Growth factor toxin fusion proteins for the treatment of leukemia

Preclinical animal studies relevant for human acute myeloid leukemia

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Groeifactor toxine fusie eiwitten voor de behandeling van leukemie.

Pre-Klinische studies in diermodellen relevant voor humane actute myeloide leukemie.

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus Prof. Dr. P.W.C. Akkermans M.A.
en volgens besluit van de College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 26 november 1997 om 11.45 uur

door

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geboren op 18 januari 1964 te Almelo

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The experiments described in this thesis were performed at the Institute of Hematology of the Erasmus University Rotterdam, The Netherlands. These investigations were supported by a grant from the Dutch Cancer Society (grant nr. EUR-93-663)

ISBN 90-9011075-5

Printed by offsetdrukkerij Haveka BV, Alblasserdam, The Netherlands.

Financial support was provided by the Dutch Cancer Society, Amgen B.V., Novartis Pharma B.V. and Harlan Nederland.
Cover design: H. Rozemuller

Aan Herma

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Chapter 1

General Introduction

1.1 General introduction and scope of this thesis

In the development of new therapeutic agents to treat malignancies, bacterial and plant toxins are being investigated. Targeting cells with these toxins has been facilitated by chemical conjugation or genetic engineering of the toxin to proteins with cellular binding potential, such as antibodies (immunotoxins) or growth factors (growth factor toxins or GF-toxins). Chimeric GF-toxins and immunotoxin proteins combine the specificity of the GF or the antibody part to bind to cellular structures with the killing potential of the toxin part. Genetically engineered toxins have been produced from two bacterial toxins: Pseudomonas exotoxin (PE) and Diphtheria toxin (DT). Such PE and DT based fusion proteins have shown potential to treat hemopoietic malignancies. Some degree of clinical benefit has already been reported in phase I and II clinical trials in leukemia and lymphoma.

Acute myeloid leukemia (AML) may also benefit from this approach. Myeloid leukemic cells express GF receptors (GFRs) which bind ligands with high affinity followed by rapid internalization of the ligand-receptor complexes. These features provide a rationale for GF-toxin treatment of AML. Because GFRs are also expressed by non-leukemic cells, preclinical animal studies are essential for the evaluation of the potential of these new therapeutic agents to treat leukemia. Animal models will provide insight into the antileukemic effects, and equally relevant, to the most prominent toxic side effects.

1.2 Toxin fusion proteins

1.2.1 Introduction to the toxins

The majority of toxins, produced by plants and bacteria, are polypeptide enzymes that catalytically inactivate protein synthesis. A few toxins have been used for the development of new therapeutic agents by coupling of the potent toxins to antibodies or other ligands (Table 1). The bacterial toxins explored in this thesis are DT and PE. Both toxins catalyze the ADP-ribosylation of a post-translationally modified histidine (diphthamide) (1, 2) in elongation factor-2 (EF-2) in the following reaction (3, 4):

$$NAD^{+} + \text{EF-2} \xrightarrow{\text{DT/PE}} \text{ADP ribose-EF-2 and nicotinamide} + H^{+}$$

ADP-ribosylated EF-2 is unable to further catalyse the transfer of peptidyl-tRNA from the aminoacyl site to the polypeptidyl site of the eukaryotic ribosome, and consequently, cells lose the ability to synthesize protein and will consequently die. One single molecule entering the cytosol is sufficient to kill a cell (5). However, the binding, internalization and the proteolytic processing of toxins are rate limiting steps of toxin activity (6).

Table 1
Powerful toxins employed for their cell killing activities as immunotoxins or growth factor toxins

source	toxin	
<u>bacteria</u>		
Pseudomonas aeruginosa	Pseudomonas exotoxin	
Corynebacterium diphtheria	Diphtheria toxin	
<u>Plants</u>		
Phytolacca americana (seeds)	type I	pokeweed antiviral protein (PAP)
Saponaria officinalis (seeds)		saporin
Momordica charantia (seeds)		momordin
Gelonium multifore (seeds)		gelonin
Ricinis communis (seeds)	type II	ricin
Abrus precatorius (seeds)		abrin

1.2.2 Diphtheria toxin

Diphtheria toxin (DT) (7-9) is synthesised and released by the bacteria *Corynebacterium diphtheria*. DT is a highly potent toxin for a variety of mammalian species, including man. *In vivo* experiments in sensitive species showed that purified DT is lethal in doses of 100 ng/kg, with no particular target organ. Cell lines derived from rats and mice are less sensitive to DT (10, 11). In these animals similar effects are achieved at concentrations several orders of magnitude higher than in sensitive species.

The toxin is a single polypeptide chain of 535 amino acids residues and has a molecular weight of 62 kilodaltons (Kd). As a complete molecule DT is enzymatically inactive. Activation is achieved by proteolytic cleavage inside the cell. The cleavage results in the formation of two fragments, i.e. fragment A of 24 Kd, and fragment B of 38 Kd, held together by two disulphide bridges (Fig 1). X-ray crystallography revealed the structure of DT (12). Fragment A is the catalytic domain (residues 1-193), and fragment B actually consist of two domains; the receptor binding domain B (residues 389-535) and the transmembrane domain T (residues 205-378).

The COOH-terminal fragment B is required for binding to specific surface proteins on the cell membrane and for transport across the lipid bilayer. The determination of an anion antiporter as a potential binding protein facilitates the study of the mechanisms of binding and transport of DT (13-20). Sensitivity for DT mainly depends on the number of functional cell surface receptors. Binding studies showed a range of a few thousand (21) up to 200, 000 per cell (22) depending on the cell type. DT-binding sites on mouse and rat cells do not lead to intoxication of cells (23-26) although the cells were fully capable of processing DT after binding via alternative receptors, such as Concanavalin A (Con A)-binding glycoproteins or transferrine-receptor (24, 27). Hence, resistance to DT might be a defect in the process between toxin binding and delivery of fragment A to the cytoplasm. However, toxin molecules entering cells by endocytosis might intoxicate insensitive cells (28). Intoxication

by this pathway plays only a role at high concentation due to the inefficient uptake and processing.

In the endosome, DT is cleaved specifically at Arg_{194} in the protease sensitive loop, formed by the disulphide bridge between Cys_{187} and Cys_{202}. The protease, which has been characterized as a furin-like enzyme (29), cleaves the protein at a low pH. The 24 Kd catalytic domain is then translocated into the cytoplasm, mediated by the putative channel-forming helices of the transmembrane domain (T) spontaneously inserting into the vesicle membrane (12, 30, 31).

Deletion studies showed that the first five amino acids of DT, GADDV, are not required for ADP ribosylation activity but are necessary for cytotoxicity (32). The current opinion is that this sequence is functioning as a recognition signal for an efficient translocation in the cytosol.

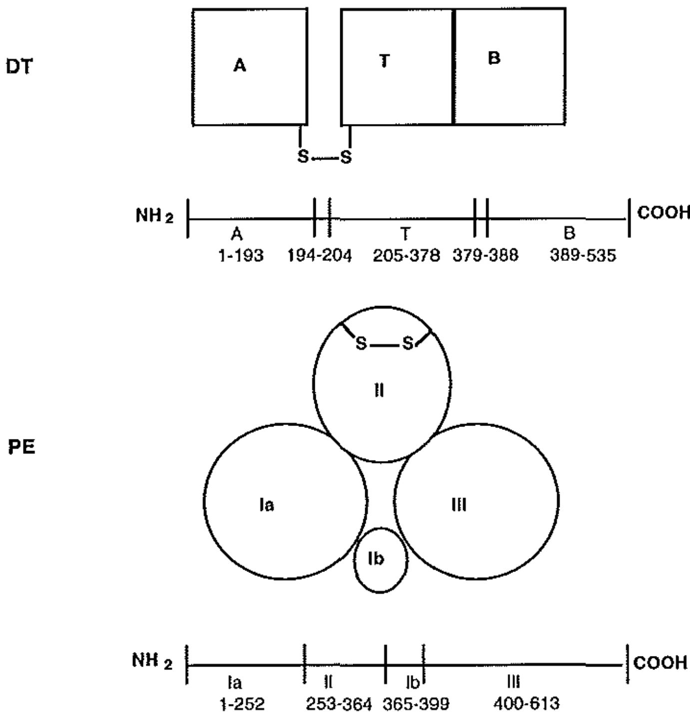

Figure1
Structure of DT and PE.

1.2.3 Pseudomonas exotoxin

Pseudomonas exotoxin A (PE) (9, 33, 34) is a 66 Kd single chain toxin secreted by *Pseudomonas aeruginosa*. The potential of PE and its chimeras has been extensively reviewed (35, 36). The PE molecule like DT is a pro-enzyme; it cannot catalyze the ADP-ribosylation of EF-2 without activation. Although minimal structural homology exists between DT and PE, they catalyze the same ADP ribosylation reaction. Using photo affinity

labeling experiments it was shown that the Glu_{148} of DT is at or near the NAD^+-binding site, which corresponds to the Glu_{553} of PE (37).

The identification of the discrete domains of PE by X-ray crystallography (38, 39) has added to the understanding of the structure of the molecule and its mode of action. PE is composed of three major structural domains (Fig 1): an NH_2 terminal cell binding domain Ia (residues 1-252); a translocation domain II (residues 253-364); and a COOH terminal enzymatically active domain III (residues 400-613). The function of domain Ib (residues 365-399) is still unknown. So, in contrast to DT, in which the ADP-ribosylating activity is located in the NH_2-terminus, while the binding domain is located in the C-terminus, the arrangement of the functional domains in PE is inverted.

PE is comprised of a cluster of basic amino acids in which Lys_{57} plays a key role in binding to a surface receptor (40). The PE receptor has been identified as the large subunit of the alpha 2-macroglobulin receptor (41-43), located both on the surface and on the intracellular membranes of all eukaryotic cell types. This protein most likely mediates internalization to the endosomal compartment.

After binding and internalization, the toxin undergoes conformational changes. At a low pH, the toxin is cleaved ("nicked") in an arginine-rich loop of domain II by a cellular protease, furin (6, 29, 44). The ubiquitously expressed protease furin is found primarily in the *trans*-Golgi apparatus and on the plasma membrane (45). This proteolytic processing of PE is a prerequisite for efficient translocation of the enzymatic domain to the cytosol. After the splicing of the protein by reduction of the disulphide bridge, 2 fragments of 28 and 37 Kd are generated. The 37 Kd fragment contains the active enzyme domain, the translocation domain, and the sequence REDLK at the COOH terminus. The sequence REDLK is similar to the eukaryotic endoplasmic retention signal and may function in a similar way (46, 47). It is thought that the interaction with the endoplasmic reticulum is an important step in toxin translocation to the cytosol. It appears that the functionally similar toxins, DT and PE, use different intracellular pathways to reach the cytoplasm.

The translocation domain II is responsible for the delivery of the enzymatic fraction to the cytosol (48). In contrast to DT, no long stretches of hydrophobic amino acids are found in native PE. However, in an acidified environment, domain II becomes globally hydrophobic and is then converted into a translocation-competent domain (38, 49).

1.2.4 Other toxins

Other toxins used for targeting therapy are mostly derived from plants. They are summarized in Table 1. These ribosome-inactivating proteins (RIPs) inactivate eukaryotic ribosomes, and are therefore lethal to animals and cultured cells (50). The RIPs are divided into two groups based on their structure (51). The proteins existing in nature as a single-chain are classified as type I and those consisting of an A (active) chain with RIP properties covalently linked to a B (binding) chain are classified as type II toxins. The latter RIPs enter

cells more easily and are therefore more potent toxins. The enzymatic A-chain of both groups hydrolyses the N-glycosidic bond between an adenine and ribose of Ala_{4324} in the 28S component of the large 60S ribosomal subunit (52, 53). Removal of this adenine residue destroys the binding site of elongation factor 2, thereby blocking the protein synthesis.

Ricin is the most commonly used toxin. Immunotoxins made of whole ricin are very potent but lack specificity because of the B-chain, which binds to galactosyl residues of glycoproteins and glycolipids present on the surface of a variety of cells. The modified immunotoxins, having only the A-chain are more specific but not as active as the whole ricin immunotoxin. Several immunotoxins based on ricin, but also saporin, have been entered into phase I/II clinical trials to evaluate their toxic and therapeutic effects in hematological malignancies, solid tumors and autoimmune disorders (54-56).

1.2.5 Growth factor-toxin fusion proteins

The term "immunotoxins" (IT) is used for all fusion proteins that use a ligand for directing the toxin to cancer cells. These proteins may be polyclonal or monoclonal antibodies, but also GFs or hormones (57). The latter cannot be named "immunotoxins" in the strict sense. Although, they are closely related to antibodies in their mode of targeting, they do not contain an antibody moiety.

The first generation of such immunotoxins were conjugates of antibodies or GFs with toxins such as ricin, DT or PE. A new approach, developed in the early nineties using recombinant DNA techniques, resulted in the production of fusion proteins without further chemical modification (58-60). The two types of immunotoxins that are being extensively studied: chemical conjugates and recombinant toxins, have properties that differ widely. The advantages of fusion proteins are that large amounts of uniform proteins can be produced and that these fusion molecules are smaller than chemical conjugates. The latter property could be beneficial with regards to tumor penetration. A factor which also influences the potency is the half-life of toxins in human serum. Larger toxins, as the first generation toxins, have a prolonged half-life in the circulation, and may be preferable to the short half-life recombinant toxins for leukemia treatment. However, smaller proteins, which rapidly leave the circulation may have reduced non-specific toxic side effects. There is not enough data available to decide which type of immunotoxin is most suitable for cancer therapy (61).

In most toxins the binding moiety of the DT or PE has been deleted and replaced by antibodies or GFs. In general GF-toxin fusion proteins offer several advantages over the immunotoxins containing antibodies: (a) receptor binding is generally of high affinity, (b) most receptors are known to be internalized by receptor-mediated endocytosis and (c) most cell types expressing the receptor are known.

Because PE and DT have an inverse structure (Fig 1), the fusion of the GF has to be at the amino terminus of PE or at the carboxyl terminus of DT. Several derivatives have been constructed from the toxins. Complete PE is used, in which domain Ia is inactivated by

replacing Lys_{57}, His_{246}, Arg_{247}, and His_{249} with glutamates, $PE66^{4E}$ (62). The major toxic effect is liver necrosis, mediated by the domain Ia. To decrease this nonspecific toxicity, domain Ia of PE was removed, resulting in PE40, which is much less toxic to the liver (63). Subsequent deletion of amino acids 365-384 results in an even smaller molecule, PE38 (64). DT can be shortened by removing a portion of the binding domain, DT_{486} (65). It can be further truncated to a molecule known as DAB_{389} or DT_{388} (32, 66), which shows less toxic side effects.

To produce recombinant toxins, a plasmid encoding a single-chain protein is introduced in *E.coli*. The bacteria are allowed to proliferate and produce the recombinant protein in insoluble inclusion bodies. After purifying the inclusion bodies the protein must be denaturated and renaturated in refolding buffer. The resulting recombinant protein is then purified to near homogeneity by standard column chromatography (67, 68)

Recombinant toxins have been produced using epidermal growth factor (EGF), transforming growth factor alpha (TGFα) and a chimeric protein consisting of the heparin binding domain and the TGFα (HB-TGFα), acidic and basic fibroblast growth factor (aFGF and bFGF), insulin-like growth factor (IGF), α-melanocyte-stimulating hormone (α-MSH), Interleukin-2 (IL-2), IL-3, IL-4, IL-6, IL-7, IL-9, IL-13, granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF). The attachment of a toxin to GFs could affect the binding capacity. Therefore, IL-4 was modified to improve its cytotoxicity. Table 2 summarizes the GF-toxins that are studied for their activity against malignant cells.

1.2.6 Clinical trials with GF-toxins

Encouraging clinical responses have been observed with GF-toxins from phase I/II studies. For instance, DAB_{486}-IL-2 and DAB_{389}-IL-2 entered phase I/II trials for evaluation of the safety, tolerance biological effects, and efficacy in lymphoma treatment (69-72), rheumatoid arthritis treatment (73, 74) and insulin-dependent diabetes mellitus treatment (75). Clinically valuable responses were reported in the treatment of lymphoma patients. Treatment of patients with cutaneous T-cell lymphoma (CTCL) showed responses in 3 trials, e.g. 3 responses in 14 CTCL patients (71), 3 responses out of 5 (70) and 3 out of 5 (76). One of 4 patients with Hodgkin's disease (HD) showed a response (69). Treatment of other non-Hodgkin's lymphomas (NHL) resulted in 3 responses out of 9 NHL patients (69) and 2 responses out of 4 (77). Additional phase I/II studies using DAB_{389}IL-2, a shortened version of DAB_{486}IL-2, showed responses in 12 out of 31 CTCL patients and 3 out of 17 other NHL patients. This toxin is now in a phase III pivotal trial for the treatment of CTCL. DAB_{486}IL-2 was also evaluated in rheumatoid arthritis (RA). A beneficial effect was reported in 9 cases out of 18 in patients with RA (75) and 4 responses out of 22 in RA patients in a double-blind phase I/II study (74). DAB_{389}-EGF was used to target carcinomas expressing the EGFR. The fusion protein is currently evaluated in phase I dose-escalation studies (78).

Table 2
Recombinant growth factor toxin fusion proteins for cancer therapy

Growth factor		toxin derivates	Receptor	target	status	ref
Epidermal Growth factor	EGF	PE40 DAB_{486}/DAB_{389}	EGFR	tumor cell lines: breast, bladder, prostate, lung and neuroglia	cultured cells phase I	(78, 84)
Transforming Growth factor α	TGFα	PE40/PE4E/PE37 /PE35	EGFR	epidermoid, adenocarcinoma, glioblastoma, bladder	cultured cells	(85-90)
Heparin binding domain and TGF α	HB-TGFα	PE40/PE38/PE4E	EGFR + heparin	smooth muscle cells	cultured cells	(91, 92)
acidic Fibroblast Growth factor	aFGF	PE40/PE4E	FGFR	tumor cell lines	cultured cells	(93-95)
basic Fibroblast Growth factor	bFGF	PE40	FGFR	tumor cell lines	cultured cells	(96)
Insulin-like Growth Factor	IGF1	PE40	IGFR	glioblastoma, carcinoma cell lines	cultured cells	(94, 97)
α-Melanocyte Stimulating Hormone	MSH	DAB_{389}	melantropin R	melanomas	cultured cells	(98, 99)
Human Interleukin-2	hulL-2	$PE40/PE66^{4glu}$ DAB_{486}/DAB_{389}	hulL-2R α or β hulL-2R α and β	lymphomas, myasthenia gravis	cultured cells phase I/II	(66, 100-106)
Murine Interleukin-3	mIL-3	DT_{390}	mIL-3R	bone marrow cells	cultured cells	(107)
Human Interleukin-3	hulL-3	DAB	hulL-3	leukemia cell lines	cultured cells	(108)
Human Interleukin-4	hulL-4	PE4E/PE37	hulL-4R	carcinoma cells, activated lymphocytes	cultured cells	(109-112)
Circular permuted human interleukin-4	CPhulL-4	PE38		glioblastoma, glioma, neuroblastoma		
Murine Interleukin-4	mIL-4	PE40 DAB_{389}	mIL-4R	mouse tumors	cultured cells	(113,115)
Human Interleukin-6	hulL-6	PE40/PE4E DAB_{389}	hulL-6R	hepatoma, myeloma, prostate, epidermoid carcinoma cells	cultured cells	(68, 116-122)
Human Interleukin-7	hulL-7	DAB_{389}	hulL-7R	lymphomas	cultured cells	(123)
Human Interleukin-9	hulL-9	PE40	hulL-9R	lymphoma and leukemia	cultured cells	(124)
Human Interleukin-13	hulL-13	PE38	hulL-13/hulL-4 common R	epithelial carcinomas	cultured cells	(125)
Murine Granulocyte-Macrophage-Colony Stimulating Factor	mGM-CSF	DT_{390}	mGM-CSFR	bone marrow cells	cultured cells	(126,127)
Human Granulocyte-Macrophage-Colony Stimulating Factor	huGM-CSF	DAB_{389}	huGM-CSFR	AML	cultured cells	(108, 128-133)
Human Granulocyte-Colony Stimulating Factor	huG-CSF	PE40 DAB_{389}	mG-CSFR	leukemia	cultured cells	(134-137)

One PE based immunotoxin targeting the Lewis-Y antigen (B3) expressed by adenocarcinoma cells has been entered for phase I evaluation (79). Objective antitumor activities were observed in 5 out of 38 patients.

A variety of dose limiting toxicities were reported in phase I/II clinical trials with immunotoxins or GF-toxins containing DT and PE. The major dose-limiting toxicities for DT were liver function abnormalities, nausea, hypersensitivity, fatigue and fever (56, 71, 74, 80-82). Vascular leak syndrome (VLS) was the dose-limiting toxicity in the treatment with PE based immunotoxins (79).

Pre-existing antibodies against immunotoxins could be an obstacle for effective clinical use. Immunity is acquired naturally during the course of an infection or artificially by vaccination. Due to mass immunization against diphtheria with a detoxified form of the toxin (a toxoid), most people in the Western world have antibodies against DT, in contrast to antibodies against PE, from which 3% of humans have pre-existing antibodies. However, in phase I/II studies with DAB_{486}-IL-2 an antitumor effect could be observed in the presence of antibodies to DT (69, 70). Therefore, it is assumed that the induced antibodies are not raised against the truncated part of DT in the immunotoxins (8). However, due to pre-existing antibodies the pharmacokinetic profiles can be altered as reported for DAB_{486}-IL-2 in animal models (83).

1.3 Acute myeloid leukemia (AML) and normal hemopoiesis

1.3.1 AML

Acute myeloid leukemias are characterized by uncontrolled proliferation and abnormal differentiation of immature myeloid cell types in bone marrow and peripheral blood (138-140). The expansion of leukemic cells results in suppression and replacement of the normal hemopoiesis. Without treatment, death of patients within 2 months, is therefore mainly due to bone marrow failure; neutropenia, thrombocytopenia, and anemia. The age-specific incidence increases from below 1 per 100, 000 per year under age 30 to 14 at age 75. Therefore, AML is predominantly a disease of the elderly. The median age of incidence is 64 years, and 60% of all cases are over 60 (141). Potential risk factors for AML as reported in the literature, besides genetic factors, are environmental exposure to radiation, chemicals, or cytostatic drugs (142).

It is now widely accepted that leukemogenesis is a multistep process. A genetic transformation occurs in a committed progenitor, defined as the preleukemic cell, and additional genetic lesions in this cell may lead to full leukemic transformation (143). These leukemic stem cells are characterized by their ability to self-renew (144) and to mature to a certain degree along the differentiation lineages (140, 145). The type of precursor cell that is affected determines the abnormal lineage (s) observed within an AML (146-148).

The basis of the classification of AMLs and the nomenclature for AML was first proposed in 1976 by the French-American-British (FAB) Cooperative Group, and modified in 1985 (149, 150). The classification is based on conventional morphological and cytochemical methods. Information on immunophenotype and cytogenetic characteristics can be added to the conventional classification (151). Alternative approaches to morphological classification emphasise the number of lineages involved in AML and the degree of maturation of leukemic cells (152).

1.3.2 Treatment

The aim of therapy is first to induce a complete remission (CR) by using a combination of cytotoxic drugs. This treatment is also harmful to normal bone marrow cells. Therefore, after treatment bone marrow failure is usually severe and prolonged and intensive supportive care is required. Nowadays, CR is obtained in most AML patients (60-80%) (153). To eliminate the residual leukemic cells, consolidation courses of intensive chemotherapy, using combinations of cytostatic drugs, are usually given post-remission. Alternatively strategies have been developed involving the use of marrow ablative treatment followed by allogeneic or autologous bone marrow transplantation (BMT). Chemotherapy and BMT cure 20-30 and 40-60% of young and middle-aged adults, respectively (154-155). Advanced age, but also certain cytogenetic abnormalities, FAB subtype, history of previous hematological disorders, and severe leukocytosis at presentation may predict a poorer outcome (156-159).

