds to said receptor, fused to a polypeptide cytotoxin wherein the binding domain of said polypeptide cytotoxin is absent and which polypeptide cytotoxin inhibits protein synthesis by said cells so that said cells are killed by apoptosis.

16. The method of claim 15 wherein the polypeptide cytotoxin is a plant toxin or bacterial toxin.

17. The method of claim 16 wherein the polypeptide cytotoxin is derived from diphtheria toxin.

18. The method of claim 15 wherein the mammalian cells are cancer cells.

19. The method of claim 18 wherein the mammalian cells are leukemia cells.

20. The method of claim 19 wherein the mammalian cells are acute or chronic myelogenous leukemia cells, mixed lineage leukemia cells or T-cell leukemia cells.

21. The method of claim 18 wherein the cancer cells are lymphoma cells.

22. The method of claim 20 wherein the cancer cells are breast cancer cells, colon cancer cells, prostate cancer cells, or lung cancer cells.

23. The method of claim 18 wherein the cancer cells are multi-drug resistant or radiation resistant.

24. The method of claim 15 wherein the cells are human macrophages.
25. The method of claim 24 wherein the proliferation is due to contact with foreign tissue antigens contained in an organ transplant.

26. A method comprising suppressing the proliferation of a population of mammalian cells which express the CD19 surface antigen by reacting said cells with a recombinant toxin comprising B43 or a subunit thereof which binds to said receptor, fused to a polypeptide toxin wherein the binding domain of said polypeptide is absent and which polypeptide toxin inhibits protein synthesis by said cells so that said cells are killed by apoptosis.

27. The method of claim 26 wherein the polypeptide toxin is a plant toxin or bacterial toxin.

28. The method of claim 27 wherein the polypeptide toxin is derived from diphtheria toxin.

29. The method of claim 26 wherein the cells are cancer cells.

30. The method of claim 27 wherein the cancer cells are leukemia cells.

31. The method of claim 30 wherein the cancer cells are B-lineage acute lymphoblastic leukemia cells, chronic lymphocytic leukemia cells, blast crisis of chronic myelosytic leukemia cells, or hairy cell leukemia cells.

32. The method of claim 29 wherein the cancer cells are lymphoma cells.

33. The method of claim 32 wherein the cancer cells are AIDS lymphoma cells, B-lineage lymphoma cells or EBV-lymphoma cells.
34. The method of claim 26 wherein the proliferation is due to an autoimmune disease, an immune response to therapeutically beneficial agents such as blood components or coagulation factors, or the rejection of an organ transplant.

35. A therapeutic method for the treatment of cancer comprising parenterally administering to a patient who is afflicted with a cancer expressing the CD19 surface antigen a therapeutically effective amount of a recombinant polypeptide cytotoxin comprising a single chain antibody subunit which binds to a CD19 receptor, wherein said antibody subunit is fused to a polypeptide cytotoxin which inhibits protein synthesis by said cell so that the cell is killed by apoptosis and which polypeptide cytotoxin does not comprise a native binding domain, and wherein the antibody subunit does not bind to a receptor expressed on normal pluripotent bone marrow progenitor cells.

36. The method of claim 35 wherein the cancer is a leukemia.

37. The method of claim 36 wherein the cancer is B-lineage acute lymphoblastic leukemia, chronic lymphocytic leukemia, blast crisis of chronic myelosytic leukemia or hairy cell leukemia.

38. The method of claim 36 wherein the cancer is a lymphoma.

39. The method of claim 38 wherein the cancer is AIDS lymphoma, B-lineage lymphoma, or EBV-lymphoma.

40. The method of claim 35 wherein the polypeptide toxin is a plant toxin or bacterial toxin.

41. The method of claim 40 wherein the polypeptide toxin is derived from diphtheria toxin.
42. The method of claim 40 wherein the polypeptide toxin is pokeweed antiviral antiviral protein or an active subunit thereof.

43. The method of claim 35 wherein the recombinant polypeptide cytotoxin is administered in combination with a pharmaceutically acceptable liquid carrier.

44. The method of claim 43 wherein the liquid carrier comprises isotonic saline.

45. The method of claim 43 wherein the recombinant polypeptide cytotoxin is administered intravenously.

46. The method of claim 35 wherein the antibody subunit is the Fv fragment of B43 or derivatives thereof.

47. The method of claim 35 wherein the amount of recombinant polypeptide cytotoxin is about 0.025 mg/kg/day to 1 mg/kg/day administered over about 3 to 5 days.

48. A therapeutic method for the treatment of cancer comprising parenterally administering to a patient who is afflicted with a cancer expressing the GMCSF receptor a therapeutically effective amount of a recombinant polypeptide cytotoxin comprising GMCSF or an active subunit thereof fused to a polypeptide toxin which inhibits protein synthesis by said cell so that the cell is killed by apoptosis and which polypeptide toxin does not comprise a native binding domain, and wherein the GMCSF or active subunit thereof does not bind to a receptor expressed on normal pluripotent bone marrow progenitor cells.
49. The method of claim 48 wherein the cancer is a leukemia.

50. The method of claim 49 wherein the cancer is acute or chronic myelogenous leukemia, mixed lineage leukemia or T-cell leukemia.

51. The method of claim 49 wherein the cancer is a lymphoma.

52. The method of claim 51 wherein the cancer is breast cancer, colon cancer, prostate cancer, or lung cancer.

53. The method of claim 48 wherein the polypeptide toxin is a plant toxin or bacterial toxin.

54. The method of claim 53 wherein the polypeptide toxin is derived from diphtheria toxin.

55. The method of claim 48 wherein the recombinant polypeptide cytotoxin is administered in combination with a pharmaceutically acceptable liquid carrier.

56. The method of claim 55 wherein the liquid carrier comprises isotonic saline.

57. The method of claim 55 wherein the recombinant polypeptide cytotoxin is administered intravenously.

58. The method of claim 48 wherein the amount of recombinant polypeptide cytotoxin is about 0.025 mg/kg/day to 1 mg/kg/day administered over about 3 to 5 days.
FIG. 3C

Viability (Percent)

Concentration (ng/ml)

Vincristine

FIG. 6A

Percent Control Recovery

DT_{ct}-GMCSF Concentration (ng/mL)

SUBSTITUTE SHEET (RULE 26)
FIG. 4D

$D_{ct} - GMCSF \times 4h$

FIG. 4E

$D_{ct} - GMCSF \times 24h$

FIG. 4F

$D_{ct} - GMCSF \times 24h$

SUBSTITUTE SHEET (RULE 26)
BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3 AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

University of Minnesota Medical School
Attention: John P. Perentesis
Box 204 UMHC
420 Delaware Street SE
Minneapolis, MN 55455

Deposited on Behalf of: Regents of the University of Minnesota

Identification Reference by Depositor: ATCC Designation
Plasmid, pET11d-DTctGMCSF 97178

The deposit was accompanied by: _ a scientific description _ a proposed taxonomic description indicated above.

The deposit was received June 1, 1995 by this International Depository Authority and has been accepted.

AT YOUR REQUEST:

X We will inform you of requests for the strain for 30 years.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one’s right to receive, or if a U.S. Patent is issued citing the strain and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strain.

If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with living culture of the same.

The strain will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested June 8, 1995. On that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Annette L. Bade, Director, Patent Depository

cc: Anthony L. Strauss

Kimberly Jordahl