Search for new therapeutic agents has focused on modulation of the immune system (160, 161), modulation of drug resistance phenotypes (162), and responses of leukemic cells to hemopoietic GFs (163-165) and differentiating agents like all-trans retinoic acid (166-168).

So far, no preclinical studies have been reported in which the efficacy of GF-toxins resulted in clinical applications to treat AML. The toxins, targeted to AML cells kill, cells by a mechanism entirely unrelated to irradiation or chemotherapy. The lack of unique immunologic markers on AML cells that distinguish them from normal hemopoietic cells hampered the targeting of AML cells by immunotoxins. However, antigens expressed by AML cells and by only the committed progenitors or mature hemopoietic cells offers the opportunity to use antibodies for treatment, such as CD15 (169), CD13 and CD14 (170). The fact that AML cells express high affinity receptors for GF, makes the receptors ideal targets for GF-toxin fusion proteins.

1.3.3 Normal hemopoiesis

The dynamic process of hemopoiesis could be divided roughly into three major components (171). Firstly, a rare population of common pluripotent stem cells which gives rise to the erythroid, granulocytic, monocytic, megakaryocytic and lymphoid lineages. This population of pluripotent stem cells is maintained by self-renewal (172, 173). Secondly, hemopoiesis occurs within a complex environment in the medullary cavity of the bone

marrow. Many stromal cells support the maintenance of hemopoiesis (174, 175). Thirdly, the regulation of maintenance, differentiation and also apoptosis of the hemopoietic cells is induced by GFs or cytokines (176, 177).

1.4 Growth factors and receptors

1.4.1 Growth factors (GFs)

The hemopoietic GFs regulate the proliferation and differentiation of hemopoietic progenitor cells, the initiation of maturation, the suppression of apoptotic death, and the stimulation of various functions of mature blood cells (176, 178-181). The major source of GFs are T lymphocytes, monocytes, macrophages, endothelial cells and fibroblasts. GF genes have been cloned and the recombinant GFs were used to characterize their properties in *in vitro* assays (182, 183). The *in vivo* situation is much more complex because of the interaction of GFs with other regulators.

Based on their biological action the GFs can be divided in several groups (176). Firstly, the "early" factors, stem cell factor (SCF) and FLT-3 ligand, acting on the stem cells and the early committed progenitors. IL-3 and GM-CSF act on a later stage of stem cells, the early multipotential cells. It seems that of these two GFs, IL-3 is acting on more primitive cells than GM-CSF.

Secondly, the "late" factors, which specifically exert their activities during the terminal differentiation stage of the lineages . For example, G-CSF for the granulocyte lineage, macrophage colony stimulating factor (M-CSF or CSF-1) for the monocyte/macrophage differentiation, IL-5 for the eosinophils, erythropoietin (Epo) for the erythroid differentiation and thrombopoietin (TPO) for the megakaryocytic differentiation into platelets. Although each cytokine is considered to be an early or a late acting factor, they all typically manifest a spectrum of effects crossing the boundaries between the early and late actions.

Finally, IL-1, IL-4, IL-6, IL-11 and IL-12 were characterized as GFs with mainly lymphopoietic activities, like IL-2, IL-7, IL-10 or IL-13, but they also play a role in the early hemopoiesis (184-187).

1.4.2 Growth factor receptors (GFR)

Most of the receptors for hemopoietic GFs are members of the hemopoietic receptor superfamily (188, 189). This family is characterized by the presence of four highly conserved cystein residues and a five-residue motif of Trp-Ser-X-Trp-Ser (WSXWS), in the extracellular domain. This region is referred to as the cytokine receptor homology (CRH) domain and is required for ligand binding. The cytoplasmic domains of the hemopoietic receptors are less conserved and do not appear to contain sequences indicative of enzymatic activities. This is in contrast to the group of receptor tyrosine kinases that possess intrinsic

tyrosine kinase activities in the cytoplasmic domain (190). C-kit, c-fms and FLT-3, receptors for SCF, M-CSF and FLT-3 ligand respectively, belong to this group of receptors.

The understanding of the structure and function of the receptors was improved after the observation that most receptors are activated by ligand induced dimerization and oligomerization (191). Most cytokine receptors undergo heterodimerization after ligand binding (192-194). Common components are associated with the low affinity ligand-binding subunits followed by a conformation of the receptor into a high affinity receptor. This allows interaction of cytoplasmic protein-tyrosine kinases that are associated with the intracellular domain of the receptors. Many membrane-bound receptors are also synthesized as soluble forms, lacking the transmembrane and cytoplasmic domains (195).

1.4.3 GF and GFR in AML

Normal hemopoiesis is a GF dependent process. Therefore a lot of studies are performed to give more insight into the role of GF and GFR in the pathogenesis of AML. There is now ample evidence that leukemia growth is also GF dependent (140, 196-198). Leukemic blasts from patients with AML proliferate in response to individual GFs *in vitro*, but the response is variable. Growth is more consistent when GFs are combined. However, the *in vivo* effects of GFs are still not completely understood (199). In 70% of all AML cases a variable degree of autonomous proliferation is observed (200, 201). Blocking with antibodies against a variety of GF, such as GM-CSF, IL-1, TNFα and IL-6 suggests the production of these GF by the AML cells in a direct (autocrine) or indirect (paracrine) way. Although AML cells respond *in vitro* to GF by proliferation they fail to enter terminal differentiation.

AML cells express receptors for a variety of GFs (see Table 3). Studies of the number of receptors, binding affinities and also the modulation of expression have shown that these features are not different from their normal counterparts (196, 202). It has been known that altered forms of receptors exist that exhibit constitutive activity, as described for C-fms (203), C-kit (204) and TPO receptor (205) and the common β subunit of the receptors for GM-CSF, IL-3 and IL-5 (206, 207). Also structural abnormalities have been identified in receptors, resulting in impaired function as described for the G-CSFR (202, 208).

Table 3
Expression of growth factor receptors by primary AML

GFR	detection method	expression level	AML-cases %	sites/cell	ref.
SCFR (c-kit)	Facs, Scatchard analysis	protein	60 - 90%	600 - 29, 000	(209 - 210)
FLT3	Facs	protein	80 - 90%	ND	(212, 213)
IL-6R	RT-PCR	mRNA	21% (IL-6R) 100% (gp130)	ND	(214)
IL-3R	Scatchard analysis	protein	85%	20 - 150	(215)
GM-CSFR	Scatchard analysis	protein	90 - 100%	40 - 1200	(215, 216)
G-CSFR	Scatchard analysis	protein	80 - 100%	60 - 1500	(217 - 219)
M-CSFR (c-fms)	Facs	protein	15 - 30%	ND	(220)
IL-4R	Scatchard analysis	protein	100%	20 - 130 (high aff.) 800 - 3300 (low aff.)	(221)
TpoR (mpl)	RT-PCR/ Facs	mRNA/ protein	40 - 80%	ND	(222)

GFR: growth factor receptor; RT-PCR: reverse transcriptase-polymerase chain reaction, ND: not determined

1.5 Animal models for AML

There is a need for animal models to translate clinical problems into laboratory experiments and to extrapolate new therapies from models to man. A particular animal model should be chosen based upon the specific question to be answered (223). To study the intervention of leukemia growth only rodent models are available.

Leukemia studies were performed using transplantable syngeneic leukemias, for example L1210 or P388 (224) but also the BNML, which will be introduced later. Xenografts in nude mice have also been used to evaluate the effect of therapeutic intervention in human leukemias. However, the establishment of human leukemias and lymphomas in nude mice has been variable and difficult. Their growth does not reflect the distribution pattern of leukemia or lymphoma in humans. The SCID mouse as a model for disseminated human diseases has therefore important advantages. To evaluate the effect of a given therapy, a model should be well-characterized. A reproducible growth pattern and a linear relationship between the number of inoculated cells and the leukemic cell load at a certain time point are prerequisites in this respect.

1.5.1 The Brown Norway acute Myeloid Leukemia (BNML)

The BNML was classified as an acute pro-myeloid leukemia with signs of diffuse intravascular coagulation as the leukemia progresses. The major characteristics of this model

have been described in detail (225-228). The BNML has widely been accepted as a relevant model for human AML. All experiments, described in this thesis, are performed using the *in vitro* growing LT12 subline of the BNML, which was originally reported as IPC81 (229). Different aspects of the growth of this cell line have been studied in detail during the last decade. The characteristics and differences of the LT12 and BNML are summarized in Table 4 (227, 230). The *in vitro* growing cell line offers the opportunity to explore the direct effect of antileukemic reagents on growth of the cells *in vitro* as well as *in vivo*. The *in vivo* studies with LT12, may give an insight on the side effects of therapy to normal tissues.

Table 4
Characteristics of the BNML and LT12 model

	BNML	LT12 parental cell line
maintenance	*in vivo*	*in vivo* and *in vitro*
clonogenicity	*in vivo*	*in vivo* and *in vitro*
survival time 10^6 cells i.v.	25-28 days	18-20 days
organ weights# spleen	3-4 gram	2-3 gram
liver	20-25 gram	12-14 gram
ED_{50}##	25 cells	300-400 cells
hind-leg paralysis#	0-20%	100%

at the time of death. ##ED_{50} is the number of cells to initiate leukemia in 50% of inoculated BN rats

1.5.2 Parameters to detect and quantify LT12 cells

(1) Cytology

Counting of total nucleated cell numbers has been used in combination with May-Grünwald-Giemsa staining for differential cell counting to enumerate LT12 cells as promyelocytes (227, 229).

(2) The dose-survival parameter

After i.v. injection of leukemic cells the survival time is directly correlated with the number of injected cells in the range of 10^3-10^7 cells. From an increase in the survival time after a therapeutic intervention in the leukemia growth, a reduction in leukemic cell load of up to 5-6 log can be determined. The growth of LT12 has been studied in detail (227, 230). Briefly, the hemopoiesis in the bone marrow is replaced by infiltrating leukemic cells. In the terminal stages of leukemia, the infiltration of leukemic cells into the peripheral nervous system leads to paralyzed hind limbs, followed by paralysis of the front limbs. This is observed in all animals.

(3) The leukemic cell load

Leukemia growth can be followed in organs such as the femur, spleen, liver, thymus or peripheral blood by quantification of genetically marked LT12 cells (232). The clonogenic

capacity of LT12 cells is used to determine the number of LT12 cells in *in vitro* culture systems, such as soft-agar cultures (233) or liquid limiting dilution assays.

(4) Immunophenotyping

Stable genetically transferred GFRs into LT12 can be used for discriminating leukemic cells from normal bone marrow cells by using antibodies or biotinylated GFs (234).

1.5.3 GFs and GFRs in LT12

In the LT12 model a variety of recombinant GFs of human and murine origin has been studied for their effect on leukemia growth. A summary of the data obtained so far is given in Table 5. It was observed that every GF stimulating rat bone marrow inhibits the growth of LT12 cells. The maximal anti leukemic effect was found with mGM-CSF which induced a reduction of 80% in colony formation in *in vitro* assays. To study the effect of a treatment method using GFRs as the target, the low number of receptors expressed by the parental LT12 cells could hamper the studies. Therefore, gene transfer of receptor genes into LT12 cells is necessary to use this model for evaluation of GF-toxins. The gene transfer into mammalian cells using the retroviral expression vectors carrying appropriate genes has already been used for genetically tagging cells without influencing tumorigenicity of LT12 cells (230, 232).

Table 5
Effect of recombinant GF on normal rat CFU-C and leukemic cell growth of LT12 *in vitro*

GF	normal rat CFU-C	LT12 cells
mouse/rat origin		
rmGM-CSF	↑↑↑	↓↓
rmIL-3	↔	↔
rrIL-3	↑↑	↓↓
rrSCF	↑	↓
human origin		
rhuGM-CSF	↔	↔
rhuM-CSF	↑↑	↓
rhuG-CSF	↑	↔
rhuIL-3	↔	↔
rhuIL-6	↑	↓

The effect on colony formation is expressed as the potency of forming CFU-C's per 10^5 rat bone marrow cells. ↑↑↑ 150-250; ↑↑ 50-150; ↑ 0-50.
The effect on clonogenicity of LT12 *in vitro* is shown as % reduction of control in forming colonies. ↓↓ 50-80% reduction, ↓ 20-50% reduction, ↔ no effect on colony formation

1.5.4 AML in Severe Combined Immunodeficient mice (AML/SCID)

The SCID mouse (235) is nowadays increasingly used as a recipient for xenografts to study human hemopoiesis (236-239). Mice homozygous for the SCID mutation are deficient

in functional B and T lymphocytes and consequently lack detectable immunoglobins and cellular immunity (235).

The SCID mice is used as *in vivo* model for the study of human leukemias (240-242). To improve the engraftment of myeloid leukemic cells experimental conditions have been varied. By implanting human fetal bone fragments (243, 244), or injecting cells into the renal capsule of the SCID mice or intraperitoneally (237, 245), reproducibility of engraftment has been observed. Alternatively, pre-conditioning of the mice with total body irradiation and macrophage depletion (246) was also successful. The support of human GFs as IL-3 (247), PIXY and mast-cell GF (248) facilitates growth in recipient SCID mice, but engraftment in the absence of GFs has also been observed.

The enumeration of the percentage engraftment of human leukemic cells in SCID mice correlates with the number of leukemic cells transplanted i.v. However, each leukemic cell sample should be evaluated for its capacity to grow in SCID mice. The biological variation is due to the varying number of leukemia initiating cells per sample, seeding capacity of leukemic cells, and the T cell leakiness in SCID mice (249).

The engraftment of human leukemic cells in SCID mice gives the opportunity of investigating homing, progression and treatment sensitivity. Therefore, this model can provide important diagnostic and prognostic information. Examples of this have already been reported for the *in vivo* effects of all-trans retinoic acid treatment on acute promyelocytic leukemia cells (243), c-myb antisense oligodeoxynecleotides activity against leukemic cells, and monoclonal antibodies (250).

SCID mice are also used for preclinical evaluation of immunotoxins. Successful treatment of disseminated human hemopoietic malignancies has been reported (251-255). Immunotoxins based on ricin, saporin or PAP against lymphoma cell lines, such as Daudi, Ramos or T-cell acute lymphoblastic leukemia, showed an antitumor effect, prolonging the mean survival time. A fusion toxin, DT_{390}-anti-CD3sFv, was capable of inhibiting graft-versus-host-disease generated across the major histocompability barrier of mice in SCID mice (256).

1.6. Outline of this thesis

PE and DT based immunotoxins or GF-toxins have revealed encouraging results as agents to treat cancer. The evaluation of the potential of GF toxins to specifically kill myeloid leukemic cells *in vitro* as well as *in vivo* will be described in this thesis. In addition, the *in vivo* studies will also elucidate the toxicity profiles for different GF toxins.

Experiments with huIL-6-PE4E in **Chapter 2** were performed to show the *in vitro* and *in vivo* potential of a GF-toxin in the treatment of myeloid leukemia. Primary AML as well as rat leukemic cells showed sensitivity to huIL-6PE4E.

In **Chapter 3** the potential of DT-huGM-CSF to eliminate AML cells in short term proliferation assays is described. The huGM-CSF responsiveness of 80 to 100% of AML cases showed to be predictive for sensitivity to the toxin in this assay. AML shows heterogeneity and it is important to analyse which subpopulation of AML progenitors are sensitive for DT-huGM-CSF treatment. In **Chapter 4** experiments are described to determine the sensitivity of different subpopulations of AML cells by *in vitro* exposure with DT-huGM-CSF. *In vitro* exposure of AML cells resulted in a reduction in the number of short term and long-term repopulating leukemic cells. In contrast to AML cells, normal hemopoietic progenitors were not sensitive for DT-huGM-CSF. The action of DT-huGM-CSF on the long-term leukemia initiating cell (LTL-IC) in SCID mice was studied in **Chapter 5**. The aim of this study was to evaluate the *in vivo* targeting-potential of DT-huGM-CSF. We confirmed with these experiments that DT-huGM-CSF selectively kills leukemic cells, among them the LTL-IC's, expressing the functional GM-CSFR. A possible therapeutic role of GM-CSF toxin has to be evaluated in an appropriate animal model by comparing the anti-leukemic effect with the toxic side effects. This was investigated in **Chapter 6**, in which experiments are described to determine the antileukemic effect of a DT murine GM-CSF in direct relation to the systemic toxicity and the bone marrow specific toxicity. In **Chapter 7** the results and relevance of the presented data are discussed and it will be argued whether there is any role for GF-toxin fusion proteins in the treatment of AML in the foreseeable future.

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Chapter 2

Treatment of acute myelocytic leukemia with interleukin-6 Pseudomonas exotoxin fusion protein in a rat leukemia model.

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Leukemia, vol 10: pp 1796-1803, 1996

Abstract

We studied the applicability of interleukin-6 *Pseudomonas* exotoxin fusion protein (IL-6PE4E) for treatment of acute myelocytic leukemia (AML). Leukemic cells from five out of ten AML patients studied expressed IL-6 receptor (IL-6R) and proliferation *in vitro* was inhibited in 4 of these cases. The potential of this approach *in vivo* was tested in a pre-clinical model for AML; the Brown Norway Acute Myelocytic Leukemia (BNML). To obtain IL-6R expression levels on BNML cells comparable to the numbers expressed on human AML, human IL-6R gene transfectants of the BNML sub-line LT12 (LT12/IL-6R) were generated. IL-6PE4E is cytotoxic *in vitro* to LT12/IL-6R expressing 1400 high affinity IL-6R per cell with 50% inhibition of DNA synthesis at 1 ng/ml. *In vivo* treatment of leukemic rats carrying LT12/IL-6R leukemia indicated that the maximal tolerated dose of IL-6PE4E was 275±25 µg/kg/day, when continuously administered for 7 days and resulted in a 90% reduction in leukemic cell load. At this dose level of IL-6PE4E no reduction of normal hemopoietic progenitors was seen in non-leukemic rats. At higher dose levels (350-1050 µg/kg/day) severe systemic toxicity was encountered. On the basis of these pre-clinical studies the feasibility of growth factor-toxins for selective *in vivo* targeting to AML cells is evaluated.

Introduction

A major problem in the treatment of acute myelocytic leukemia (AML) is relapse of leukemia, usually accompanied by the increased resistance of AML cells to cytotoxic drugs (1). Surface molecules, such as growth factor receptors and differentiation antigens or tumor cell antigens, are being considered as potential targets for new cytotoxic agents with increased antitumor specificity (2-4). Among the cytotoxic agents that have been chemically coupled or fused to ligands or antibodies, Pseudomonas exotoxin (PE) has been extensively employed (5, 6). Thus far, no results of in vivo treatment of AML with growth factor (GF) -toxin fusion proteins have been reported. The numbers of receptors for a variety of hemopoietic GF expressed by human (hu) AML cells are typically in the order of 100 to 1000 receptors per cell (7). Whether these numbers of receptors are sufficient to kill AML cells in vivo with GF-toxin fusion proteins has not been established.

In order to investigate this new approach we studied the chimeric protein interleukin-6 Pseudomonas exotoxin (IL-6PE4E), a fusion molecule of huIL-6 and the full-length mutated form PE4glu, which is not capable of binding to the ubiquitous expressed PE receptor (8). The cytotoxic properties of IL-6PE4E in vitro and in vivo have been established for a number of different cell types, e.g., prostate, hepatic, epidermal and myeloma cell lines have been shown to be sensitive to the cytotoxic action of IL-6PE4E (8-14). Even cells expressing 200 to 600 IL-6 receptors (IL-6R) per cell could be killed. However, besides receptor density the

efficacy of cell kill also depends on factors such as receptor complex internalisation, processing of the toxin into its active form and translocation into the cytoplasm (13).

IL-6R are expressed on AML blasts of a subgroup of patients (14, 15). These receptors bind IL-6 with a high affinity followed by rapid internalisation of the ligand (16, 17). In the present study, we first investigated whether IL-6PE4E can selectively kill human AML cells expressing IL-6R in vitro . Subsequently, we have used the BNML rat leukemia model (18-20) for comparative studies in vitro and for determination the therapeutic value of IL-6PE4E in vivo.

Materials and Methods

Human AML cells

Bone marrow cells were obtained from AML patients following informed consent. AML cases were classified cytological according to the criteria of the French-American-British Committee (FAB) (21). Purified AML cell suspensions were obtained as described (22). Briefly, AML cells were isolated by Ficoll-Isopaque centrifugation. Subsequently, T-lymphocytes and monocytes were removed from the leukemic cell suspension by erythrocyte-rosette ficoll sedimentation using 2-aminoethyl isothiouronium bromide (AET)-treated sheep red blood cells, and adherence to plastic, respectively. Cells were used after cryopreservation using a controlled freezing apparatus and storage in liquid nitrogen.

Experimental Animals

Brown Norway inbred rats (BN/RijHsd) were purchased from Harlan CPB, Rijswijk, The Netherlands. Female rats 12-14 weeks of age were used (body weight 120-150 g). Animals were bred under specified pathogen free (SPF) conditions and maintained under clean conventional conditions. FI(B6xCBA) mice were bred in the Central Animal Facility of the Erasmus University, Rotterdam, The Netherlands, and maintained under conventional conditions. All animal experiments have been approved and carried out according to the guidelines set by the Animal Research Ethics Committee of the Erasmus University Rotterdam.

Growth factors and growth factor toxins

Recombinant human IL-6PE4E and IL-6 (glycosylated, recombinant human IL-6, Sigosix ™) were kindly supplied by Dr A. Ythier, Ares Serono, Geneva, Switzerland. A mutated form of IL-6PE4E lacking the ADP ribosylating activity was previously described (13). A second rhuIL-6 was obtained from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (CLB) Amsterdam, The Netherlands. For receptor studies using flow cytometry, IL-6 was biotin-labeled, as described for growth factors (23, 24).

In vitro suspension culture

The BNML subline LT12 was grown in RPMI 1640 medium (GIBCO, Paisley, UK) supplemented with 10 % (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

DNA-synthesis/^{3}H-Thymidine (^{3}H-TdR) incorporation

DNA synthesis was measured as described (25). Briefly, human AML cells (2×10^4) were cultured for 3 days in 96-well round-bottom microtiter plates in 100 μl serum-free medium, with the addition

of IL-6PE4E. Eighteen hours before harvesting, 0.1 μCi ^{3}H-TdR (Amersham International, Amersham, UK) was added. Cells were collected using an automatic cell harvester (Skatron, Lier, Norway), and the cell-associated radioactivity was measured in a liquid scintillation counter (Pharmacia-LKB, Bromma, Sweden).

For rat LT12 and LT12/IL-6R cell lines, $1x10^4$ cells/well were plated in 96-well flat-bottom microtiter plates in 200 μl of supplemented medium as described for LT12. All cultures were performed in triplicate and data are expressed as mean counts per minute (CPM) or as percent of control.

Leukemic colony assay (CFU-L)

LT12 cells were cultured in 1 ml mixture of Dulbecco's Modified Eagles Medium (GIBCO), 0.3% (w/v) agar, 20% (v/v) FCS, $5x10^{-7}$ M β-mercaptoethanol, 2 mM L-glutamine, 50 U/ml penicillin and 50 ml streptomycin. Triplicate dishes were cultured at 37°C and 10% CO_2 and colonies were counted at day 10.

DNA transfection

The retroviral vector pXM6/IL-6R/Neo (26) was transfected into LT12 cells using the Ca-phosphate precipitation method (27). Transfected cells were cultured for 48 hours and then selected in an agar culture system containing G418 (600 μgml GIBCO). G418-resistant colonies were isolated and expanded in liquid culture medium and tested for IL-6R expression by flowcytometry.

Flow cytometric analysis of IL-6R expression

After washing in PSA (phosphate buffered saline containing 1% (v/v) fetal calf serum and 0.02% (w/v) sodium azide), AML cells or LT12 cells (10^5-10^6 per sample) were incubated with biotinylated huIL-6 (50 nM) in a volume of 50 μl for 1 hr at room temperature. The cells were then stained with streptavidin-phycoerythrin (1:200 v/v, Molecular Probes, Eugene,
OR, USA) for 30 minutes on ice. Each incubation was followed by a wash step in PSA. Fluorescence signals of the cell labeling were amplified by incubating the cells for 30 minutes on ice with alternate layers of biotinylated goat-anti-streptavidin (1:100 v/v, Vector Laboratories, Burlingame, CA, USA) and streptavidin-phycoerythrin. Specificity of binding of biotin-IL-6 was verified by including parallel incubations of the cells with biotin-IL-6 in the presence of a 100-fold molar excess of IL-6. Cells were analyzed using the FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

[^{125}I]-IL-6 binding

Purified recombinant IL-6 was labeled with ^{125}I according to the Bolton and Hunter procedure (28). Binding studies were performed exactly as described (26). Mean receptor numbers per cell and binding affinities were derived by Scatchard analysis (29). Experiments were conducted in duplicate.

Treatment of leukemic and non leukemic rats with IL-6PE4E

Leukemic rats were obtained by injecting $5x10^5$ LT12 cells, expressing IL-6R, (LT12/IL-6R cells) intravenously (i.v.) on day 0. On day 3, or day 10, Alzet mini-osmotic pumps type 2001 (Charles River, Sulzfeld, Germany) were implanted i.p. for continuous delivery of IL-6PE4E. The pumps released 1.0 μl/hour for 7 days. IL-6PE4E was diluted in sterile PBS containing 1 mM Nicotinamide adenine dinucleotide (NAD^+) as the stabilizing agent (13). The pumps were removed 10 days after implantation. Each group consisted of 6 animals. Blood samples were taken from alternate animals during and after the treatment. Rats that died during the dosage-escalation treatment with IL-6PE4E

were subjected to gross pathology and histological examination. Statistical analysis of differences in survival time was performed by Kaplan-Meier survival analysis and the Peto-Peto-Wilcoxon test using Abacus Concepts, Survival tools for StatView 1994 (Abacus Concepts, Inc., Berkeley, CA, USA).

In two separate experiments BN rats, not injected with leukemic cells, were treated for 7 days with the maximal tolerated dose of IL-6PE4E (275 μg/kg/day) to determine the effect on liver and on the number of peripheral blood cells during the treatment. The animals were sacrificed on day 8 and the effect on the hemopoietic progenitor cell compartment in bone marrow was evaluated by using clonogenic assays for the hemopoietic progenitors.

Blood analysis

Blood was collected in heparinized tubes (Sarstedt, Nümbrecht, Germany) by tail clipping and immediately analyzed using a Sysmex F-800 hemocytometer (TOA Medical Electronics, Hamburg, Germany). The differential white blood cell count was determined after May-Grünwald-Giemsa staining. Reticulocyte percentages were determined by standard counting after staining with thiazol orange and measured with a FACScan and Retic-count computer software.

Heparinized plasma was collected after centrifugation and kept frozen at -20°C. Biochemical parameters were analyzed in plasma, using Diagnostica Merck-kits (Darmstadt, Germany) and measured with a multi-test analyser (Eppendorf Merck, type Elan, Hamburg Germany) at 37°C and included alanine aminotransferase (ALAT), aspartate amino-transferase (ASAT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and total bilirubin (TBIL). A sensitive ELISA developed for huIL-6 (30) was validated for the detection of IL-6PE4E to determine IL-6PE4E concentrations in the plasma.

Quantification of normal hemopoietic progenitors

Femoral bone marrow cell suspensions were prepared by flushing rat femora with α MEM (GIBCO) and the total number of nucleated cells was determined. For the colony assays of normal bone marrow progenitors, the cells were cultured in triplicate 35-mm tissue culture dishes (Costar, Cambridge, MA, USA) in 0.8% methylcellulose, 20% horse serum, 10^{-6} M β-mercaptoethanol, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were supplemented with recombinant murine GM-CSF (500 U/ml, Behring Werke A.G., Marburg, Germany) for granulocyte-macrophage colony formation (CFU-GM) and cultured in a humidified incubator at 37°C at 10% CO_2. Colonies were counted on day 8.

For the rat-to-mouse colony forming unit-spleen assay (CFU-S), B_6CBA mice, 12 weeks of age, were lethally irradiated with 8.75 Gy γ rays (Gammacell 40, Atomic Energy of Canada, Ottawa, Canada) and injected with graded numbers of rat bone marrow cells i.v. The mice were sacrificed on day 8 or day 12. The number of macroscopically visible spleen colonies was counted. The assay has been described in detail elsewhere (31). For determination of the marrow repopulating ability, to generate CFU-GM (MRA(CFU-GM)), BN rats were lethally irradiated with 10 Gy γ rays one day before they were injected with graded femur fractions from treated rats and were sacrificed on day 11. The production of granulocyte-macrophage progenitors (CFU-GM) in the femoral bone marrow is taken as a measure of marrow repopulating ability essentially as described by Hodgson and Bradley (32).

Table 1
Effects of IL-6PE4E on 10 AML samples in relation to IL-6R expression

Patient	FAB	IL-6R	^{3}H-TdR incorporation			ID50[f] (ng/ml)
	type[a]	expression[b]	no IL-6[c]	huIL-6[d]	IL-6PE4E[e]	IL-6PE4E
1	M1	+	1,582	1,662	824	1,000
2	M2	+	2,562	3,420	701	400
3	M5	+	4,136	7,001	2,386	>5,000
4	M5	++	1,006	1,267	91	70
5	M5	+	3,149	3,147	983	300
6	M2	-	1,866	1,338	1,247	>5,000
7	M4	±	4,925	11,847	3,368	3,000
8	M5	±	969	1,030	797	>5,000
9	M5	-	1,051	1,350	839	>5,000
10	M5	-	1,785	1,738	1,241	>5,000

([a]) FAB: French-American-British Committee classification of leukemia; ([b]) IL-6R expression using biotinylated huIL-6 and flow cytometry; ([c]) samples were incubated without growth factor; or ([d]) with 2,000 U/ml IL-6 (CLB); or ([e]) 5,000 ng/ml IL-6PE4E; ([f]) the ID_{50} is the concentration of IL-6PE4E required for 50% inhibition of DNA synthesis and is derived from dose-effect experiments with IL-6PE4E.

Results

Effects of IL-6PE4E on human AML cells in vitro

Human AML cells were first tested *in vitro* for their sensitivity to IL-6PE4E in DNA synthesis assays (Table 1). Five AML samples expressing IL-6R (Fig 1A) and five samples with no detectable expression of the IL-6R (Fig 1B) were selected. The AML cells were exposed to graded amounts of IL-6PE4E and specificity of cytotoxicity was determined by performing parallel competition assays in which 2,500 ng/ml of huIL-6 was added. The cytotoxic effects are expressed as ID_{50} values, i.e. the concentration at which a 50% reduction in DNA synthesis is observed. AML cells from patient 4 (AML-M5) were most sensitive, with an ID_{50} of 70 ng/ml (8×10^{-10} M) (Fig 2). The AML cells from this patient showed a relatively high IL-6R expression (Table 1, patient 4). AML cells of patients 1, 2 and 5 were less sensitive to IL-6PE4E, with an ID_{50} of 1000, 400 and 300 ng/ml, respectively. This correlated with a lower expression of IL-6R, as determined by flow cytometric analysis (data not shown). Although AML cells from patient 3 expressed IL-6R, the cells were insensitive to IL-6PE4E, at a dose of 5,000 ng/ml. In the group of AML patients without detectable IL-6R expression (patients 6-10), no inhibitory effects of IL-6PE4E were seen (Table 1).

Cytotoxic effects of IL-6PE4E on LT12 parental and LT12/IL-6R cells in vitro

Parental LT12 and LT12/IL-6R were first tested for their sensitivity to IL-6PE4E in a DNA synthesis assay. Although less than 100 IL-6R are expressed on the parental LT12 cell

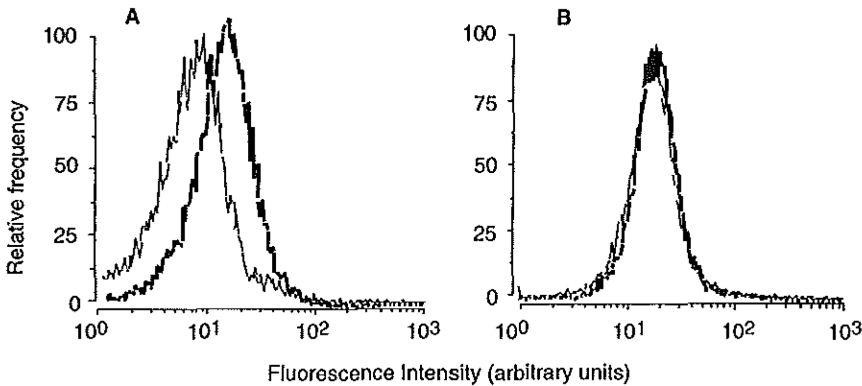

Figure 1
Fluorescence histograms of IL-6 receptor expression measurements. Panel A shows a representative patient from the group with detectable expression of IL-6 R. Panel B shows a patient from the group with no detectable expression of IL-6R. The cells were incubated with biotinylated IL-6 followed by streptavidine FITC (thick line), or in the presence of a 100 x molar excess of nonbiotinylated IL-6 (thin line).

line, the ID_{50} value of IL-6PE4E was 20 ng/ml ($3x10^{-10}$ M). The ID_{50} value of IL-6PE4E for the LT12/IL-6R cell line, expressing approximately 1400 high affinity (K_d 160 pM) and 2450 low affinity IL-6R (K_d 1.4 nM) per cell, was 1.3 ng/ml ($1.5x10^{-11}$ M) (Fig 3A). Toxicity of IL-6PE4E to LT12/IL-6R cells could be blocked by adding an excess of recombinant huIL-6, indicating that the toxicity was mediated specifically through interaction with the IL-6R (Fig 4). When an IL-6PE4E fusion protein lacking enzymatic activity (13) was added to the cells, only minimal reduction in proliferation was found. This was also the case when recombinant huIL-6 was added (Fig 3B). The inhibition of DNA synthesis was directly correlated with loss of proliferative potential, as determined by the capacity of the parental LT12 and the LT12/IL-6R cells to form colonies *in vitro* (Fig 4). Toxicity of IL-6PE4E to CFU-GM in normal bone marrow was approximately 1000 fold lower as compared to LT12/IL-6R cells.

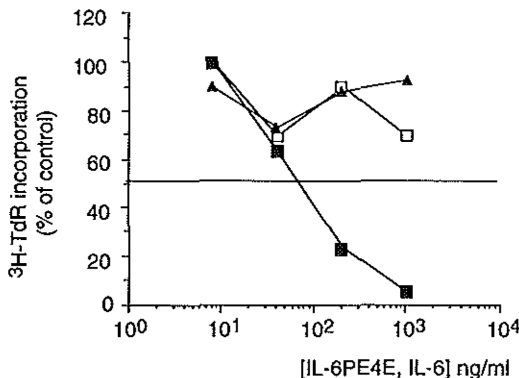

Figure 2
Cytotoxicity of IL-6PE4E to AML cells (patient 4 table 1) using the ^{3}H-TdR incorporation assay. Specificity of IL-6PE4E (■) was tested in comparison to IL-6 (▲) or with IL-6PE4E (□) with an excess of IL-6 (2.5 μg/ml) added to the culture medium.

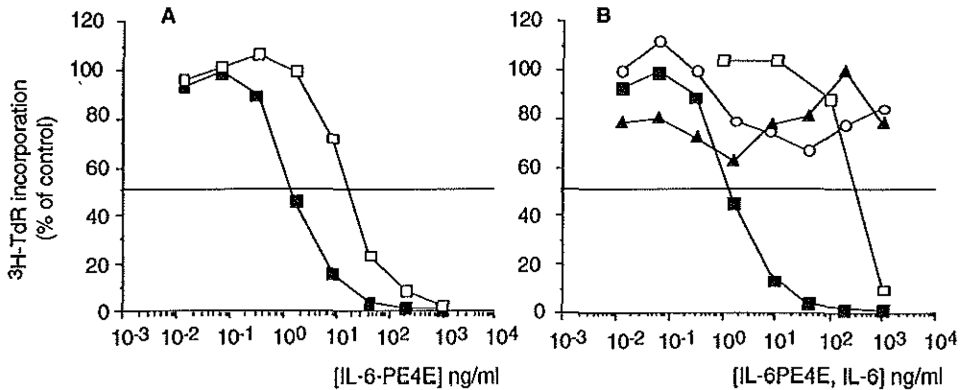

Figure 3
A Cytotoxic effects of IL-6PE4E on LT12/IL-6R cells (■) compared to the parental LT12 cell line (□) measured using the ^{3}H-TdR incorporation assay.
B Specificity of cytotoxic effect of IL-6PE4E on LT12/IL-6R (■) was tested by comparing the response to incubation with IL-6PE4Emutant (○), or IL-6 (▲) or IL-6PE4E with an excess IL-6 (2.5 μg/ml)(□).

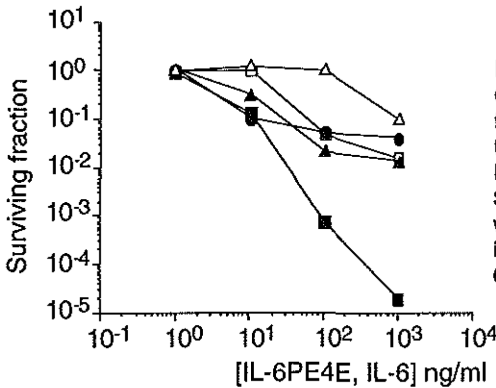

Figure 4
Cytotoxic effects of IL-6PE4E on colony formation of LT12/IL-6R cells (■) compared to parental LT12 cells (□) and to normal bone marrow progenitor cells (CFU-GM) (Δ). Specificity of IL-6PE4E for LT12/IL-6R cells was tested by comparing the response to incubation with IL-6PEmutant (●) or with IL-6 (▲).

Treatment of leukemic rats with IL-6PE4E

After i.v. injection of LT12/IL-6R cells into BN rats, all animals developed leukemia with total marrow infiltration and the characteristic symptoms at the terminal stage of the disease, i.e. a moderate increase in liver and spleen weight and paralysis of the hind legs as previously described (33). The *in vivo* growth characteristics of the genetically modified LT12 cells were similar to those of the non-transfected parental cells. LT12/IL-6R cells isolated from bone marrow, spleen, and peripheral blood showed unaltered expression of the IL-6R. The relation between the number of leukemic cells inoculated and the corresponding survival time was established. For each factor of 10 (1 log) reduction in leukemic cell number an increase

Table 2
Mortality of leukemic rats at various dose levels of continuous administration of IL-6PE4E

IL-6PE4E dose level*	rats per group	% mortality
1050	3	100
350	14	86
310	12	34
275	16	6
180	6	0
90	12	0

*in μg/kg/day delivered by osmotic minipumps placed i.p.

in the survival time of 1.9 days was found (data not shown). The survival time assay is used as an evaluation parameter for the therapeutic efficacy of IL-6PE4E.Treatment of rats with IL-6PE4E started 3 days after inoculation of the LT12/IL-6R cells, at which time-point the leukemic cells had disappeared from the blood and settled in the bone marrow (34). Undisturbed leukemia development after inoculation of $5x10^5$ cells leads to death on day 17. A dose range from 90 to 1050 μg/kg/day was tested to determine the maximal anti-leukemic effect. Animals treated with 275 μg/kg/day showed a significant increase of 2 days in the survival time when compared to PBS/NAD treated leukemic controls ($P = 0.002$ Kaplan-Meier survival analysis) (Fig 5). From the cell dose-survival relationship, it could be calculated that a 1 log reduction in the leukemic cell load was obtained.

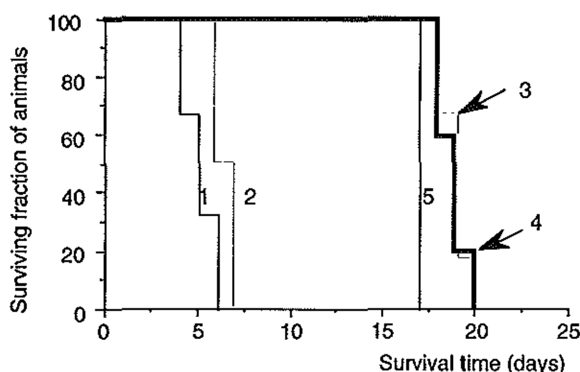

Figure 6
Survival curves of leukemic animals receiving IL-6PE4E at various dose levels. On day 0 rats were injected with $5x10^5$ LT12/IL-6R cells (6 rats per group). A 7 day treatment period (continuous infusion using osmotic minipumps placed i.p.) was started on day 3 (groups 1, 2 and 3) or on day 10 (group 4). The dose levels tested were 1050 μg/kg/day (line 1), 350 μg/kg/day (line 2), 275 μg/kg/day from day 3 (line 3) or 275 μg/kg/day from day 10 (4). The control (line 5) received the vehicle PBS/NAD only.

Treatment with IL-6PE4E (275 µg/kg/day) was also given in advanced stage of leukemia, from day 10 to 17. The increase in survival time was comparable with that of the animals treated from day 3 to 10 with 275 µg/kg/day (Fig 5). This was confirmed by quantitation of the leukemic cell content of the femoral bone marrow after the treatment with an *in vitro* clonogenic CFU-L assay in semi-solid agar (data not shown). Thus, the anti-leukemic effect of IL-6PE4E appeared to be independent of the leukemic cell load.

At dosages of 310 µg/kg/day or higher animals died of acute toxicity typically on the third day of treatment (Table 2). Gross pathology findings were hydrothorax and hepatomegaly. Histopathological analysis revealed pulmonary edema (Fig 6) and mild liver cell degeneration. In the bone marrow a normal hemopoiesis was observed with particularly active megakaryopoiesis (Fig 6). Treatment of equimolar dosages of huIL-6 did not result in any sign of side toxicity to the lungs. This suggests that toxicity observed for IL-6PE4E is caused by the toxin portion of the molecule (data not shown).

To determine toxicity towards normal tissues, non-leukemic rats were treated for 7 days using osmotic mini-pumps, placed i.p., releasing 275 µg/kg/day. An average level of 12 ng IL-6PE4E/ml was measured in plasma, with a range of 2 to 30 ng/ml. Table 3 shows that treatment with IL-6PE4E had no effect on the absolute numbers of white blood cells (WBC), but differential counts indicated an increase in granulocyte numbers (in normal BN rats 2% of WBC are granulocytes), indicative of inflammatory reactions. The latter was in agreement with the observed iron accumulation in macrophages of spleen and bone marrow (data not shown). Platelet levels increased during the treatment with IL-6PE4E and returned to normal after the treatment was completed. Red blood cell counts decreased and the percentage of reticulocytes was increased. Analysis of the liver function revealed a mild hepatotoxicity showing only a slight elevation of aspartate aminotransferase (ASAT) at day 7 (data not shown).

Table 3
Peripheral blood counts in normal rats during IL-6-PE treatment

Day of treatment	WBC ($x10^9/L$)	platelets ($x10^9/L$)	RBC ($x10^{12}/L$)	Reticulocytes %
0	16.7 ± 3.6*	692 ± 92	8.5 ± 0.4	2.8 ± 0.1
3	12.1 ± 1.3	789 ± 90	7.8 ± 0.1	5.0 ± 0.6
5	18.3 ± 0.6	1011 ± 88	7.3 ± 0.2	6.7 ± 0.5
7	18.9 ± 2.9	1164 ± 93	6.5 ± 0.1	nd

WBC, white blood cells; RBC, red blood cells; nd, not done
* Mean number of blood cells (3 rats per group ± SEM)

To determine the effect of IL-6PE4E on hemopoietic progenitors in the bone marrow, rats were sacrificed one day after the treatment period of 7 days. IL-6PE4E did not lead to a

reduction of the numbers of CFU-GM, CFU-S day 8, CFU-S day 12 or in the number of the progenitors measured with the marrow repopulating ability (MRA) (Table 4).

Table 4
Effect of IL-6PE4E on the femoral bone marrow content of normal hemopoietic progenitor cells after 7 days IL-6PE4E treatment compared to controls

progenitor cell type	rats		
	control	PBS/NAD	IL-6PE4E
exp 1			
CFU-GM	7020 ± 290	7920 ± 270	8370 ± 90
CFU-S day 8	57 ± 9	54 ± 7	64 ± 9
CFU-S day 12	25 ± 5	21 ± 7	24 ± 3
MRA CFU-GM	392 ± 32	240 ± 30	580 ± 81
exp 2			
CFU-GM	1080 ± 54	820 ± 106	1150 ± 190
CFU-S day 8	37 ± 3	38 ± 2	61 ± 4
CFU-S day 12	6 ± 1	10 ± 1	13 ± 1
MRA CFU-GM	228 ± 14	520 ± 30	490 ± 30

Mean number of progenitors per femur (x 100) ± SEM (3-5 rats per point)

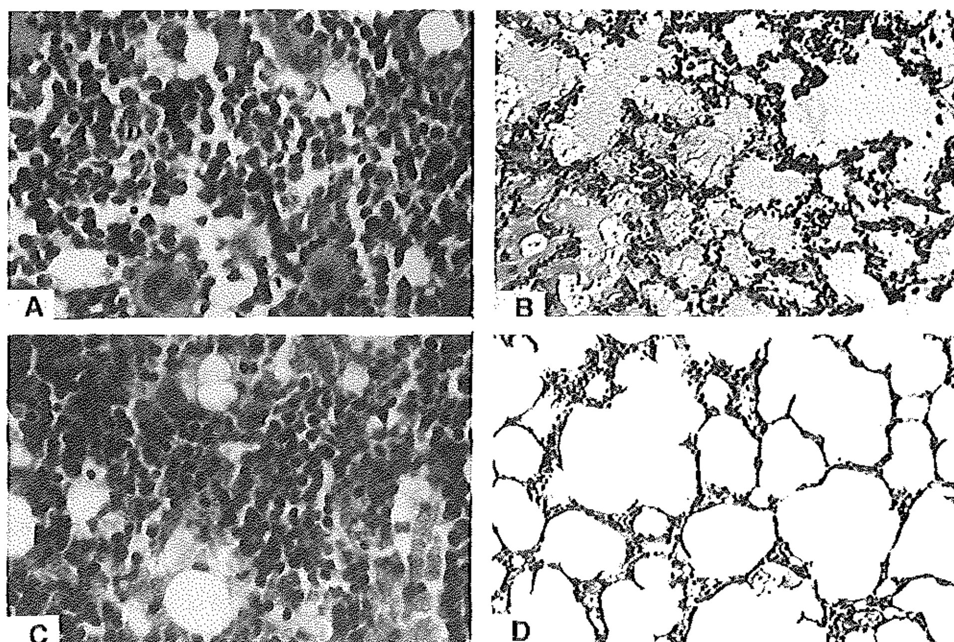

Figure 6
Histological analysis of treated rat organs. (A) Femur section of a rat treated with 275 μg IL-6PE4E/kg/day for 7 days using osmotic pump administration i.p. This section showed stimulated megakaryopoiesis. (C) Femur section of an untreated rat (x630) (B) Lung section of a treated rat. The peribronchovascular spaces contain edema fluid. (D) Lung section of an untreated rat (x100)

Discussion

In this study we have presented both *in vitro* and *in vivo* data indicating that proliferation of human AML blast can be inhibited by IL-6PE4E. The rat LT12/IL-6R cell line, in which we transfected the huIL-6R α unit, expressed 1400 high affinity IL-6 receptors, whereas nontransfected LT12 cells expressed less than 100 IL-6 binding sites. The higher numbers of IL-6 receptors expressed by the LT12/IL-6R cell line, increased the specific sensitivity of this cell line to IL-6PE4E 10 fold when evaluated with the ^{3}H-TdR incorporation assay (Fig 3A) and a 1000-fold (3 log) in the colony culture assay (Fig 4). This indicates that the number of growth factor receptors correlates with the sensitivity of leukemic cells for GF-toxins. However, expression of functional IL-6R *per se* does not predict that AML cells are sensitive to IL-6PE4E. This was evident in a case of AML cells (patient 3) in which the DNA synthesis of leukemic cells was not inhibited, despite the presence of the IL-6R. This could be due to the fact that the IL-6R expression was measured on the whole leukemic cell population, which may not be representative for the small fraction of the proliferating cells. Alternatively, these cells could have other kinetics in toxin processing.

The continuous administration of IL-6PE4E *in vivo,* at a dosage of 275 µg/kg/day, resulted in a significant increase in survival time of 2 days, which reflects a 90% (1 log) reduction in the leukemic cell load (Fig 5). Higher dosages caused acute death. The sites at risk for side effects of IL-6PE4E, besides hemopoietic tissues, are liver, endothelial cells, and the neural system (35). Evidence for a mild liver cell damage was indeed found in the IL-6PE4E-treated rats. In contrast to others, who reported hepatoxicity as the major cause of death (13, 36-38) for high dosage IL-6PE4E treatment, our autopsy findings indicated that the rats died of vascular leak syndrome (VLS). This has also been observed in rats treated with the immunotoxin BR96 sFv-PE40 by Siegall et al (39).

The toxicity of IL-6PE4E towards the normal hemopoietic progenitor cells could also be evaluated in the rat leukemia model LT12, because human IL-6 cross reacts with rat cells (40). The observed effects of IL-6PE4E at the MTD level on the blood cellularity were similar to that of IL-6, i.e. thrombopoiesis and anemia (41-44). Recent studies have shown that i.p. administrated IL-6 can induce anemia in rats (45). The anemia was caused by intestinal blood loss associated with inflammation. Because hemolysis could also be one of the causes of reduction in RBC, it is difficult to study the nature of IL-6PE4E induced anemia in rats. No significant effect of IL-6-PE treatment on myelopoiesis was seen, as was evident from the fact that the number of CFU-GM, CFU-S day 8, CFU-S day 12 or MRA-CFU-GM progenitor cells were not reduced. The lack of toxicity on these myeloid progenitors would suggest that the number of IL-6R on these cells is too low or even not present to mediate toxicity. Others have shown in humans that the IL-6R was only present on a smal subpopulation of the CD34^{+} cells (46), implying that only this minor fraction of progenitors

would be susceptible to IL-6PE4E. This leaves enough room for the speculation that the pluripotent stem cell might lack IL-6R expression.

To achieve a better therapeutic effect, higher levels of IL-6PE4E may be required to eliminate all leukemic cells in the bone marrow. IL-6PE4E plasma levels reached an average of 12 ng/ml, sufficient to kill rat leukemic cells *in vitro* (Fig 4), but IL-6PE4E concentration in bone marrow remains unknown. However, it is likely that IL-6PE4E reached the bone marrow and binds cells, since a 90 % reduction in leukemic cell number was observed and, in addition, histopathological analysis of the femur showed a stimulated megakaryopoiesis as compared with control rats. This dual activity of IL-6PE4E should be recognized as a possible general characteristic of GF-toxins, i.e. a stimulation of cell proliferation induced by low concentrations of GF-toxins , while a reduced proliferation is only achieved at higher concentrations.

The plasma levels of IL-6PE4E are dependent on the number of potential binding sites. It can also not be excluded that the soluble IL-6R α and gp130 proteins (47, 48) act as mediaters for IL-6PE4E clearance. Owing to the pleiotrophic acting of IL-6, the IL-6PE4E is less suited for therapeutic applications. To minimize the number of potential targets we infer that a better therapeutic index may be reached with GF-toxins based on GF, that have less pleiotrophic activities.

In conclusion, our studies show that GF-toxins in principal, can be targeted to AML cells that express the corresponding receptor and that this leads to inhibition of leukemic cell growth both *in vivo* and *in vitro*. These pre-clinical studies warrant further investigation of GF-toxins as therapeutic agents in the treatment of AML.

Acknowledgements

We thank Dr. A. Ythier and Dr. M. Gascon, Ares Serono (Geneva, Switzerland) for providing rhuIL-6 and IL-6PE4E, Dr. R. Yun-Fang and Dr. Y.Z. Xi for their collaboration in transfecting the rat leukemic cell lines, J.F. Gaiser for performing the ^{125}I-labeling of IL-6 and binding experiments, Drs. M. Jongen-Lavrencic for performing the IL-6 ELISA assay, W.P. van Schalkwijk for performing the serum chemistries and Dr. A.H. Mulder from the Department of Pathology, Erasmus University, for performing the histologic examinations.

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Chapter 3

Sensitivity of human acute myeloid leukemia to Diphtheria toxin-GM-CSF fusion protein.

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British Journal of Haematology, accepted for publication

Abstract

The potential to selectively eliminate acute myeloid leukemia (AML) cells with the huGM-CSF-*Diphtheria* Toxin fusion protein (DT-huGM-CSF) was studied under conditions of autonomous proliferation *in vitro* with no growth factors (GFs) added and after growth stimulation with a mixture of huG-CSF, huFIL-3 and huSCF. DNA synthesis was maximally inhibited after a 48 hours exposure to DT-huGM-CSF. Cell viability and AML colony forming ability *in vitro* were reduced. Eighteen out of 22 samples were found to be sensitive to DT-huGM-CSF, with 50% inhibition of DNA synthesis (ID_{50}) at concentrations ranging from 0.1 to 16 ng/ml, and four samples were minimally or not sensitive to DT-huGM-CSF ($ID_{50} \geq 99$ ng/ml). From the 15 samples that showed autonomous proliferation 13 were sensitive to inhibition of proliferation by DT-huGM-CSF. The level of GM-CSF receptor (GM-CSFR) expression was determined by flow cytometry after labeling with specific antibodies for the alpha and beta subunits. Although the toxicity to DT-huGM-CSF was specifically mediated by the GM-CSFR, no correlation was found between the level of expression of the GM-CSFR alpha or beta subunit and the sensitivity for DT-huGM-CSF. These *in vitro* studies show that the DT-huGM-CSF fusion protein can be used for specifically targeting and eliminating leukemic cells of the majority of AML cases.

Introduction

Growth factor receptors (GFR) expressed on malignant cells can serve for specific targeting of toxic molecules, e.g. bacterial toxins (1, 2). To exploit this principle in the treatment of hemopoietic malignancies, truncated forms of *Diphtheria* toxin (DT) were fused to human interleukin-2 (IL-2) (3), IL-6 (4), human granulocyte-colony stimulating factor (G-CSF) (5), murine IL-4 (6), murine granulocyte-macrophage colony stimulating factor (mGM-CSF) (7) and mIL-3 (8). To achieve toxicity with the DT-cytokine fusion proteins it has been shown that receptor binding and internalisation by receptor-mediated endocytosis is required. Following processing of the DT into its active form and delivery of the NH_2-domain associated ADP-ribosyltransferase to the cytoplasm (9-12), elongation factor 2 is blocked leading to protein synthesis inhibition, a decrease in cell proliferation and cell death.

Human acute myeloid leukemia (AML) cells frequently express receptors for multiple hemopoietic GFs, including the GM-CSF receptor (GM-CSFR) (13, 14) High affinity GM-CSFR are heteromeric complexes consisting of α and β subunits (15-17). Whether huGM-CSF-toxin fusion proteins can be used to eliminate human AML cells has not yet been established. The fact that the number of high affinity GM-CSFR on AML cells are in general low, i.e., in the order of 100 to 1000 receptors per cell (18) might be a potential limitation of this approach.

In the present study, we evaluated the antiproliferative effects of DT-huGM-CSF, a fusion molecule of huGM-CSF with the cytotoxic fragment A of DT, on AML cells *in vitro* in relation to the levels of expression of GM-CSFR α and β subunits.

Materials and Methods

Human AML cells

Bone marrow cells were obtained from patients with AML or myelodysplastic syndrome (MDS) following informed consent. AML cases were classified cytologically according to the criteria of the French-American-British (FAB) scheme (19, 20). Purified AML cells were obtained as described (21). Briefly, low density cells were isolated by Ficoll-Isopaque centrifugation. Subsequently, T-lymphocytes and monocytes were removed from these cell samples by erythrocyte-rosette ficoll sedimentation using 2-aminoethyl isothiouronium bromide (AET)-treated sheep red blood cells, and adherence to plastic, respectively (22). The purified cell samples contained less than 1% T-lymphocytes and monocytes. Cells were frozen using a controlled freezing apparatus and storage in liquid nitrogen. After thawing, viability ranged from 78 to 98%.

Preparation of DT-huGM-CSF

To construct pRKDTGM, encoding DT_{388}-huGM-CSF, a 0.41 Kb NdeI-HindIII fragment encoding huGM-CSF was ligated to the 4.2 Kb NdeI-HindIII fragment of pVCDT1-IL-2 (21). Thus pRKDTGM encodes the amino terminus of diphtheria toxin fused to the 127 amino acid huGM-CSF mature protein, with the amino acids RPHM between amino acid Thr386 of DT and Ala1 of huGM-CSF, and Ala following Glu127 of huGM-CSF just before the stop codon, as illustrated in Fig 1. The plasmid pRKDTGM was expressed in E.coli, and the pure monomeric protein was purified as described previously for IL-6-PE4E (24) and IL-4-PE4E (25).

^{3}H-Thymidine (^{3}H-TdR) incorporation assay

Cells (2 x 10^4) were cultured for 48 hours in 96-well round-bottom microtiter plates in 100 µl serum-free culture medium (SFM) (26) containing DT-huGM-CSF. The effects of DT-huGM-CSF were tested without addition of GFs, or in the presence of human stem cell factor (huSCF 100 ng/ml; Amgen Biologicals, Thousand Oaks, CA, USA), huIL-3 (25 ng/ml; Gist Brocades, Delft, The Netherlands) and huG-CSF (100 ng/ml; Behring Werke A.G., Germany). Eighteen hours before harvesting, 0.1 µCi ^{3}H-TdR (2 Ci/mmol, Amersham International, Amersham, UK) was added. Cells were collected using an automated cell harvester (Skatron, Lier, Norway), and the cell-associated radioactivity was measured in a liquid scintillation counter (Pharmacia-LKB, Bromma, Sweden). In competition experiments an excess amount of huGM-CSF (200 ng/ml) (Behring Werke A.G., Germany) was added simultaneously with DT-huGM-CSF. All cultures were performed in triplicate. Data are expressed as percentages of control.

Flow cytometric analysis of GM-CSFR expression

After washing in PSA (phosphate buffered saline containing 1% (v/v) fetal calf serum and 0.02% (w/v) sodium azide), leukemic cells (10^6 per sample) were incubated with mouse monoclonal antibodies against either the huGM-CSFR α chain or the huGM-CSFR β chain (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) in a volume of 50 µl for 45 min on ice. The cells were then labeled with biotinylated goat anti mouse antibody F(ab')2 (Tago Inc., Burlingame, CA, USA) for 30 minutes on ice and stained with phycoerythrin-conjugated streptavidin (1:200 v/v, Molecular

Probes, Eugene, OR, USA) for 30 minutes on ice and analysed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). In the FACScan analysis cells were stained with propidium iodide (1 µg/ml) and the dead cells were gated out. To compare expression of the GM-CSFR subunits between AML cell samples, the ratio of the mean specific fluorescence intensity of the cells labeled with the GM-CSFR α or β monoclonal antibody and the mean auto fluorescence intensity of the control cells were calculated.

Colony culture assay for leukemic progenitors

Cells were plated in 35-mm dishes (Becton Dickinson) in 1 ml Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD, USA) containing 0.9% methylcellulose, 1.5% BSA (Sigma), insulin (10 µg/ml, Sigma), linoleic acid (1.5×10^{-5} M, Merck, Darmstadt, Germany), cholesterol (1.5×10^{-5} M, Merck), sodium selenite (1×10^{-7} M, Merck), β-mercaptoethanol (1×10^{-4} M, Merck), hu transferrin (0.62 mg/ml Behring Werke) penicillin (100 U/ml, Gibco), and streptomycin (850 µg/ml, Gibco). Assays were performed in triplicate in the presence of huGM-CSF (5 ng/ml, Behring Werke), huIL-3 (10 ng/ml, Sandoz BV, Basel, Switzerland), and huG-CSF (100 ng/ml, Amgen Biologicals, Thousand Oaks, CA, USA). Cells were incubated in a humidified chamber containing 5% CO_2 at 37°C. Colonies of more than 50 cells were scored at day 14. Each estimate is based on the data of triplicate cultures.

Statistical analysis

Statistical significance of differences in response to DT-huGM-CSF between samples was determined using the paired Student *t* test. Correlations were calculated using the Spearman Rank Correlation test.

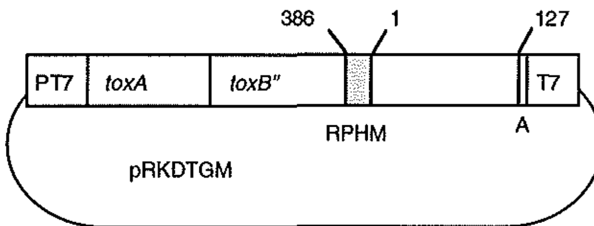

Figure 1
Expression vector of the pRKDTGM. toxA; active moiety of DT (fragment A), toxB"; translocation moiety (truncated fragment B), PT7; T7 promotor, T7; transcription terminator.

Results

Time course study

A time course experiment was carried out with AML cells from two patients (patients A and B). Cells were treated with DT-huGM-CSF at 100 ng/ml for up to 72 hours in SFM supplemented with exogenous huIL-3, huSCF and huG-CSF. At several time points aliquots were taken, washed and cultured in media with exogenous GFs but devoid of DT-huGM-CSF for the remaining incubation time until termination of the experiment at 72 hours (Fig 2). The

ID_{50} reference point was reached after 6 hours in patient A and after 24 hours in patient B. Viable cell counts after 48 hours of culture showed that in DT-huGM-CSF containing cultures the number of viable cells had decreased to 6 % for both patients, measured by eosin uptake, whereas viability in control cultures was 75%. In subsequent experiments, cells were exposed to DThuGM-CSF for 48 hours, followed by 18 hours of culture in the presence of ^{3}H-TdR.

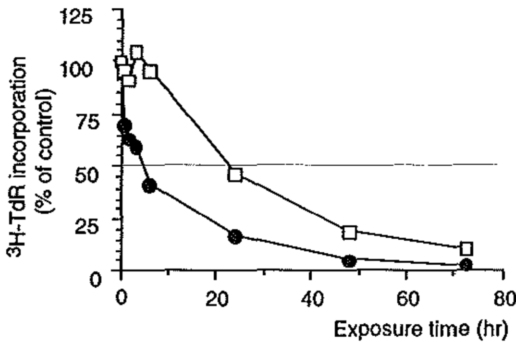

Figure 2
Effect of exposure time of DT-huGM-CSF to stimulated AML cells of patient A (●) and patient B (❑). After the incubation time the cells were washed and cultured in the presence of huSCF, huIL-3 and huG-CSF, but without DT-huGM-CSF. The relative ^{3}H-TdR incorporation as compared to untreated cells was measured.

Effect of DT-huGM-CSF on AML cells

Four AML cases were studied to compare the overall effect of DT-huGM-CSF on the total cell proliferation (^{3}H-Thymidine incorporation) with the effect on total cell survival (viability) and with the effect on the cell population with clonogenic potential (AML-CFU). Within the total leukemic cell population the fraction of cell capable of DNA synthesis is much higher than the fraction of cells with clonogenic potential. The ^{3}H-TdR incorporation assay therefore measures the total population of cells with proliferative potential whereas the AML-CFU assay measures a subfraction defined by its ability to form colonies *in vitro*. Cells from 4 patients were incubated in SFM in the absence or presence of DT-huGM-CSF. Compared to control cultures the percentage of viable cells was decreased in 3 out of the 4 AML's after exposure to DT-huGM-CSF for 48 hours (sample 11, 12 and 17, Table 1) but was hardly effected in the non-responsive AML (sample 22, Table 1). DT-huGM-CSF exposure reduced the cell fraction with clonogenic potential in samples 11, 12 and 17 compared to the control cultures; i.e. per 10^{5} cells 44 AML-CFU versus 6300 (0.7%), 0.5 versus 1.5 (33%) and 58 versus 2660 (2.2%), respectively (Table I). Although the clonogenic capacity of AML cells of sample 22 was minimal it was not further reduced after exposure to DT-huGM-CSF. These experiments showed that the inhibition of proliferation, measured with the ^{3}H-Thymidine incorporation, concurred with a decrease in cell viability and AML colony formation.

Table 1
Effect of DT-huGM-CSF on cell proliferation, viability and colony forming ability of AML cells

patient no.	ID_{50}[a] (ng/ml)	DT-huGM-CSF (ng/ml)	Viable cell[b] recovery	AML-CFU[c]
11	0.6	0	58	6300
		100	3	44
12	1.1	0	46	1.5
		100	15	0.5
17	0.4	0	68	2660
		100	10	58
22	>500	0	38	0.5
		100	32	0.5

Cells from 4 patients were incubated in serum-free medium with 100 ng/ml DT-huGM-CSF for 48 hours. ([a]) ID_{50} is the concentration in ng/ml DT-huGM-CSF required for 50% inhibition of ^{3}H-thymidine incorporation (See Table 3). ([b]) Viable cell recovery is the percentage of viable cells relative to the starting number at day 0. c: Number of colonies scored after a culture period of 2 weeks. The value is the average number of colonies per 10^5 original cells from day 0.

Effects of DT-huGM-CSF on spontaneous and hemopoietic GFs induced proliferation of AML cells.

Dose-response titration curves with DT-huGM-CSF were carried out for twenty-two AML patient samples. For each sample the effect of DT-huGM-CSF was investigated on the autonomous proliferation and on the proliferation after exposure to an exogenous GF mixture of huG-CSF, huIL-3 and huSCF. Two examples are shown in Fig 3 (patients 4 and 20). The proliferation of cells from patient 20 was not influenced by DT-huGM-CSF, either in the absence or presence of exogenous GFs. In contrast, both autonomously and GF stimulated proliferating cells from patient 4 were sensitive to DT-huGM-CSF. There was no difference between the respective ID_{50} values for the AML cells with these conditions. The toxic effects of DT-huGM-CSF on the proliferation of AML cells could be reduced by the addition of an excess amount of huGM-CSF (200 ng/ml), confirming specificity of the action of DT-huGM-CSF.

The sensitivity for DT-huGM-CSF of cells stimulated by the GF-mixture of IL-3, G-CSF and SCF is shown in Table 2. The stimulatory effect of the GF-mixture is shown as the absolute counts per minute (cpm) and the stimulatory index (S.I.). The S.I. reflects the degree of stimulation relative to the controls in which no GF are added. Eighteen of the 22 patients were sensitive with ID_{50} values ranging form 0.1 to 16 ng/ml. Table 2 shows that in the case of patients 19, 20 and 21, higher concentrations of toxin were needed to inhibit DNA synthesis (ID_{50} values >99 ng/ml). In one sample no sensitivity to the highest concentration DT-huGM-CSF tested was observed. There was no correlation found between the ID_{50} values and the effect of mixed cytokine stimulation (r=-0.10, p=0.26).

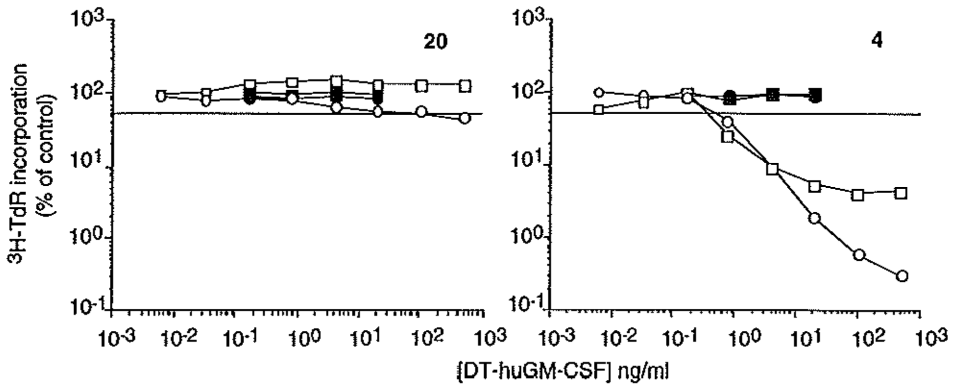

Figure 3
Antiproliferative effect of DT-huGM-CSF on AML cells of patients 20 and 4 in the ^{3}H-TdR incorporation assay. The sensitivity of cells was examined for inhibition of the autonomous growth in cytokine-free medium (□) and of stimulated growth with huSCF, huIL-3 and huG-CSF (○). Specificity of DT-huGM-CSF was tested with an excess of huGM-CSF (200 µg/ml) added stimultaneously with DT-huGM-CSF to cytokine-free medium (■) or stimulatory medium (●).

Table 2
Effect of DT-huGM-CSF on the proliferation of AML cells in the presence of huSCF, huIL-3 and huG-CSF

patient no.	FAB	^{3}H-TdR uptake cpm	S.I.	ID$_{50}$ (ng/ml)
1	M5	14,351	7.8	0.1
2	M5	15,585	1.1	0.3
3	M5	8,338	6.5	0.5
4	M5	25,634	11.0	0.7
5	M1	49,066	87.6	0.7
6	M5	2,652	3.4	1.2
7	M5	33,334	5.6	1.9
8	M4	43,400	8.3	2.2
9	M5	3,646	9.1	2.2
10	M5	9,060	3.7	2.2
11	M1	35,469	29.2	3.5
12	M5	11,829	8.3	3.5
13	MDS	34,120	148.3	5.5
14	M4	18,372	22.1	7.1
15	M5	8,335	2.7	8.9
16	M4	25,200	120.0	9.1
17	M2	33,509	24.5	10.2
18	M0	6,383	10.6	16.0
19	M5	10,052	15.7	99.0
20	M2	20,015	6.1	350.0
21	MDS	7,133	16.2	362.0
22	M0	12,235	23.5	>500

Cells from 20 AML and 2 MDS patients diagnosed according to the criteria of the French-American-British committee were incubated in serum-free medium in the presence of growth factors. Stimulation index (S.I.) is the ratio of ^{3}H-TdR uptake in growth factor stimulated cells divided by the nonstimulated proliferation.

Fifteen of the 22 cases that were studied showed intermediate or high spontaneous proliferation. Spontaneous proliferation of thirteen AML samples was reduced in the presence of DT-huGM-CSF whereas in two patients cell proliferation was not affected (Table 3), although one of these non-sensitive samples could be stimulated by exogenous huGM-CSF (number 20 in Table 3). No correlation was found between the response to exogenous huGM-CSF stimulation and the ID_{50} values of the sensitive AML samples ($r=0.08$, $p=0.34$). The effect of DT-huGM-CSF on the autonomous proliferation was not essentially different after exogenous GFs were used to induce cell proliferation for the same AML samples ($r=0.998$). When cells are exposed to DT alone i.e., DT_{388}, which lacks a binding domain but has full ADP-ribosylating activity, there was no effect on cell viability, cell proliferation and colony formation up to a concentration of 1000 ng/ml, suggesting that DT-huGM-CSF specifically kills cells by binding to the GM-CSF receptor (Data not shown).

Table 3
Effect of DT-huGM-CSF on the spontaneous proliferation of AML cells

patient	FAB	^{3}H-TdR uptake (cpm)		ID_{50} (ng/ml)	
no.		no growth factor	huGM-CSF	autonomous	(+GF's)
6	M5	780	1,370	0.1	(1.2)
14	M4	830	3,190	12.0	(7.1)
11	M1	1,214	9,768	0.6	(3.5)
3	M5	1,290	3,450	0.4	(0.5)
17	M2	1,367	8,217	0.4	(10.2)
12	M5	1,421	11,829	1.1	(3.5)
22	M0	1,563	2,026	>500	(>500)
1	M5	1,840	9,420	0.1	(0.1)
10	M5	2,430	9,850	13.0	(2.2)
15	M5	3,130	4,400	3.6	(8.9)
20	M2	3,280	16,740	>500	(350)
8	M4	5,200	13,120	22.0	(2.2)
7	M5	5,910	23,720	1.8	(1.9)
2	M5	14,080	15,290	0.2	(0.3)

Cells from 15 patients, which had sufficient autonomous proliferation, were incubated in serum-free and cytokine-free medium, or with recombinant huGM-CSF. The ID_{50} is the concentration in ng/ml DT-huGM-CSF required for 50% inhibition of autonomous proliferation. >500: ID_{50} not reached at the highest concentration tested. In parenthesis: ID_{50} of growth factor stimulated cells (derived from Table II)

Antiproliferative effect of DT-huGM-CSF in relation to GM-CSFR expression level.

To determine a possible correlation between the receptor expression of the GM-CSFR α and β subunits and the susceptibility to DT-huGM-CSF elimination we first used specific antibodies against the subunits to quantify receptor expression. This was measured using flow cytometry. Most samples showed a homogeneous expression of the GM-CSFR α and the GM-CSFR β, although the expression of the β unit was in general lower or even undetectable. Some examples of homogeneous expression are shown in Figure 4, i.e. AML samples 1, 5 and 18. Heterogeneous expression was observed on cells of AML sample 9. To compare the expression of the GM-CSFR subunits between AML cell samples the ratio of

the mean specific fluorescence and the mean autofluorescence was determined. There was no correlation found between the level of expression of the α subunit and the sensitivity to DT-huGM-CSF either in the cytokine-free (r=-0.45, p=0.33) or GF-stimulated culture (r=-0.51, p=0.061) (Figure 5). Also for the GM-CSFR β subunit no correlation was found, i.e. r=-0.52, p=0.31 in cytokine-free culture and r=-0.47, p=0.056 for GF-stimulated culture.

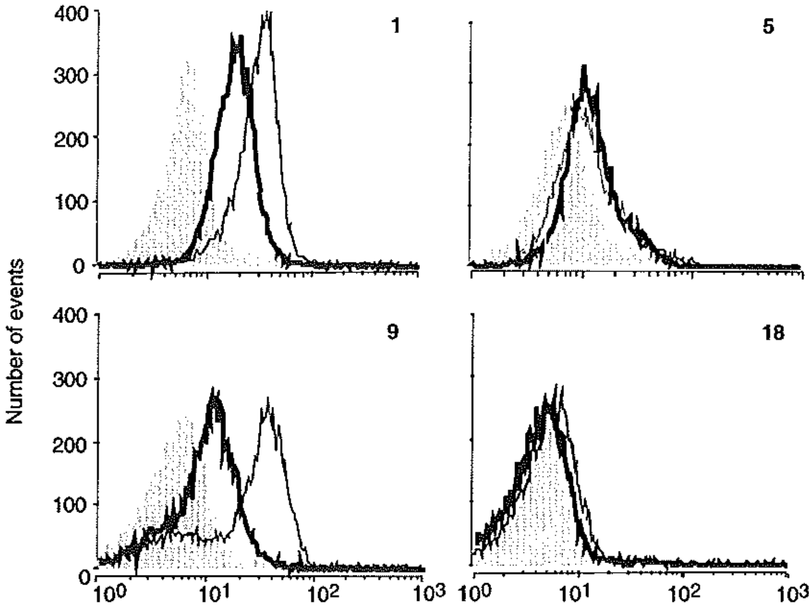

Figure 4
Flow cytometric analysis of huGM-CSF α and β subunit expression. The cells were incubated with monoclonal antibodies against the huGM-CSFR α (thick line) and β (thin line) subunit or in the absence of monoclonal antibodies (grey shade).

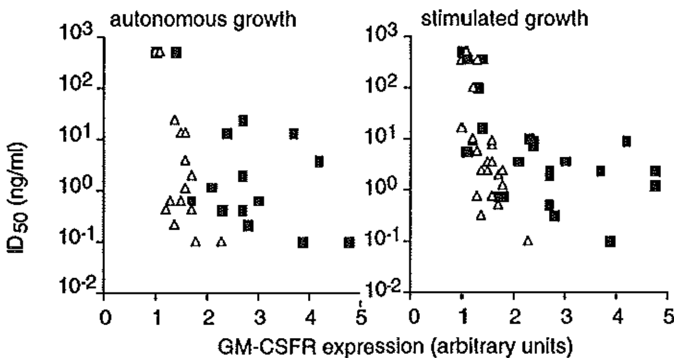

Figure 5
Relation of the huGM-CSFR α (■) and β (Δ) subunit expression of the AML samples determined by flow cytometric studies using monoclonal antibodies and the sensitivity of the AML samples to DT-huGM-CSF, measured as the ID_{50} in the cytokine-free or stimulatory medium.

Discussion

In this study we determined the sensitivity of hu AML cells to *in vitro* treatment with DT fused to huGM-CSF. In 18 of the 22 AML patients studied, proliferation of AML blasts was reduced by DT-huGM-CSF. That this proliferation inhibition of DT-huGM-CSF led to cell death was confirmed by viability cell counting and the inhibition of the colony forming ability of the AML cells (CFU-AML) after a 48 hours exposure. Our experiments provide evidence that toxicity of DT-huGM-CSF is mediated through binding of the huGM-CSF part of the fusion protein to the relevant receptor and are in agreement with data obtained by others e.g. with DT fused to murine GM-CSF (7) and saporin coupled to huGM-CSF (27).

In 50% of AML cases, proliferation may occur without adding GFs to the cultures. This may be due to autocrine mechanisms or to loss of GF dependence (18). DT-huGM-CSF for AML treatment will only be effective if both the leukemic progenitor cells and the leukemic stem cells are affected. Evidence is accumulating that leukemic stem cells with long term proliferative potential exist (28-30). Studies are in progress to test whether the leukemic stem cell can be targeted with GF-toxins. In these studies DT-huGM-CSF treated AML suspensions are transplanted into severe combined immune deficient (SCID) mice. This is a well established assay for the measurement of the proliferation and survival of AML stem cells (28, 29).

Although subpopulations of the normal hemopoietic progenitor cells express the GM-CSFR it is not possible to directly measure the expression of GM-CSFR on the hemopoietic stem cells, since these cells cannot be purified to homogeneity. If stem cells express the GM-CSFR the application of GF toxins in the treatment of leukemia will be limited. However, using *in vitro* assays to distinguish normal hemopoietic stem cells with long and short term repopulating ability (31) we found no toxic effect (manuscript in preparation).

As well as the leukemic and normal hemopoietic cells, non-hemopoietic tissues might also be potential targets for DT-huGM-CSF. For instance, high affinity complexes of the huGM-CSFR are reported to be present on normal endothelial cells (32). Furthermore, the presence of huGM-CSFR on colon adenocarcinoma, small cell carcinoma of the lung, osteogenic sarcoma and breast carcinoma cells (33-35), raised the possibility that GM-CSFR are also expressed on the normal tissues from which these tumors originate. We are currently evaluating the toxic side-effects of DT-huGM-CSF *in vivo* in a rat model to address this issue.

We investigated whether screening for GM-CSFR expression on AML cells and stimulation by GM-CSF could be predictive of which AML patients will be sensitive to DT-huGM-CSF. Although cytotoxicity was mediated by the huGM-CSFR we found that neither the level of expression of the GM-CSFR α and β subunit nor the S.I. of GM-CSF appeared to correlate with the degree of sensitivity. However, in the group of AML samples studied cells from patient 20 responded to huGM-CSF, indicating high affinity complex formation, but

were still minimally sensitive to DT-huGM-CSF. An explanation could be that in DT-huGM-CSF resistant AML samples, an autocrine production of huGM-CSF (36-38) interferes with DT-huGM-CSF binding. A variability in the efficiency of DT processing is also a possibility as a cause of the differences in sensitivity (39). Concerning the GM-CSFR expression, it should be realized that the receptor expression was measured on the total leukemic cell population which may not be representative for the small fraction of proliferating cells.

In conclusion, these *in vitro* results show that a GF toxin fusion protein can be utilised to eliminate short-term proliferating AML cells. These studies form the basis for further investigations of DT-huGM-CSF as a potential therapeutic agent in the treatment of AML.

Acknowledgements

The authors thank Hans Hoogerbrugge for his technical assistance and Jan Hendrikx and Hazel Gowing for critical reading of the manuscript.

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Chapter 4

Diphtheria toxin fused to huGM-CSF eliminates AML cells with the potential to initiate leukemia in immunodeficient mice, but spares normal hemopoietic stem cells.

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Blood, accepted for publication

Abstract

We studied the cell kill induced by human GM-CSF fused to *Diphtheria* Toxin (DT-huGM-CSF) in acute myeloid leukemia (AML) samples and in populations of normal primitive hemopoietic progenitor cells. AML samples from three patients were incubated *in vitro* with 100 ng/ml DT-huGM-CSF for 48 hours, and AML cell kill was determined in a proliferation assay, a clonogenic assay (CFU-AML) and a quantitative long-term bone marrow culture i.e. the leukemic-cobblestone area forming cell assay (L-CAFC). To measure an effect on cells with *in vivo* leukemia initiating potential DT-huGM-CSF exposed AML cells were transplanted into immunodeficient mice. In two out of three samples it was shown that all AML subsets, including those with long term abilities *in vivo* (SCID mice) and *in vitro* (L-CAFC assay) were reduced in number by DT-huGM-CSF. Cell kill induced by DT-huGM-CSF could be prevented by coincubation with an excess of GM-CSF, demonstrating that sensitivity to DT-huGM-CSF is specifically mediated by the GM-CSF receptor (GM-CSFR). Therefore binding and internalization of GM-CSF probably occur in immature AML precursors of these two cases of AML. The third AML sample was not responsive to either GM-CSF or DT-huGM-CSF.

The number of committed progenitors of normal bone marrow (BFU-E, CFU-GM and CAFC week 2) and also the number of cells with long term repopulating ability, assayed as week 6 CAFC, were unchanged after exposure to DT-huGM-CSF (100 ng/ml, 48 hours). These studies show that DT-huGM-CSF may be used to eliminate myeloid leukemic cells with long-term potential *in vitro* and in immunodeficient mice, whereas normal hemopoietic stem cells are spared.

Introduction

Human acute myeloid leukemia (AML) cells generally express receptors for hemopoietic growth factors. The granulocyte-macrophage colony stimulating factor receptor (GM-CSFR) is expressed as a heteromeric complex containing an α and a β subunit (1-3). In AML this receptor is mostly of high affinity. The GM-CSFR of AML cells is functional, because cells from more than 80% of AML cases respond to GM-CSF with proliferation (4-5). Such receptors with a narrow tissue distribution and a high affinity are promising targets to selectively deliver toxins to malignant cells (6, 7).

A truncated form of the potent *Diphtheria* toxin (DT) has been fused to a number of cytokines (8-12). It has been shown before that such fusion proteins may be used for the elimination of malignant cells. The deleterious effects of DT-fusion proteins require receptor binding and subsequent internalisation by receptor-mediated endocytosis. This is followed by processing of the DT into its active form and delivery of the NH_2-domain associated ADP-ribosyltransferase to the cytoplasm. It kills the cell by catalyzing the irreversible ADP

ribosylation and subsequent inactivation of elongation factor 2. The number of internalized DT molecules required for cell kill are quite low (13-16).

Recently we established that DT-huGM-CSF may be used to target primary human AML as determined in an *in vitro* proliferation assay (17). Limiting dilution experiments of AML cells in immunodeficient mice showed that the frequency of the cell with the ability to initiate leukemia in immunodeficient mice is low and varies between 0.2 and 100 per 10^6 AML cells (18). Whether these immature AML cells, with the ability to maintain AML *in vivo*, express functional GM-CSFR or respond to huGM-CSF is unknown. Probably, the sensitivity of this AML subset determines the antileukemic efficacy of a therapeutic intervention. The immunodeficient mouse model is an established system for investigation of immature human AML cells (19-21) and might serve as a useful tool to investigate the effect of DT-huGM-CSF to AML cells with long term repopulating capacity.

Applicability of DT-huGM-CSF could be hampered by the toxic effect on normal hemopoietic cells. It has been shown that the GM-CSFR is probably not expressed by the phenotypically most immature subsets of CD34 positive cells (22), and GM-CSFR mRNA was not observed in 5-Fluorouracil resistant bone marrow cells (23). This evidence suggests that normal hemopoietic stem cells may escape cell death induced by DT-huGM-CSF.

Here we report on the efficacy of DT-huGM-CSF for elimination of AML cells with long-term repopulating abilities *in vivo*, using transplantation of DT-huGM-CSF exposed AML cells into SCID mice. In addition we used the cobblestone area forming cell (L-CAFC) assay, a quantitative long-term bone marrow culture system that we have applied to the investigation of AML before (21, 24). The toxicity of DT-huGM-CSF to normal hemopoietic cells under identical conditions was determined in the clonogenic assay for committed progenitors, and in the CAFC assay for hemopoietic stem cell subsets (25).

Materials and Methods

Human AML cells

Samples were obtained, following informed consent, from untreated patients with AML. The cases were classified cytologically according to the criteria of the French-American-British Committee (FAB) (26). Mononuclear cells were isolated as a buffy coat, without T cell depletion and frozen using a controlled freezing apparatus followed by storage in liquid nitrogen. After thawing by stepwise dilution, cell viability assessed by trypan blue staining ranged from 62 to 91%.

Incubations with DT-huGM-CSF

The construction and purification of DT-huGM-CSF has previously been described (21). Normal bone marrow (nBM) cells from healthy donors and AML cells were exposed to DT-HUGM-CSF (100 ng/ml) in serum free medium (SFM) (27) at a density of 2.5×10^6 cells/ml at 37°C in a humidified 5% CO_2 atmosphere for 48 hours. Control incubations in SFM without DT-huGM-CSF and the incubation with a equimolar concentration of human GM-CSF (huGM-CSF; a gift from Sandoz BV, Basel, Switzerland) were performed simultaneously. For competition experiments an excess of huGM-CSF (2 μg/ml) was added to the cultures containing DT-huGM-CSF. Equivalent

proportions of the flasks based on the input values were used in the assays. No correction for cell loss or viability was applied.

^{3}H-Thymidine (^{3}H-TdR) incorporation assay

Cells ($2x10^4$) were cultured for 72 hours in 96-well round-bottom microtiter plates in 100 µl SFM containing DT-huGM-CSF (100 ng/ml). The effects of DT-huGM-CSF were tested in the absence of growth factors, and in the presence of the combination of human stem cell factor (SCF 100 ng/ml; a gift from Amgen Biologicals, Thousand Oaks, CA, USA), human interleukin-3 (IL-3 25 ng/ml; a gift from Gist Brocades, Delft, The Netherlands) and human granulocyte-colony stimulating factor (G-CSF 100 ng/ml; Amgen). Eighteen hours before harvesting, 0.1 µCi ^{3}H-TdR (2 Ci/mmol, Amersham International, Amersham, UK) was added to each well. Cells were collected using an automatic cell harvester (Skatron, Lier, Norway), and the cell-associated radioactivity was measured in a liquid scintillation counter (Pharmacia-LKB, Bromma, Sweden). In competition experiments an excess of GM-CSF (2 µg/ml) was added simultaneously with DT-huGM-CSF. All cultures were performed in triplicate. Data are expressed as percentage of control.

SCID mice and transplantation of AML

Female specific pathogen-free CB17 SCID/SCID mice (5-8 weeks of age) were obtained from Harlan CPB, Austerlitz, The Netherlands, and housed under pathogen free conditions in a laminar air flow unit. The mice were supplied with sterile food and acidified drinking water with 100 mg/L ciprofloxacine (Bayer AG, Leverkusen, Germany). The mouse plasma Ig level was determined with an ELISA using a sheep anti-mouse antibody reacting with mouse IgG and IgM (Boehringer Mannheim Biochemica, Penzberg, Germany). Mice with plasma Ig levels over 40 µg/ml were excluded. SCID mice were pretreated with 0.2 ml dichloromethylene diphosphonate (CL_2MDP) liposome stock solution, injected into the lateral tail vein, on the day before transplantation of the leukemic cells to eliminate the macrophages in spleen and liver (28). In addition, total body irradiation at a dose of 3.5 Gy was delivered by a [^{137}Cs] source (Gammacell, Atomic Energy of Canada, Ottawa, Canada) adapted for the irradiation of mice. The AML graft size was the equivalent of $30x10^6$ AML input cells for all three samples. The grafts, suspended in 300 µl Hanks balanced salt solution (HBSS) (GIBCO, Breda, The Netherlands), 16 U/ml Heparin and 0.1% bovine serum albumen (BSA, Sigma, St Louis, MO, USA), were injected into the lateral tail vein.

Tissue collections.

The experiments were carried out following consent of the Institutional Ethics Committee for animal experiments. SCID mice were sacrificed using CO_2 inhalation followed by cervical dislocation in accordance with institutional animal research regulations. Cell suspensions were prepared from the BM and analysed by flow cytometry.

Flow cytometric analysis of SCID mouse derived AML cells

To quantify AML growth and to compare the immunophenotype with the initial graft samples, cells recovered from the bone marrow of SCID mice were incubated with the following (combinations of) mouse monoclonal antibodies: CD34-FITC, CD34-PE, CD38-PE, CD34-FITC/IgG1-PE, CD34-FITC/HLA-DR-PE, CD34-FITC/CD38-PE, CD34-FITC/C-Kit-PE, CD34-FITC/CD33-PE, CD45-FITC/CD33-PE. All antibodies were obtained from Becton Dickinson, San Jose, CA, with the exception of c-kit-PE (Immunotech, Marseille, France). Cells recovered from SCID mouse bone marrow staining with two antibodies specific for human hemopoietic cells were counted as human cells (29). Mouse IgG1-FITC and mouse IgG1-PE conjugated antibodies and

samples from non-transplanted SCID mice were used as controls. Samples were analysed using the FACScan flow cytometer and Lysis II software (Becton Dickinson, Mountain View, CA). Erythrocytes and dead cells were excluded by gating on forward and orthogonal light scatter.

Serum free colony assay for leukemic progenitors

Cells were plated in 35-mm dishes (Becton Dickinson) in 1 ml Dulbecco's modified Eagle's medium (DMEM, GIBCO, Gaithersburg, MD) containing 0.9% methylcellulose, 1.5% BSA (Sigma), insulin (10 μg/ml, Sigma), linoleic acid ($1.5x10^{-5}$ M, Merck, Darmstadt, Germany), cholesterol ($1.5x10^{-5}$ M, Merck), sodium selenite ($1x10^{-7}$ M, Merck), β-mercaptoethanol ($1x10^{-4}$ M, Merck), human transferrin (0.62 mg/ml, Behring Werke, Marburg, Germany), penicillin (100 U/ml, GIBCO), and streptomycin (850 μg/ml, GIBCO). Assays were performed in triplicate in the presence of GM-CSF (5 ng/ml), IL-3 (10 ng/ml), and G-CSF (100 ng/ml). Colonies were scored after 14 days of incubation at 37°C in humidified 5% CO_2 atmosphere.

Colony assay for normal hemopoietic progenitors

Colony-forming unit-granulocyte macrophage (CFU-GM) and burst-forming unit-erythroid (BFU-E) were enumerated to test toxicity of DT-huGM-CSF to normal committed progenitor cells. DT-huGM-CSF exposed nBM cells were plated at $1x10^4$ cells per dish in 1 ml of semisolid medium, (1.2% methylcellulose in Iscove's modified Dulbecco's medium (IMDM); GIBCO) containing 30% fetal calf serum (Hyclone, Logan, UT, USA) supplemented with 0.75% BSA (Sigma), penicillin (100 U/ml), streptomycin (100 μg/ml), β-mercaptoethanol ($5x10^{-5}$ M), erythropoietin (Epo 1 U/ml; Boehringer, Mannheim, Germany), IL-3 (15 ng/ml), G-CSF (50 ng/ml), GM-CSF (5 ng/ml) and murine SCF (100 ng/ml; Genetics Institute, Cambridge, MA) all at final concentrations. Additional studies were performed in semi-solid medium methyl cellulose cultures (MC), either serum containing (30% FCS) or serum-free, supplemented with only huGM-CSF (5 ng/ml) for colony formation induction. Cultures were kept at 37°C and 5% CO_2 in a humidified atmosphere. CFU-GM included CFU-G, CFU-M and CFU-GM. CFU-GM and BFU-E colonies were counted on day 14 of culture in the same dish.

CAFC Assay.

The CAFC assay was performed as described (25), using similar conditions for normal and leukemic samples. Briefly, confluent stromal layers of FMBD-1 cells in 96-wells plates were overlaid with AML cells or normal bone marrow cells in a limiting dilution setup. The cells were cultured in IMDM (GIBCO) supplemented with 20% horse serum (GIBCO) and hydrocortisone 21-hemisuccinate (10^{-6} M, Sigma). IL-3 (12.5 ng/ml) and G-CSF (20 ng/ml) were added weekly to the cultures. Input values were the equivalent of 50,000 nucleated cells (NC) per well in the lowest dilution. Twelve dilutions two-fold apart were used for each sample, with 15 replicate wells per dilution. The percentage of wells with at least one phase-dark hemopoietic clone of at least five cells beneath the stromal layer was determined about every 14 days and CAFC frequencies were calculated using Poisson statistics as described (30).

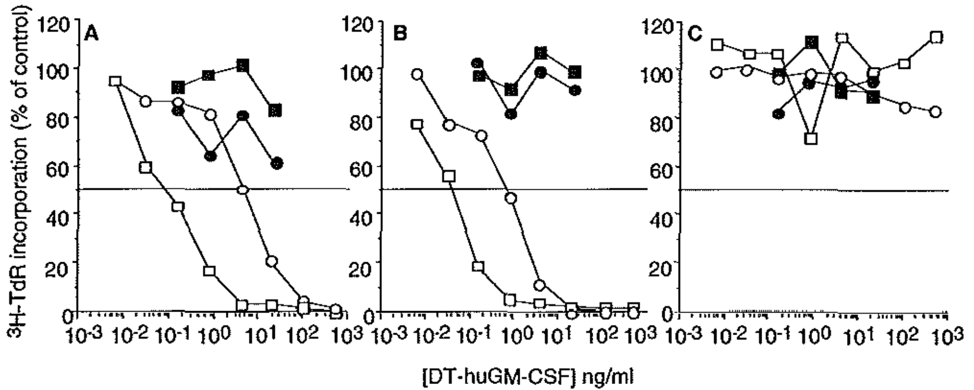

Figure 1
Proliferation inhibition of AML cells by DT-huGM-CSF measured in the ^{3}H-TdR incorporation assay. Cells were examined for inhibition of proliferation in cytokine-free medium (□) and in conditions of stimulated growth by SCF, IL-3 and G-CSF (○). Specificity of DT-huGM-CSF was tested by adding an excess of GM-CSF (2 μg/ml) simultaneously with DT-huGM-CSF in cytokine free medium (■) or SCF, IL-3 and G-CSF containing medium (●).

Results

Antiproliferative effects of DT-huGM-CSF in a proliferation assay

The response of AML samples to huGM-CSF or a mix of cytokines (G-CSF, IL-3 and SCF) was determined in a proliferation assay (Table 1). AML cells from patients A and B proliferated when exposed to huGM-CSF, indicating the presence of functional GM-CSFR. AML cells from patient C showed no proliferative response to huGM-CSF. All samples proliferated when incubated with the combination of G-CSF, IL-3 and SCF.

To determine the effect of DT-huGM-CSF on unstimulated proliferation we incubated the AML cells for 72 hours at DT-huGM-CSF concentrations ranging from 0.001 to 500 ng/ml without growth factors. Similar experiments were performed in the presence of the combination of G-CSF, IL-3 and SCF (Fig 1). Table 1 shows the ID_{50}, i.e. the DT-huGM-CSF dose required to induce a 50% inhibition of DNA synthesis of the AML cells. The AML cells from patients A and B were sensitive to the toxin under both conditions and the toxic effect could be inhibited by an excess amount of GM-CSF. The ID_{50} varied from 0.04 to 5 ng/ml. AML cells from patient C were insensitive to DT-huGM-CSF; even a concentration of 500 ng/ml failed to induce an antiproliferative effect. These results correlated with AML cell viability after incubation with DT-huGM-CSF for 48 hours as assessed by trypan blue staining: the numbers of viable cells were reduced to 18% for patient A and 3% for patient B as compared to the control incubations. The viability of AML cells from patient C was unchanged at 97%.

Restimulation of the AML cells of patients A and B recovered after incubation with DT-huGM-CSF revealed that the cells surviving DT-huGM-CSF exposure were unable to proliferate in response to GM-CSF or the combination of G-CSF, IL-3 and SCF. The proliferation pattern of AML cells from patient C was unchanged: no response to huGM-CSF and proliferation in response to the combination of growth factors (data not shown). Culturing of the cells in a clonogenic assay revealed that after DT-huGM-CSF incubation CFU-AML numbers were reduced to 2.2% (pat A) and 0.7% (pat B) as compared to controls. AML cells from patient C did not produce colonies at all.

The concentration of 100 ng/ml of DT-huGM-CSF was selected as an effective dose in later experiments, because maximal efficacy in the proliferation assay was already achieved at considerably lower concentrations. Exposure for longer periods of time (up to 72 hours) did not result in a change in the viability of the exposed AML samples (data not shown).

Table 1
Effect of DT-huGM-CSF on AML cells in the ^{3}H-TdR uptake assay

AML	FAB	^{3}H-TdR uptake (cpm)			ID_{50} (ng/ml)	
		no GF	GM-CSF	SCF/IL-3/G-CSF	autonomous growth	stimulated growth
A	M2	1,300	8,200	33,500	0.1	5
B	M1	1,200	9,800	35,500	0.04	0.7
C	M1	1,600	2,000	31,400	>500	>500

Cells from 3 AML patients diagnosed according to the criteria of the French-American-British committee were incubated in serum-free medium with or without a mixture of SCF, IL-3 and G-CSF. The ID_{50} is the concentration of DT-huGM-CSF required for 50% inhibition of the autonomous proliferation or the proliferation under stimulatory conditions.

Outgrowth of DT-huGM-CSF exposed, huGM-CSF exposed and control AML cells in SCID mice

The effect of the described interventions on *in vivo* leukemia initiating capacity of the AML cells was investigated using transplantations into SCID mice. Groups of 5 SCID mice were evaluated 30-48 days after transplantation. The mice that had received a DT-huGM-CSF treated graft of AML A and B, showed a much lower percentage of leukemic cells as compared to control mice (Table 2). In mice transplanted with DT-huGM-CSF exposed cells from AML B, all grafts failed (graft failure was defined as less than 0.5% of AML cells proliferating in the SCID mouse bone marrow). DT-huGM-CSF treatment of AML cells from patient C did not lead to a significant reduction in leukemic cell load in the mice. The results also show that exposure of AML cells to GM-CSF (48 hours, SFM) did not result in an appreciable increase of the leukemic cell load in SCID mice (Table 2).

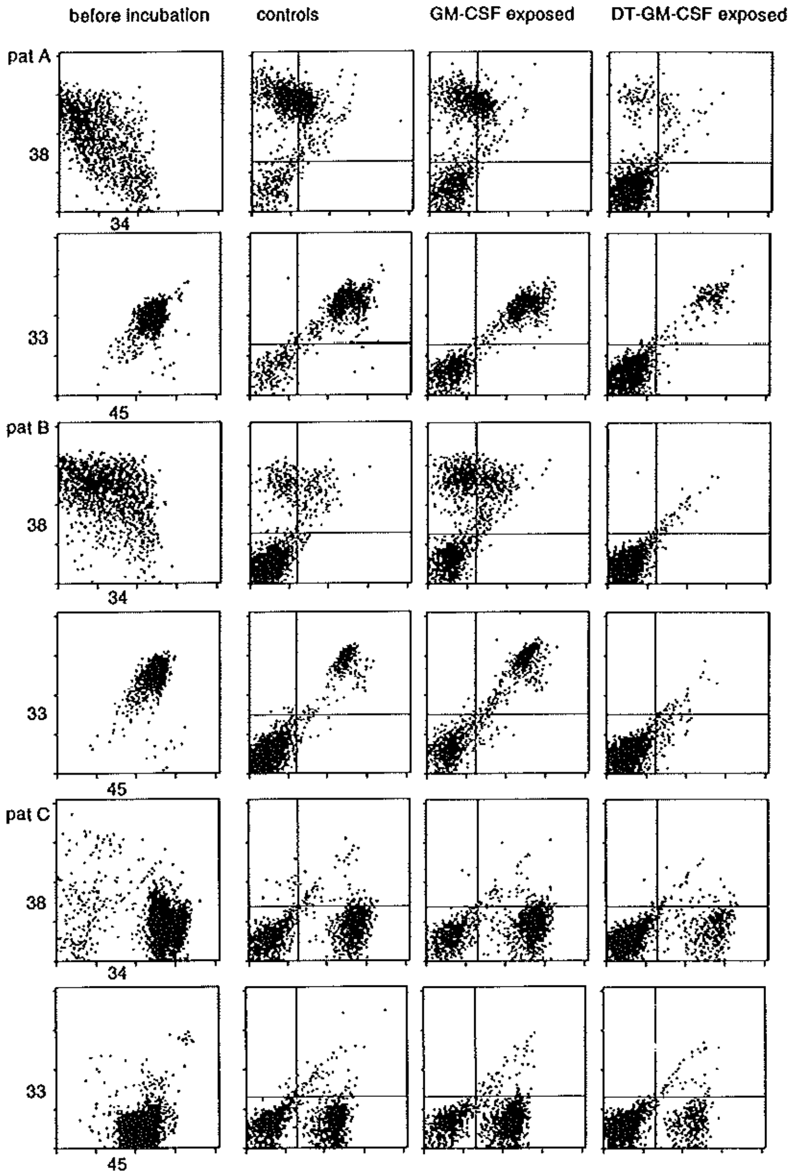

Figure 2
Double labeling dot plots of three AMLs. The first lane shows the CD38 (Y-axis) versus CD34 (x-axis) and CD33 (y-axis) versus CD45 (x-axis) of the original cell suspensions before incubation and injection into the SCID mice. Lanes 2, 3 and 4 show CD38/CD34 and CD33/CD45 double labeling dot plots of bone marrow derived from SCID mice that were killed around day 40 after injection of AML cells that were either incubated for 48 hours in serum-free medium (SFM) lane 2; in SFM containing huGM-CSF only, lane 3; or in SFM containing DT-huGM-CSF at 100 ng/ml, lane 4. Murine cells are found in the lower left quadrant and depending on the characteristics of AML A, B or C, human cells are found in the other three quadrants.

Table 2
Outgrowth of DT-huGM-CSF exposed, huGM-CSF exposed and control AML cell populations in SCID mice.

AML	Incubation	% human cells in SCID mouse BM#
A	control	75 ± 13
	huGM-CSF	64 ± 11
	DT-huGM-CSF	12 ± 8.7
B	control	21 ± 9.2
	huGM-CSF	31 ± 21
	DT-huGM-CSF	0
C	control	42 ± 9
	huGM-CSF	50 ± 19
	DT-huGM-CSF	21 ± 7

Mean number of percentage of human cells in SCID mouse bone marrow ± SD. Values calculated on the basis of data from five engrafted mice.

Flow cytometric analysis of AML cells from the SCID mouse bone marrow.

Cells recovered from the SCID mouse bone marrow were investigated for leukemic origin. Flow cytometry with the described panel of monoclonal antibodies showed that the phenotypes of the AML cells recovered from the SCID mice were identical to the grafts, except in AML case A (Figure 2). In case A the phenotype converted from 75% CD34 negative AML cells to a phenotype entirely negative for CD34 expression. Additional support for the leukemic nature of the cells proliferating in the SCID mice comes from the observation that nBM does not proliferate extensively in SCID mice under the experimental conditions used (unpublished observation).

Proliferation assay of AML cells recovered from the SCID mouse bone marrow

Cells recovered from SCID mice that had received a DT-huGM-CSF exposed graft from patient A proliferated in response to huGM-CSF. Renewed incubation with DT-huGM-CSF reduced proliferation (data not shown). Because such AML cells could not be demonstrated in the initial graft, directly after the incubation, these data indicate that huGM-CSF and DT-huGM-CSF responsive cells regenerated in the mouse.

Outgrowth of DT-huGM-CSF exposed AML cells in the CAFC assay

Long term growth of the DT-huGM-CSF exposed AML cells from patient A, B and C was investigated in a quantitative long-term bone marrow culture system, the L-CAFC assay. It was shown that the frequencies of week 7 L-CAFC were 6060 ± 1318 and 3828 ± 834 per 10^5 NC in case A and B respectively (Fig 3), whereas this frequency in unfractionated nBM always varies between 0.1 and 10 per 10^5 NC. Therefore the origin of the great majority of the cobblestone areas produced by case A and B must be leukemic. The frequency analysis of leukemic progenitors after 7 weeks of culture (late L-CAFCs) showed a 7 and 87 fold

reduction in the DT-huGM-CSF treated cell populations as compared to the control cells from patient A and B, respectively. The L-CAFC assay of the DT-huGM-CSF exposed cell sample of patient C showed that week 7 L-CAFC frequencies were $3.2 \pm 0.6/10^5$ as compared to $4.0 \pm 0.7/10^5$ for control cells from patient C, and was therefore unchanged. The L-CAFC data indicates that the great majority of leukemic stem cells of patient A and B were sensitive to DT-huGM-CSF, which is in agreement with the *in vivo* leukemia initiating capacity of these samples.

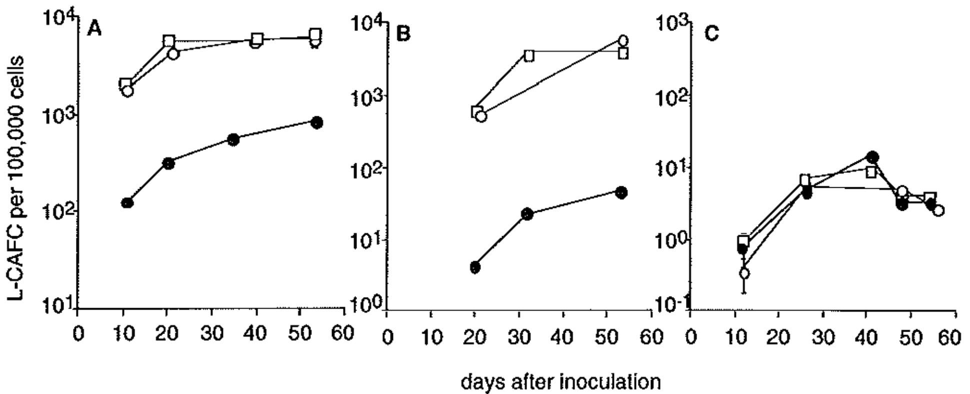

Figure 3
L-CAFC frequencies (mean ± SEM) of DT-huGM-CSF exposed (●), huGM-CSF exposed (○) and control AML cell populations (□). Frequencies are based on the number of input cells.

Coincubation of DT-huGM-CSF with excess concentrations of GM-CSF

To test whether the toxicity of DT-huGM-CSF is conferred by the GM-CSFR. We incubated AML cells of patient A with DT-huGM-CSF in the presence of an excess amount of huGM-CSF and compared the result of incubation with the effect of excess huGM-CSF alone. The frequency of week 5 L-CAFC ($1254 \pm 295/10^5$ and $1027 \pm 41/10^5$ (mean ± SEM)) and the engraftment in SCID mice (in groups of 5 mice 69 ± 14% and 64 ± 11% (mean ± SD)) of AML cells in the SCID mouse BM, respectively) were similar, showing that the toxicity of DT-huGM-CSF could be blocked by coincubation with an excess of GM-CSF. This implies that the toxicity to the AML stem cells is mediated via the GM-CSFR. We showed earlier that the effect of DT-huGM-CSF on AML cells as determined in ^{3}H-TdR incorporation assay could be prevented by high concentrations of huGM-CSF as well (21).

Table 3
Relative effect of DT-huGM-CSF exposure on hemopoietic progenitors

	CFU-GM#	BFU-E#	CAFC week 2##	CAFC week 6##
mean	90.2 ± 19.2	147.6 ± 32.5	119.2 ± 32.9	113.1 ± 14.8
range	73 - 127	100 - 190	76 - 157	88 - 126

Data represent the mean relative effect as the percentage of control of DT-huGM-CSF exposure on normal bone marrow of 5 individuals.
Mean number of progenitors ± SEM.
Mean number of percentage ± SEM

Effect of DT-huGM-CSF on normal hemopoietic progenitors

To investigate the cytotoxic effect of DT-huGM-CSF to normal hemopoietic progenitor cells, five samples of nBM cells were exposed to 100 ng/ml DT-huGM-CSF for 48 hours in liquid culture. The relative effect on committed progenitors was determined in clonogenic assays by enumerating the number of CFU-GM and BFU-E's, and compare this with non-exposed nBM cells. Cells with the ability to form CFU-GM colonies were found after incubation with DT-huGM-CSF. To specifically test whether under these conditions CFU-GM that were responsive to huGM-CSF only as the inducer of colony formation could survive, the following experiment was performed. Two additional nBM samples were incubated in serum-free liquid cultures in the presence or absence of DT-huGM-CSF (100 ng/ml) and the survival of committed progenitor cells was measured using methyl cellulose (MC) cultures in which colony formation was induced using three different conditions, 30% FCS plus G-CSF, IL-3, SCF, Epo and huGM-CSF, 30% FCS plus huGM-CSF only or huGM-CSF only in a serum free MC culture (Fig 4). In the MC cultures supplemented with all GF's a moderate reduction in the number of CFU-C was found, i.e. in the order of 25 %. No differences in colony numbers were found in cultures supplemented with huGM-CSF only, either under serum containing or under serum free conditions. This indicates that after an exposure period for 48 hours equal numbers of huGM-CSF responsive CFU-C survive a concentration of DT-huGM-CSF sufficient for the elimination of AML progenitor cells including the CFU-AML and the leukemia initiating cell in SCID mice.

Hemopoietic stem cell subsets were evaluated in the CAFC assay (Table 3) in which the week 2 CAFC correlates with the number of short-term repopulating progenitor cells (25, 30) and week 6 CAFC correlates with long term repopulating stem cells. All frequencies of nBM subsets were unchanged after the *in vitro* DT-huGM-CSF exposure. These data indicate that all hemopoietic progenitor cells, including the most primitive ones (week 6 CAFC), escape DT-huGM-CSF induced cell kill.

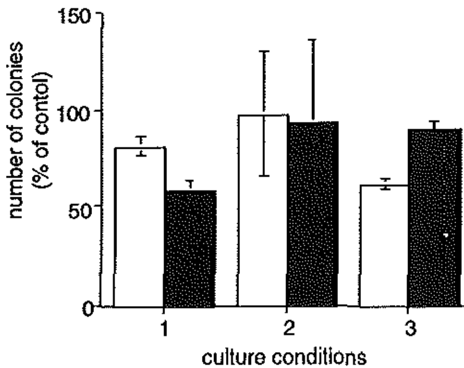

Figure 4
CFU-GM colony formation in MC cultures after an initial 48 hours period of exposure to DT-GM-CSF (100 ng/ml) of 2 normal bone marrow samples. Culture condition 1 is MC supplemented with GM-CSF, G-CSF, IL-3, SF and Epo in 30% FCS; condition 2 is MC supplemented with FCS 30% and GM-CSF only, condition 3 is MC in serum free medium with GM-CSF only. The number of CFU-GM surviving in DT-GM-CSF containing medium is expressed relative to the number of CFU-GM that survived in the respective "medium only" control group.
White bars nBM 1; black bars nBM 2.

Discussion

Transplantation of AML cells into SCID mice may be used to determine *in vivo* growth of primitive leukemic cells (19, 20, 31). In this model we evaluated the sensitivity of human AML progenitor cells to *in vitro* treatment with DT fused to huGM-CSF. In 2 out of 3 AML samples, the long term repopulating AML cells, defined as the leukemia initiating cells in SCID mice, were reduced in number by DT-huGM-CSF. In one of the cases (case B) AML growth in SCID mice was completely prevented by DT-huGM-CSF. Although the reduction of AML cell proliferation in case A and B was obvious, exact quantification of SCID mouse transplantation results requires limiting dilution experiments (18). In the L-CAFC assay the DT-huGM-CSF induced a reduction in the number of AML cells with long-term abilities (CAFC week 6-7), 8 fold and 87 fold in case A and B, respectively.

An excess of unlabeled huGM-CSF blocked the toxic action of DT-huGM-CSF, indicating that leukemic stem cell reduction was mediated by the specific binding of DT-huGM-CSF to the GM-CSFR, which is consistent with the specificity observed in experiments with murine GM-CSF fused to DT (12) and huGM-CSF coupled to saporine (32). This suggests that elimination of the primitive AML cells from case A and B by DT-huGM-CSF requires the expression of GM-CSFRs, that are at least able to bind DT-huGM-CSF.

The applicability of growth factor toxins *in vivo* could be limited by side-effects to non-leukemic cells bearing the same receptor, e.g. cells from the normal hemopoietic system that express GM-CSFR. However, exposure of normal bone marrow to a high dose of DT-huGM-CSF for 48 hours did not result in a reduction of the numbers of erythroid (BFU-E), myeloid (CFU-GM) and CAFC week 2 progenitor cells. Moreover, primitive hemopoietic progenitors (CAFC week 6) remained unaffected by high concentrations of DT-huGM-CSF.

The survival of bone marrow progenitors that could be induced to form colonies upon stimulation with huGM-CSF as the only GF in the serum-free culture medium containing high concentrations of DT-huGM-CSF was somewhat surprizing. This suggests that the

number of receptors that are expressed on the progenitor cells are sufficiently high to induce a growth response but apparently not high enough to achieve cell killing with DT-huGM-CSF. The short duration of the culture i.e. 48 hours and because the SFM culture only contains DT-huGM-CSF makes it unlikely that the progenitors are derived from the immature stem fraction present in the culture.

In murine hemopoiesis the GM-CSF receptor is expressed on about 55% of the committed progenitor cells and estimated to be in the order of 560 receptors per cell which is about tenfold higher than on mature stem cells (33); the GM-CSF-R is not expressed on immature stem cells. On maturing cells of the myeloid and macrophage lineages the number of receptors ranges from 500-1500 which is in the same range as reported for AML blasts (1, 4, 33). This numerical difference might be the basis for the differential effect of DT-huGM-CSF on normal and leukemic stem cells. On the other hand one should not exclude the possiblity that the efficacy of cellular processing of internalized toxins depends on the cellular kinetic activity which might be different for the various stem cell subpopulations. Based on the lack of toxicity induced by the DT-huGM-CSF to these cells we conclude that primitive normal hemopoietic progenitor cells do not express functional GM-CSFR, which is consistent with data obtained by others (22, 23, 32). In addition to leukemic and normal hemopoietic cells, non-hemopoietic tissues might be affected by DT-huGM-CSF. High affinity complexes of the GM-CSFR have been identified on normal endothelial cells (34). Because GM-CSFR are also expressed on tumor cells e.g. colon adenocarcinoma cells, small cell lung carcinoma cells, osteogenic sarcoma cells and breast carcinoma cells (35-37), this might suggest that GM-CSFR is expressed on their normal counter parts. Therefore, it will be essential to evaluate the toxic side-effects of DT-huGM-CSF in preclinical animal models, as we described earlier (38).

DT-huGM-CSF could be clinically useful; it may induce sufficient cell kill when applied *in vivo*, while *in vitro* autologous bone marrow grafts might be treated. DT-huGM-CSF induces AML cell kill via an alternative mechanism than cytostatic drugs. This provides the means to eliminate leukemic cells that are resistant to cytostatic drugs. Experiments with 5-FU (25) showed that AML stem cells are resistant to the antimetabolite 5-FU, whereas in the same system a substantial reduction of week 6 CAFC and SCID mouse leukemia initiating cells was observed as a consequence of exposure to DT-huGM-CSF. The optimal conditions for the use of DT-huGM-CSF and its maximal efficacy have yet to be determined.

In conclusion, these results show that DT-huGM-CSF may be utilised to eliminate AML cells with the ability to initiate AML in vivo. The lack of toxicity to normal primitive progenitor cells suggests an exploitable therapeutic window. These preclinical studies warrant further investigations of DT-huGM-CSF as a potential therapeutic agent in the treatment of AML.

Acknowledgements

We thank T. Visser for technical assistance and E. van Bodegem for taking care of the mice.

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Chapter 5

Successful treatment of human AML in SCID mice using Diphtheria toxin fused to huGM-CSF

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Submitted for publication

Abstract

The severe combined immunodeficient (SCID) mouse model may be used to evaluate new approaches for the treatment of acute myeloid leukemia (AML). We have previously demonstrated the killing of SCID mouse leukemia initiating cells by *in vitro* incubation with human GM-CSF fused to *Diphtheria* Toxin (DT-huGM-CSF). In this report, we show that *in vivo* treatment with DT-huGM-CSF eliminates AML growth in SCID-mice. Seven cases of AML were studied. SCID mice were treated intraperitoneally with the maximal tolerated dose of 75 µg/kg/day for 7 days. Antileukemic efficacy was determined at days 40 and 80 after transplantation, by enumerating the percentages of human cells in SCID mouse bone marrow using flow cytometry and patient-specific short tandem repeat-polymerase chain reaction (STR-PCR) analysis.

Four out of seven AML cases were sensitive to *in vivo* treatment with DT-huGM-CSF at both evaluation-time points. In three of these 4 cases elimination of human cells was demonstrated by flow cytometry and STR-PCR. One AML was moderately sensitive to DT-huGM-CSF and growth of the two remaining AMLs was not influenced by DT-huGM-CSF.

Our data show that *in vivo* treatment with DT-huGM-CSF has the potential to reduce long-term growth of AML and warrant further development of DT-huGM-CSF for the treatment of human AML.

Introduction

The severe combined immunodeficient (SCID) mouse may be used as a model to study normal and abnormal hemopoiesis (1-6), providing an unique opportunity to evaluate therapeutic interventions of hemopoietic malignancies (5, 7-9). Among the new agents developed to treat neoplastic diseases, immunotoxins were shown to be very effective (10-12). Encouraging results have been reported in preclinical studies using tumor models in immunodeficient mice (13-17).

Leukemic cells of more than 80% of patients with acute myeloid leukemia (AML) express functional granulocyte-macrophage colony stimulating factor receptors (GM-CSFR) (18-20). This provides a rationale to explore the utility of huGM-CSF fused to a truncated form of Diphtheria toxin (DT-huGM-CSF) for AML treatment. We showed earlier that *in vitro* exposure of AML cells to DT-huGM-CSF eliminates leukemic cells with long-term leukemia initiating potential in long-term bone marrow cultures and in immunodeficient mice, whereas normal hemopoietic stem cells were spared (21, 22). This apparent discrepancy could be explained by low levels of GM-CSFR expression on primitive normal cells (23, 24) as compared to higher levels on AML cells. From these studies we concluded that GM-CSFR on leukemia initiating cells are functional in that they specifically bind the

DT-huGM-CSF, which is then followed by internalisation. However, the antileukemic potential of DT-huGM-CSF as an antileukemic agent *in vivo* is as yet unknown.

In the current study we show that DT-huGM-CSF is effective for the elimination of a small leukemic cell load, in a setting resembling minimal residual disease. The antileukemic effect was evaluated by measuring the degree of human chimerism in SCID mouse bone marrow using flow cytometric analysis and PCR of the short tandem repeat sequences (STR). The latter are polymorphic tandem repetitive elements which are described in humans (25), and may be used to assess chimerism following bone marrow transplantation (26) and have been shown to be a highly sensitive method to assess chimerism following allogeneic bone marrow transplantation (27).

Materials and Methods

Human AML cells

Samples were obtained, following informed consent, from 7 untreated patients with AML. The cases were classified cytologically according to the criteria of the French-American-British Committee (FAB) (28, 29). Mononuclear cells were isolated as a buffy coat, without T cell depletion. Cells were frozen using a controlled freezing apparatus and stored in liquid nitrogen. After thawing by stepwise dilution, cell viability assessed by eosin staining ranged from 80 to 95 %.

Growth factor toxins

The construction of the chimeric DT-huGM-CSF was described by us before (21). The construct pRKDTMGM, encoding DT_{388}-murine GM-CSF (DT-mGM-CSF) was constructed by ligation of a 0.38 Kb NdeI-HindIII fragment encoding mGM-CSF into the 4.2 Kb NdeI-HindIII fragment of pVCDT1-IL-2 (30). The construct pRKDT388, encoding DT_{388} was constructed by blunting and ligation of the 4.2 Kb NdeI-HindIII fragment of pVCDT1-IL-2. The pRKDT388 encodes amino acids 1-388 of DT, and the amino acids SLNSAANKARKEAELAAATLEQ at the C-terminus of DT. Plasmids were expressed in E.coli, and the pure monomeric proteins were purified as described previously optimized to guarantee endotoxin free preparations (31, 32).

ADP ribosylation assay

Toxin was nicked with 0.04 µg trypsin for 15 minutes at 37°C. The reaction was stopped with soybean trypsin inhibitor (Sigma, St Louis, MO, USA). Duplicate samples of nicked fusion proteins and DT were examined for their ADP ribosyl transferase activity as previously described (33). Briefly, ADP-ribosylation was performed in 80 µl reaction mixtures containing 50 µl of 0.01 mol/l Tris-HCl buffer with 1.0 mmol/l dithiothreitol, pH 8.0, 10 µl of rabbit reticulocyte lysate (Promega Corporation, Madison, WI, USA), and 10 µl of toxin sample. The reaction was initiated by the addition of 10 µl of 0.5 µmol/l [^{32}P] nicotinamide adenine dinucleotide (Amersham International, Amersham, UK). Reaction mixtures were incubated at room temperature for 1 hour and the reaction was stopped by the addition of 1 ml 10% trichloroacetic acid (TCA). The precipitate was collected by centrifugation and washed with 1 ml 10% TCA. The radioactivity was counted by standard scintillation techniques.

^{3}H-Thymidine (^{3}H-TdR) incorporation assay

Cells (2x10^4) were cultured for 72 hours in 96-well round-bottom microtiter plates in 100 µl serum free medium (SFM) containing DT-huGM-CSF, DT_{388} or DT-mGM-CSF. The effects of the toxins on cell proliferation were tested in the absence of growth factors, as well as in the presence of the combination of human stem cell factor (SCF 100 ng/ml; a gift from Amgen Biologicals, Thousand Oaks, CA, USA), human interleukin-3 (IL-3 25 ng/ml; a gift from Gist Brocades, Delft, The Netherlands) and human granulocyte-colony stimulating factor (G-CSF 100 ng/ml; Amgen). Eighteen hours before harvesting, 0.1 µCi ^{3}H-TdR (2 Ci/mmol, Amersham) was added to each well. Cells were collected using an automatic cell harvester (Skatron, Lier, Norway), and the cell-associated radioactivity was measured in a liquid scintillation counter (Pharmacia-LKB, Bromma, Sweden). In competition experiments an excess of huGM-CSF (2 µg/ml; a gift from Sandoz BV, Basel, Switzerland) was added simultaneously with DT-huGM-CSF. All cultures were performed in triplicate. Data are expressed as percentage of control.

SCID mice and transplantation of AML

Female specific pathogen-free CB17 scid/scid mice (5-8 weeks of age) were obtained from Harlan CPB, Austerlitz, The Netherlands, and housed under pathogen free conditions in a laminar air flow unit. The mice were supplied with sterile food and acidified drinking water with 100 mg/L ciprofloxacin (Bayer AG, Leverkusen, Germany). The mouse plasma Ig level was determined with an ELISA using a sheep anti-mouse antibody reacting with mouse IgG and IgM (Boehringer Mannheim Biochemica, Penzberg, Germany). Mice with plasma Ig levels > 40 µg/ml were excluded. SCID mice were pretreated with 0.2 ml dichloromethylene diphosphonate (CL_2MDP) liposomes (34, 35), injected into the lateral tail vein (i.v.), on the day before transplantation of the human cells to eliminate the macrophages in spleen and liver. In addition, total body irradiation at a dose of 3.5 Gy was delivered by a ^{137}Cs source (Gammacell, Atomic Energy of Canada, Ottawa, Canada) adapted for the irradiation of mice. The graft size was 30x10^6 AML cells for all leukemia samples. The grafts, suspended in 300 µl Hanks balanced salt solution (HBSS) (Gibco, Breda, The Netherlands), 16 U/ml Heparin and 0.1% bovine serum albumen (BSA, Sigma), were injected i.v. AML A, C, D and G were established to proliferate in SCID mice without support of human hemopoietic growth factors, whereas AML samples B, E and F were IL-3 dependent in the SCID mice. The SCID mice with AML B, E and F received 60 µg of human IL-3 (Gist Brocades, Delft, The Netherlands) in 200 µl HBSS and 1% BSA (Sigma) intraperitoneally (i.p.), three days a week as described (6).

Treatment of SCID mice with DT-huGM-CSF

DT-huGM-CSF, DT-mGM-CSF and DT_{388} were injected i.p. in a volume of 0.1 ml PBS. Each group consisted of 3 to 6 mice. SCID mice were treated daily with DT-huGM-CSF, DT_{388}, huGM-CSF or DT-mGM-CSF starting at day 3 or day 40 after transplantation. The treatment duration was 7 days. As a control for the specificity of DT-huGM-CSF, an excess of huGM-CSF was administered simultaneously with DT-huGM-CSF. DT_{388} and huGM-CSF were administered in equimolar concentrations, and DT-mGM-CSF at a lower dosage, i.e. 10 µg/kg/day.

Histologic examination

Mice that died during the dose-escalation treatment with DT-huGM-CSF were subjected to gross pathology and histological examination. The femur, liver, kidney, lung and intestines were fixed in 4 % formalin, embedded in paraffin, and 4 µm sections were cut and stained with hematoxylin and

eosin. The stained sections were examined by light microscopy for the detection of damage to these organs by the treatment with the toxin.

Tissue collections.

The experiments were carried out following consent of the Institutional Ethics Committee for animal experiments. SCID mice were sacrificed using CO_2 inhalation followed by cervical dislocation in accordance with institutional animal research regulations. Cell suspensions were prepared from the bone marrow and analysed by flow cytometry.

Flow cytometric analysis of SCID mouse derived AML cells

To quantify AML growth and to compare the immunophenotype with the initial graft samples, cells recovered from the bone marrow of SCID mice were incubated with the following (combinations of) mouse monoclonal antibodies: CD34-FITC, CD34-PE, CD38-PE, CD34-FITC/IgG1-PE, CD34-FITC/HLA-DR-PE, CD34-FITC/CD38-PE, CD34-FITC/C-Kit-PE, CD34-FITC/CD33-PE, CD45-FITC/CD33-PE. All antibodies were obtained from Becton Dickinson, San Jose, CA, with the exception of c-kit-PE (Immunotech, Marseille, France). Cells recovered from SCID mouse bone marrow staining with two antibodies specific for human hemopoietic cells were counted as human cells (36). Mouse IgG1-FITC and mouse IgG1-PE conjugated antibodies and labeling of bone marrow cells from non-transplanted SCID mice were used as controls. Samples were analysed using the FACScan flow cytometer and Cell Quest software (Becton Dickinson, Mountain View, CA). Erythrocytes and dead cells were excluded by gating on forward and orthogonal light scatter.

PCR analysis of SCID mouse bone marrow

For the STR-PCR analysis DNA was extracted from human peripheral blood samples and from the SCID mouse bone marrow samples using the modified proteinase K, sodium dodecyl sulphate (SDS) protocol (37). A panel of human short tandem repeat (STR) markers which we have used previously to assess chimaerism following allogeneic BMT (27) and a panel of mouse STR sequences which we have developed to evaluate engraftment in mouse models of transplantation (37) were used to identify informative marker (s) between human and SCID mouse cells. In this way the percentage contribution of human cells to engraftment could be evaluated. STR-PCR was performed as previously described (26, 27). Briefly, approximately 20 ng purified DNA was amplified in a 25 µl reaction with the addition of 2-5 µCi [^{32}P] dCTP (Amersham, UK) to aid in subsequent detection of minor cell populations by autoradiography. Conditions for amplification cycling were: 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute with an additional 5 min at 72°C on the final cycle to ensure complete extension of all amplification products. Hot start PCR was performed to avoid nonspecific amplification. Samples were amplified for 30 cycles on a Trioblock thermal cycler (Biometra, Maidstone, UK). One tenth of amplified product was mixed with an equal volume of formamide dye mix and electrophoresed through 6% denaturating polyacrylamide sequencing gels. Gels were dried down, and exposed to autoradiographic film for 4-24 hrs at -70°C.Dilution experiments were performed to test the sensitivity of detection of minor cell populations. PCR was performed on material isolated from various cell dilutions using both human and mouse STR primers.

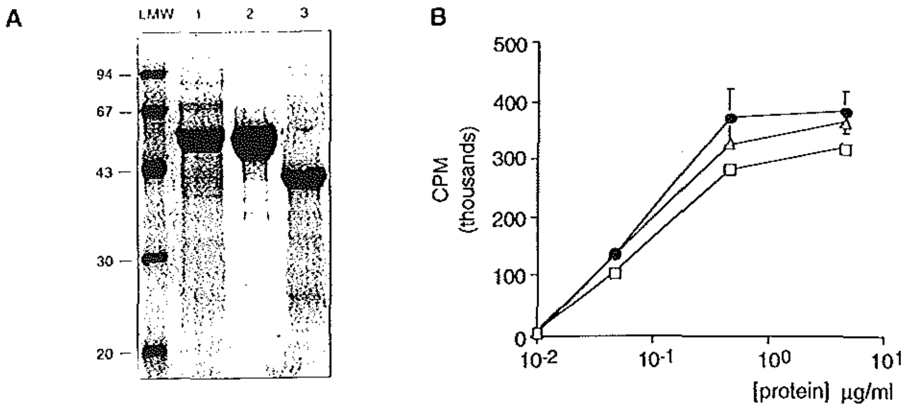

Figure 1
A SDS-PAGE of the purified proteins stained with Spyro Orange and detected with UV. LMW, low molecular weight standards of 97, 67, 43, 30 and 20 kD; lane 1, DT-mGM-CSF; lane 2, DT-huGM-CSF; lane 3, DT_{388}. All proteins were runned through a 10% gel.
B ADP ribosyl transferase activity of trypsin-nicked DT-huGM-CSF (□), DT-mGM-CSF (●) and DT_{388} (Δ) were studied in a cell-free assay. Protein was added at various concentrations to the reaction system. The activity was measured as cpm of [^{32}P]-ADP ribose bound to rabbit reticulocyte lysate (EF-2).

Results

Characterization of the fusion proteins

DT-huGM-CSF, DT_{388} and DT-mGM-CSF were purified by the method as described by Kreitman (31) to avoid contamination of endotoxins in the final preparation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis confirmed the purity of the proteins. The fusion proteins DT-mGM-CSF (57 kD), DT-huGM-CSF (56 kD), and DT_{388} (40 kD) migrated in the gel corresponding to their expected size (Fig 1A). All fusion toxins used in this study were greater than 95% pure. The ADP-ribosylation assay showed a comparable dose-dependent increase in [^{32}P] incorporation for all proteins (Fig 1B).

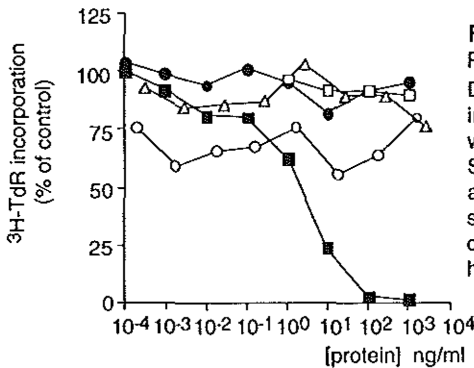

Figure 2
Proliferation inhibition of AML cells of case A by DT-huGM-CSF measured in the ^{3}H-TdR incorporation assay. Toxins were added to cells with a mix of SCF, IL-3 and G-CSF (■). Specificity of DT-huGM-CSF was tested by adding an excess of huGM-CSF (2 μg/ml) (□) simultaneously with DT-huGM-CSF. Other control proteins tested are DT-mGM-CSF (●), huGM-CSF (Δ) and DT_{388} (○).

Antiproliferative effects of DT-huGM-CSF in a proliferation assay

The effects of the toxins on *in vitro* proliferation of AML cells were evaluated in the ^{3}H-TdR incorporation assay. Cells were stimulated with the combination of G-CSF, IL-3 and SCF. DT-huGM-CSF was added in concentrations ranging from 0.001 to 1,000 ng/ml for 72 hours. A representative AML sample is shown in Fig 2. The cytotoxic effect of DT-huGM-CSF could be prevented by an excess of huGM-CSF, showing the specificity of the toxin. AML cells were resistant to DT_{388} and DT-mGM-CSF (Fig 2). Addition of huGM-CSF to the combination SCF, IL-3 and G-CSF did not result in an additive proliferative effect.

Table 1 shows the ID_{50} values found for the various AMLs. The ID_{50} is the DT-huGM-CSF dose required to induce a 50% inhibition of DNA synthesis. In 5 of the 7 AMLs, DT-huGM-CSF proved to be cytotoxic. The 5 sensitive samples had a mean ID_{50} of 2.8 ng/ml, ranging from 0.7 to 5.0 ng/ml. AML cells from patient F and G were insensitive to DT-huGM-CSF; even a concentration of 1, 000 ng/ml failed to induce an antiproliferative effect. AML cells in these 2 cases were unresponsive to huGM-CSF, indicating the absence of functional GM-CSFR.

Table 1
Effect of DT-GM-CSF *in vitro* on AML cells in the ^{3}H-TdR uptake assay

AML	FAB	^{3}H-TdR uptake (cpm)			ID_{50} (ng/ml)
		no growth factor	GM-CSF	SCF/IL-3/G-CSF	
A	M1	1,200	9,800	35,500	0.7
B	M1	1,400	3,700	11,800	3.5
C	M2	1,300	8,200	33,500	5.0
D	M5	1,800	8,700	22,300	1.5
E	M1	420	7,900	28,400	3.4
F	M5	4,000	5,700	16,000	>1000
G	M1	1,600	2,000	31,400	>1000

Cells from 7 AML patients diagnosed according to the FAB criteria were incubated in serum-free medium without growth factors added or with GM-CSF alone or a mixture of SCF, IL-3 and G-CSF.The ID_{50} is the concentration of DT-huGM-CSF required for 50% inhibition of the proliferation with SCF, IL-3 and G-CSF.

In vivo effect of DT-huGM-CSF administration on SCID-mice

To determine the maximal tolerated dose (MTD), mice, conditioned with 3.5 Gy gamma radiation and macrophage depletion by Cl_2MDP liposomes, were treated with graded doses of DT-huGM-CSF. DT-huGM-CSF was administered by i.p. injection for a period of 7 days starting three days after conditioning. Table 2 lists the mortality found at the different dose levels. SCID mice receiving a daily dose of 400 μg/kg/day i.p. died on the fourth day of treatment. Three out of 4 mice treated with 200 μg/kg/day and 1 out of 8 treated with 100

µg/kg/day died on day 7 of the treatment period. The remaining mice recovered completely. In subsequent experiments the mice were treated with the MTD of 75 µg/kg/day.

Morphological examination of the mice that died of toxicity showed signs of degeneration of the renal proximal tubule cells and fatty degeneration of the liver. The other organs showed no abnormalities. Vascular leakage, and associated pulmonary edema, as typically observed side effects of immunotoxins based on ricin or Pseudomonas exotoxin, were not observed.

Table 2
Determination of the maximally tolerated dose of DT-huGM-CSF in SCID mice administered daily by i.p. injections for 7 days

Dose (µg/kg/day)	no. of mice	mortality (%)
50	6	0
75	4	0
100	8	12.5
200	4	75
400	4	100

Effect of in vivo DT-huGM-CSF administration on outgrowth of AML

The effect of DT-huGM-CSF treatment on the outgrowth of leukemia *in vivo* was investigated for 7 cases of AML. Five were sensitive to huGM-CSF in the ^{3}H-TdR incorporation assay and 2 were not sensitive (Table 1). Three days after transplantation daily treatment with DT-huGM-CSF (75 µg/kg/day) was started for a period of 7 days. The SCID mice were evaluated 40 days after transplantation (Table 3) by flow cytometry. Whereas in control mice injected with cases A, B, D and E, 1.6, 10.9, 3.8 and 3.0% human cells were detected, no human leukemic cells could be detected at this time point in mice treated with DT-huGM-CSF. At day 80 of evaluation the cases A, D and E were still negative. Case B showed a reduced percentage of human cells in comparison to control mice at day 80. DT-huGM-CSF treatment of mice grafted with AML cells of patient F and G did not lead to a reduced leukemic cell load in comparison to control mice. Treated AML-SCID mice from sample C, showed a slight reduction in the percentage of leukemic cells in comparison to control mice (58.5 versus 95.5%). Cases C, F and G were not analyzed at day 80.

Phenotypic analysis of AML cells from the SCID mouse bone marrow.

Cells recovered from the SCID mouse bone marrow were investigated using flow cytometry with the antibodies CD34, CD38, CD33, CD45, HLA-DR and anti c-kit. The phenotypes of the AML cells recovered from the SCID mice were almost similar to the original grafts (Table 4). In case C the phenotype converted from 20% CD34 positive AML cells to a phenotype negative for CD34 expression, which has been reported earlier (38). In case D the phenotype converted from 30% c-kit positive AML cells to a phenotype >90%

positive for c-kit expression. Additional support for leukemic cells in SCID mice was obtained by the morphologic evaluation of cytospin preparations (data not shown).

Table 3
Outgrowth of DT-huGM-CSF exposed versus control AML cells in SCID mice

AML	Treatment	% AML cells in mouse BM# (no. of mice)	
		day 40	day 80
A	control	1.6 ± 0.8 (4)	16.6 ± 14.3 (3)
	DT-huGM-CSF	0 - 0 (2)	0 (6)
B	control	10.9 -10.9 (2)	54.0 - 42.8 (2)
	DT-huGM-CSF	0.3 ± 0.1 (5)	2.4 ± 2.0 (5)
C	control	95.5 ± 12.5 (5)	ND
	DT-huGM-CSF	58.5 ± 25.5 (8)	ND
D	control	3.8 ± 5.5 (4)	14.8 - 62.4 (2)
	DT-huGM-CSF	0 (3)	0 (3)
E	control	3.0 ± 2.0 (4)	95.7 - 0 (2)
	DT-huGM-CSF	0 (4)	0 (3)
F	control	46.8 ± 22.5 (4)	ND
	DT-huGM-CSF	51.0 ± 12.0 (3)	ND
G	control	17.4 ± 8.2 (5)	ND
	DT-huGM-CSF	31.1 ± 31.2 (4)	ND

SCID-AML mice were treated for 7 days starting at day 3. #The percentage ± SD of human cells proliferating in the SCID mouse bone marrow was determined by flow cytometry at days 40 and 80. ND: not determined.

Table 4
Immunophenotype of AML cells before transplantation and after growth in SCID mice

Phenotype	A		B		C		D		E		F		G	
	pat.	SCID	pat.	SCID	pat.	SCID	pat.	SCID	pat.	SCID	pat.	SCID	pat.	SCID
CD34	40	30	-	-	20	-	-	-	20	20	30	30	+	+
CD38	+	+	+	+	+	+	+	+	70	+	+	+	-	-
HLA-DR	+	+	+	+	+	+	+	+	80	+	+	+	+	+
c-kit	+	+	+	+	+	+	20	+	+	+	30	-	+	+
CD33	+	+	+	+	+	+	+	+	+	+	75	60	-	-

Percentages of positive cells in AML identified as CD45 positive cells (+) > 90% of the AML cells stained with the antibodies as determined by flow cytometry and compared to a IgG1-FITC or IgG1-PE control. (-) < 10% of the AML cells stained with the antibodies.

Effect of DT-huGM-CSF on overt AML in SCID mice

Treatment of SCID mice engrafted with AML was also performed in advanced stages of leukemia, at days 40 to 47 after transplantation (Table 5). We examined cells from patient A, which were sensitive to the early treatment with DT-huGM-CSF. We determined the direct effect on AML cells by measuring AML cells the day after the treatment of 7 days, i.e. on

day 47. At this time point the number of leukemic cells was significantly reduced. After 33 days, i.e. 80 days after transplantation, significant leukemia was observed.

Dose titration of DT-huGM-CSF on the outgrowth of AML

Thus far we observed that, at the MTD level, most AML cells can be eliminated. Next, we investigated the dose-effect relation for DT-huGM-CSF on the outgrowth of AML A by administering doses in a range from 6.25 to 75 μg/kg/day (Table 6) to determine the lowest effective dose level. At every dose level used we achieved elimination of leukemic cells except in the lowest dose level group, in which in 1 out of 3 mice leukemic cells (0.2%) were detected by flow cytometric analysis. This indicates that 1/10 of the MTD dose level is already sufficient to achieve an antileukemic effect. We also investigated whether an *in vivo* AML cell killing effect was achieved by administering huGM-CSF, DT_{388} and DT-mGM-CSF. Since the outgrowth of AML cells was not reduced significantly in all these groups it can be concluded that the toxicity to the AML stem cells is specifically mediated via the huGM-CSFR.

Table 5
Outgrowth of DT-huGM-CSF exposed versus control AML cells of sample A in SCID mice treated from day 40 to 47

day of analysis	% AML cells in mouse BM#	
	control	DT-huGM-CSF
day 40	4.7	
	39.5	
	10.0	
day 47		0
		0.7
		0
		0.9
day 80	41.8	70.1
	55.2	9.5
	0	12.6
		4.7

SCID-AML mice were treated for 7 days starting at day 40. #The percentage of human cells present in the SCID mouse bone marrow was determined by flow cytometry. Controls were measured at day 40 and the treated mice were measured at day 47. At day 80 SCID mice were evaluated for the long term outgrowth of treated and untreated SCID-AML.

DNA analysis

The SCID mice marrow samples in which we could not detect human cells with flow cytometry were further analyzed by PCR for human STRs in DNA extracts of bone marrow. For each patient we identified both human and mouse STR-PCR primers which could be used to distinguish between human and mouse cells on the basis of their electrophoretic mobility

through polyacrylamide gels (Table 7). The sensitivity of human specific PCR was assessed using a cell dilution assay of SCID mouse BM cells into AML cells. Likewise, the sensitivity of mouse specific STR-PCR was assessed using a cell dilution assay of AML cells into SCID mouse bone marrow. PCR allows detection of the minor cell population, either human or mouse at dilutions between between 1/1,000 and 1/10,000. This level of detection of the minor cell population is below the limit of detection by conventional FACScan analysis for chimerism detection in SCID BM. All amplifications of mouse samples from day 40 post treatment with mouse STR-PCR primers were negative for human cells. Analysing the bone marrow samples of the dose titration of DT-huGM-CSF on the outgrowth of AML case A, human STR primers indicated the presence of low numbers of human cells at the lower doses (Table 6). STR-PCR of the bone marrow samples of treated SCID mice at day 80, transplanted with AML cells of cases A, D and E showed the presence of human cells in one mouse out of 3 mice transplanted with AML cells from case A (chimerism <0.01%), one out of 3 mice transplanted with AML cells from case D (chimerism <0.01%) and in 1 out of 3 mice transplanted with AML cells from case E (chimerism <1%), respectively.

Table 6
Dose-effect of treatment with DT-huGM-CSF from day 3 to 10 on the outgrowth of AML sample A in SCID mice

agent	Treatment µg/kg/day i.p.	no. of mice positive for AML/total mice analysed flow cytometry	STR-PCR
-	-	3/3 (8.2 ± 5.9)	nd
DT-huGM-CSF	75	0/3	0/3
	37.5	0/4	1/4 (<0.01%)
	12.5	0/2	1/2 (<1%)
	6.25	1/3 (0.2%)	1/3 (<1%)

SCID-AML mice were treated for 7 days starting at day 3. The percentage of human cells proliferating in the SCID mouse bone marrow was determined by flow cytometry and STR-PCR at day 40. In parenthesis the percentage (±SD) of AML cells.

Table 7
Human and mouse STR primer pairs

Hu AML cells	human specific STR	mouse specific STR
A	Fes (15q)	1036 (D4Mit 13)
D	vWF (12)	1036 (D4Mit 13)
E	ACPP (3q21)	1036 not informative

The table above indicates which STRs (mouse and human) were specifically informative markers between SCID BM cells and human AML cells. In parenthesis the chromosomal location.

Discussion

In this study we evaluated the utility of DT fused to huGM-CSF for *in vivo* treatment of human AML using the SCID/AML mouse model. We selected 7 cases of AML for evaluation, from which 5 cases (A, B, C, D and E) showed a proliferative response to huGM-CSF *in vitro* and were sensitive to DT-huGM-CSF exposure *in vitro*. Cases F and G were non responsive to huGM-CSF and insensitive to DT-huGM-CSF. The long-term repopulating AML cells of 4 out of the 5 huGM-CSF responsive AMLs surveyed in this study, were eliminated by a 7-days treatment with DT-huGM-CSF at the MTD of 75 µg/kg/day. Regrowth of leukemia of case A, D and E did not occur as late as day 80 as confirmed by flow cytometry, suggesting effective reduction of AML growth. However, human STR-PCR showed the presence of low numbers of AML cells in all groups. At day 80 a low percentage of human cells was found in mice transplanted with cells of case B. Cell dose titrations of case A indicated that at least a 3 log AML cell kill was achieved. DT fused to murine GM-CSF, DT_{388} or huGM-CSF did not interfere with AML growth *in vitro*, neither with AML outgrowth, indicating that leukemic stem cell reduction was mediated by specific binding of DT-huGM-CSF to the GM-CSFR of the primitive AML cells.

The response to huGM-CSF was predictive for sensitivity to DT-huGM-CSF in 6 out of 7 cases. In our study for the cause of insensitivity to DT-huGM-CSF, we observed that $CD34^+$ phenotype subpopulation of AML cells from case F (30% of all cells) was associated with a clear expression of the α subunit but lacked the β subunit of GM-CSFR (data not shown). Often SCID mouse leukemia initiating cells are restricted to the $CD34^+$ fraction (4, 39). AML cells from case G lack both GM-CSFR subunits. We suggest that the failure of DT-huGM-CSF to eliminate cells of F and G and the moderate sensitivity of case C was due to the absence or low expression of functional GM-CSFR on the rare population of SCID mouse leukemia initiating cells.

Mice were treated with the MTD of DT-huGM-CSF applied as a daily i.p. administration for 7 constitutive days. The schedule of prolonged delivery of growth factor toxins showed less side effects while it retained a maximal antileukemic effect (40-43). In addition, a treatment duration of 7 days is conventional in human AML treatment and has been shown to be adequate for AML tumor reduction by cytostatic drugs. Lower doses of drugs are in general better tolerated and the duration of the treatment can thus be extended without an increased risk of treatment related toxicity. However, it can also lead to a reduced antileukemic effect. Additional experiments with DT-huGM-CSF show antileukemic effect at the 10-fold reduced dose of 6.25 µg/kg/day. The efficacy seems to be dose-dependent. Prolonged treatment at lower dose levels would increase antileukemic effects. The toxic deaths at dose levels above the MTD in the SCID mice occurred as a consequence of liver failure. This concurs with localisation studies of DT-huGM-CSF performed in SCID mice (44) which suggest that the liver is a major site of uptake and clearance.

Treatment of mice with overt leukemia at day 40 was effective, as demonstrated by reduced leukemia loads after completion of treatment. However, significant AML regrowth was observed by day 80, suggesting that the number of primitive leukemic cells with proliferative capacities had increased in the interval between transplantation and day 40 and could not be eliminated by one course of DT-huGM-CSF treatment. This is in agreement with the observation that the numbers of primitive and committed progenitors of normal bone marrow increase after transplantation in immunodeficient mice (45-47). Prolonged treatment or repeated treatment cycles may increase the efficacy of DT-huGM-CSF.

The data on antileukemic activity presented here in combination with the lack of toxicity to normal primitive hemopoietic progenitor cells (22) give support for further development of clinical application of these novel agents. However, before entering a clinical phase I study in humans we suggest prior evaluation of toxic side effects of DT-huGM-CSF in other animal models. In the mouse there is no cross reactivity between huGM-CSF and the murine GM-CSFR. Therefore, a possible therapeutic window for DT-GM-CSF could be determined in rodents using murine GM-CSF as the targeting agent. Alternatively and preferably, the rhesus monkeys employed earlier for evaluation of huGM-CSF could be used as a suitable preclinical animal model (48, 49). In addition, DT-huGM-CSF binding studies using human tissues could give insight *in vivo* toxicity.

Acknowledgements

We thank E. van Bodegem for taking care of the mice and Dr. L.M. Budel from the Department of Pathology, Erasmus University, Rotterdam, for performing the histologic examinations.

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Chapter 6

In vivo targeting of leukemic cells using *Diphtheria* toxin fused to murine GM-CSF

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Submitted for publication

Abstract

We have previously demonstrated that Diphtheria Toxin (DT) fused to human GM-CSF effectively eliminates human long-term leukemia initiating cells in SCID mice. However, because huGM-CSF does not react with the murine GM-CSF receptor possible side effects to non-leukemic tissues could not be analysed in the AML/SCID model. To overcome this problem, we used murine GM-CSF fused to DT and studied the therapeutic index in the rat leukemia model BNML/LT12. In DT-mGM-CSF dose escalation experiments, severe dose dependent toxicity to organs as liver, kidney and lung was observed. Therefore, the antileukemic effects were evaluated with the lower doses. Daily intraperitoneal bolus injections of 75 μg/kg/day for 7 days induced a 3 log leukemic cell kill. The dose of 75 μg/kg/day had no effect on the hemopoietic progenitor cell subsets.

Introduction

Diphtheria toxin (DT) is a potent bacterial toxin, widely used for the construction of fusion proteins (1-3). By replacing the binding domain of DT by a growth factor, potential novel therapeutic agents for malignancies have been developed. The toxins are targeted to cell surface receptors expressed by neoplastic cells. After receptor mediated internalization, toxins are cleaved, which generates active fragments that are competent to translocate across the intracellular membrane from the endocytic vesicles into the cytoplasm. Intoxication of cells is through inhibition of protein synthesis by adenosine diphosphate ribosylation of elongation factor 2 (4).

The fusion protein DT-huGM-CSF is one of the growth factor toxins currently under investigation for the treatment of myeloid malignancies, in particular acute myeloid leukemia (AML). GM-CSF receptors (GM-CSFR) are found on leukemic cells of more than 80% of the patients with AML (5, 6). We have previously reported that human AML cells with long-term leukemia initiating potential in Severe Combined Immunodeficient (SCID) mice could be eliminated by *in vitro* or *in vivo* targeting (7, 8). The absence of toxicity to the normal progenitor cell compartment, when exposed to the DT-huGM-CSF under similar conditions, suggested an exploitable therapeutic window. Since DT-huGM-CSF used in these studies reacts with human but not with murine cells (9), the murine tissues were not at risk. Thus, these experiments had a limited predictive value for toxic side effects of therapy in human. Non hemopoietic tissues that express GM-CSFR include placenta (10), endothelium (11, 12), and the central nervous system (13, 14). Expression of GM-CSFR on various non-hemopoietic tumor cells (15-18) might also be indicative of the presence of GM-CSFR on their normal counterparts.

In the current study we have used the BNML rat leukemia model for AML (19, 20) to study the antileukemic effects of DT fused to murine GM-CSF in direct relation to the bone marrow specific toxicity and to the systemic toxicity.

Materials and methods

Experimental animals

Brown Norway inbred rats (BN/BiRij) were purchased from Harlan CPB, Zeist, The Netherlands. Female rats 12-14 weeks of age were used (body weight 150-170 g). Animals were bred under specified pathogen free (SPF) conditions and maintained under clean conventional conditions. F1 (B_6xCBA) mice were bred in the Central Animal Facility of the Erasmus University, Rotterdam, The Netherlands, and maintained under conventional conditions. All animal experiments have been carried out in accordance with institutional animal research regulations.

Cell lines and reagents

The BNML subline LT12, HL60, P815, BA-F3, 32D, FDC-P1 and CTLL-2 were grown in RPMI 1640 medium (GIBCO, Pailey, UK). The 3T3, J774, MO7e and K562 cell lines were grown in Dulbecco's Modified Eagles Medium (GIBCO). Both media were supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Some of these cell lines were growth factor dependent; CTTL-2 cultures were supplemented with 20 U/ml huIL-2 (Biogen, Geneva, Switzerland), FDC-P1 cultures were supplemented with 10% WEHI-3B conditioned medium as a source of mIL-3, and BA-F3 and 32D were supplemented with 10 ng/ml murine IL-3. MO7e culture was supplemented with 5 ng/ml rhuIL-3 (a gift from Gist Brocades, Delft, The Netherlands) and 10% (v/v) 5637 conditioned medium as a source of huGM-CSF. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

The construction and characterisation of DT-mGM-CSF, DT_{388} and DT-huGM-CSF were described elsewhere (8). The plasmids were expressed in E.coli, and the pure monomeric protein was purified as described previously for IL-6-PE4E (21).

Murine GM-CSF was radiolabeled using the method of Bolton and Hunter (22). The mGM-CSF binding characteristics were determined as previous described (5, 23).

In vitro cytotoxicity assays

^{3}H-Thymidine (^{3}H-TdR) incorporation assay and colony assay for the LT12 cell line (CFU-L) was performed as described (24).

The CFU-L was used to determine the exposure time required for DT-mGM-CSF to induce maximal inhibition of colony formation. 0.5x10^6 LT12 cells were incubated for the indicated period in 0.5 ml medium with variable doses of DT-mGM-CSF, washed intensively thereafter, and resuspended in complete medium and the number of colony forming cells were enumerated by performing the CFU-L assay.

Treatment of leukemic and nonleukemic rats with GM-CSF toxins

Leukemic rats were obtained by injecting 5x10^5 LT12 intravenously (i.v.). On day 3, Alzet mini-osmotic pumps type 2001 (Charles River, Sulzfeld, Germany) were implanted intraperitoneally (i.p.). The pumps released 1.0 µl/hour for 7 days. The DT-mGM-CSF was diluted in sterile PBS containing 1 mM NAD^+ as the stabilizing agent (25). The pumps were removed 10 days after implantation. Alternatively, toxins were administered by repeated i.p. bolus injections. DT-mGM-CSF dilutions were freshly made in PBS and immediately injected.

Quantification of the leukemic cell load in vivo

Exact quantification of the leukemic cell load in bone marrow can be determined by performing limiting dilution assays. Bone marrow cells of three treated or untreated leukemic rats were obtained by flushing the femural shafts with α MEM (GIBCO). The bone marrow cells were cultured in medium without exogenous growth factors in 96 micro-well culture dishes. Input values were the equivalent of 500,000 nucleated cells (NC) per well in a volume of 200 µl. Twelve to sixteen dilutions, three-fold apart, were used for each sample with 24 replicate wells per dilution. The percentage of wells containing at least five leukemic cells was determined after 14 days. The frequency of clonogenic leukemic cells was calculated using Poisson statistics as described (26).

Evaluation of DT-mGM-CSF related toxicity

Rats that died during the treatment with DT-mGM-CSF were subjected to pathological and further microscopical histological examination. Femur, liver, spleen, heart, kidney, lung, intestines, and brain from treated rats were fixed in 4% formalin, paraffin-embedded and 4 µm sections were cut and stained with hematoxylin and eosin. The sections were examined by light microscopy for signs of damage to these organs by the treatment with the toxin.

Blood samples for measuring blood cellularity and biochemical parameters were taken during the treatment as described (24).

Effect of DT-mGM-CSF on normal hemopoietic progenitor and stem cells subsets

To evaluate the effects of DT-mGM-CSF on normal bone marrow, nonleukemic rats were treated with a dose of 75 µg/kg/day for 7 days by i.p. bolus injections. Bone marrow cells of 3 treated and 3 untreated rats were harvested and the number of progenitor cells from the pooled fractions determined. Clonogenic assays (CFU-C) (27, 28), the rat-to-mouse Colony Forming Unit Spleen (CFU-S) assay (29) and the rat cobble stone area forming cell (CAFC) assay (30) were applied to quantitate progenitor and stem cells of different primitivity.

To determine the effect of continuous exposure of DT-mGM-CSF *in vitro* (CFU-C), cultures from rat bone marrow cells were supplemented with 5% IL-3 (supernatant from monkey COS-1 cells transfected with the rat IL-3 gene (30)), 10 ng/ml recombinant rat SCF (Amgen, Thousand Oaks, CA, USA) and 50 U/ml huM-CSF (Genetics Institute, Cambridge, MA, USA).

Results

Toxicity of DT-mGM-CSF in vitro

First, the toxicity of DT-mGM-CSF towards a variety of human and murine hemopoietic cell lines, known to possess or lack GM-CSF receptors (31-33), was studied (Table 1). Parental LT12 cell line express less than 50 GM-CSFR/cell and show an ID_{50} value of DT-mGM-CSF of 13 ng/ml. The murine myeloid cell lines FDC-P1 and J744 were also sensitive to DT-mGM-CSF. Excess mGM-CSF blocked the cytotoxicity of DT-mGM-CSF, indicating that the toxic effects of the fusion proteins were mediated specifically through interaction with the GM-CSFR (data not shown). DT_{388}, devoid of its cell binding domain, and DT-huGM-CSF were not cytotoxic for the murine cell lines. In agreement with the species specificity of mGM-CSF, it was found that human myeloid cell lines MO7e and HL60 were

not sensitive to DT-mGM-CSF. As expected these cell lines were sensitive to DT-huGM-CSF

Table 1
Sensitivity of hemopoietic tumor cell lines to DT-GM-CSF

Cell line		ID_{50} (ng/ml)			GM-CSFR	[ref]
		DT-mGM-CSF	DT-huGM-CSF	DT_{388}		
LT12	rat promyelocytic cell	13	>1000	>1000	+ #	
FDC-P1	murine myelocytic cell	12	>1000	>1000	+ #	[31]
J774	murine monocytic cell	1	>1000	>1000	+	[31]
32D	murine myeloblastic cell	>1000	>1000	>1000	±	[31]
P815	murine mastocytoma cell	>1000	>1000	>1000	-	[31]
CTLL-2	murine cytotoxic T cell	>1000	>1000	>1000	ND	
BA-F3	murine pro-B cell	>1000	>1000	>1000	ND	
MO7E	human megakaryocytic cell	>1000	3	>1000	300-400	[33]
HL60	human myeloblastic cell	>1000	10	>1000	<50	[32]
K562	human erythroblastic cell	>1000	>1000	>1000	-	[32]

ID_{50} is the concentration of the DT-GM-CSF needed to induce a 50% reduction in cell proliferation in the ^{3}H-TdR incorporation assay. # specific binding of [^{125}I]-mGM-CSF was observed, but binding is too low for exactly quantitating the number of receptors. ND: not determined

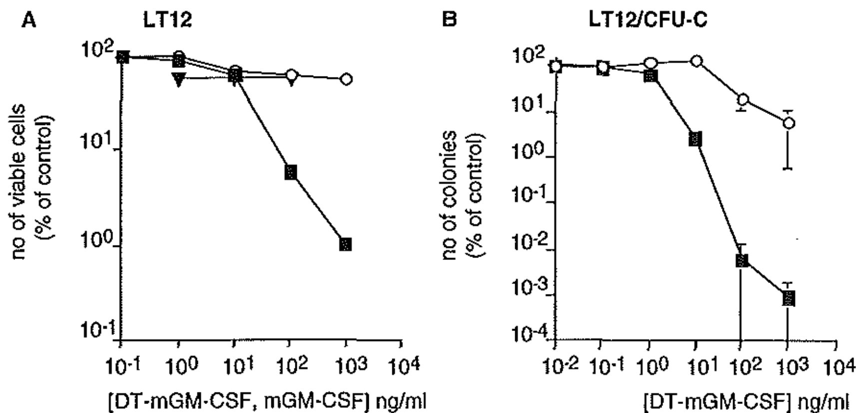

Figure 1
A The viability of LT12 cells was examined after exposure to DT-mGM-CSF for 2 days (■). Specificity of DT-mGM-CSF was tested with mGM-CSF (O) and an excess of mGM-CSF (2 µg/ml) added stimultaneously with DT-mGM-CSF (▼). B The effect of DT-mGM-CSF on the clonogenic capacity ± SD of leukemic (■) and normal bone marrow cells (O) measured in clonogenic assays. Cells were continuously exposed to DT-mGM-CSF during the culture period.

The inhibitory effect of DT-mGM-CSF on the proliferation of LT12 cells in the ^{3}H-TdR assay was examined in further detail by studying the cell viability and colony forming ability. Exposure to DT-mGM-CSF resulted in a dose dependent reduction of viability of LT12 cells as measured with eosin staining (Fig 1A). Outgrowth of LT12 colonies in semi solid medium was severely affected. A 5 log reduction of colony formation was observed at a concentration

of 1000 ng/ml DT-mGM-CSF (Fig 1B). In contrast, outgrowth of normal CFU-C was reduced only one log at this concentration (Fig 1B).

To establish the time-effect relationship for DT-mGM-CSF to intoxicate LT12 cells, cells were incubated with several dosages of the toxin (10-100-1000 ng/ml) for varying lengths of exposure time. As shown in Fig 1C, we observed a clear correlation between the dose and exposure time *in vitro*.

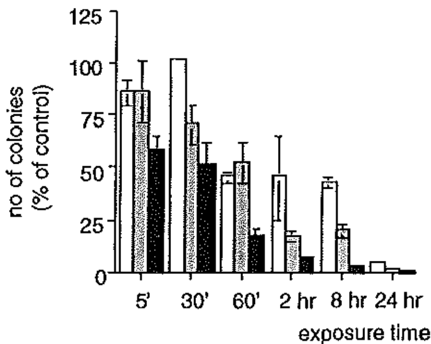

Figure 2
Effect of exposure time of LT12 cells to various concentrations of DT-mGM-CSF in liquid culture. Cells were incubated with 10 ng/ml □, 100 ng/ml ▒ and 1000 ng/ml ■. After the indicated incubation time the cells were washed and a CFU-L assay was performed. The number of colonies is expressed as the % of CFU-L ± SD of untreated control cells.

Toxicity of DT-mGM-CSF in vivo

To determine the maximal dose of DT-mGM-CSF that could be given to rats, DT-mGM-CSF was administered at dose levels of 50 to 600 µg/kg/day for 7 days (Table 2). Treatment of rats started three days after inoculation of leukemic cells as described before (24), in a setting resembling minimal residual disease. To determine the most optimal schedule for administration of DT-mGM-CSF, we compared the effect of i.p. osmotic pumps for the continuous delivery of the toxin with daily i.p. injections of the DT-mGM-CSF (Table 2).

Continuous delivery of DT-mGM-CSF at dose levels of 50 and 100 µg/kg/day was well tolerated. At higher doses, mortality increased in a dose dependent manner. I.p. bolus injections were given in doses of 50, 75, 100, 150 and 300 µg/kg/day. In all groups toxicity related deaths were observed.

Postmortem examination was performed on animals that died during treatment. Histopathologic examination of rats revealed moderate toxicity of liver, spleen, lungs and kidney. A substantial number of hepatocytes showed vacuolated cytoplasm but no cellular necrosis was observed. At higher doses, i.e. rats receiving 150-600 µg/kg/day either by daily bolus injection or by osmotic pumps i.p., the spleen architecture was disrupted by a complete depletion of the white and red pulp. In the lungs occasionally bleeding was observed. Kidneys showed moderate damage of the tubuli. No abnormalities were observed in the heart, intestines or brain. Rats treated by daily bolus injection of 50 or 75 µg/kg/day for 7 days of DT-mGM-CSF and killed one day after the treatment, did not show histopathological abnormalities.

Table 2
Survival of rats treated with DT-mGM-CSF for 7 or 4 days

administration	dose (μg/kg/day)	number of days	number of rats	survival (%) day 3	day 5	day 7	day 9	day 11	day 13
i.p. pump	50	7	11	100	100	100	100	100	86
(days 3-10)	100	7	17	100	100	100	100	100	86
	150	7	5	100	100	40	0		
	200	7	8	100	50	50	38	25	25
	300	7	7	100	29	14	14	0	
	450	7	3	100	67	33	33	0	
	600	7	3	100	33	0			
i.p. bolus inj.	50	7	14	100	100	100	91	82	73
(days 3-10)	75	7	16	100	100	81	58	47	47
	100	7	5	100	100	80	80	40	40
	150	7	5	100	100	60	0		
	300	7	2	100	50	0			
i.p. bolus inj.	75	4	8	100	100	100	100	100	80
(days 3-7)	150	4	8	100	100	60	0		

Pooled data from 3 experiments. Leukemic rats were treated for 7 or 4 days. Both treatment schedules started at day 3 after inoculation of LT12 cells.

In vivo antileukemic effect of DT-mGM-CSF

Next, DT-mGM-CSF could be evaluated for its potential therapeutic role in the treatment of leukemia. Because of the severe systemic toxicity of dosages exceeding 100 μg/kg/day, we studied the antileukemic effects in the lower dose range (50 to 100 μg/kg/day) by determining the effect on the leukemic cell population in bone marrow from 3 rats.

Compared to untreated controls a 3 log leukemic cell kill was observed after i.p. bolus injections of 75 μg/kg/day given for 7 days. The i.p. bolus injections of 50 μg/kg/day resulted in a 1 log cell kill. A 10 to 25 fold reduction was found in the group treated with DT-mGM-CSF when this was delivered continuously at a dose of 50 and 100 μg/kg/day for 7 days (Table 3). To establish the effect of shorter treatment duration, rats were i.p. injected with doses of 75 and 150 μg/kg/day for 4 days. The antileukemic effect in the bone marrow compartment was reduced from 3 log to 1 log leukemic cell kill for the 75 μg/kg/day treatment compared to the 7 days treatment (Table 3). The higher dose of 150 μg/kg/day revealed an antileukemic effect of 3 log leukemic cell reduction. Toxicity to the rats indicated that a 4 days treatment was better tolerated than the 7 days treatment schedule at the 75 μg/kg/day dose level (Table 2).

Rats treated with DT-mGM-CSF either by i.p. bolus injections or with osmotic pumps revealed a maximal increase in the survival time of 3-4 days in comparison to the untreated controls (p= 0.02 Kaplan-Meier survival analysis). Based on the cell dose-survival relationship, it could be calculated that an overall reduction of 1.5-2 log in the leukemic cell load was achieved.

Table 3
Effect of DT-mGM-CSF treatment on leukemia growth in BN rats

group	dose (μg/kg/day)	number of days	number of rats	leukemic cells/femur # ($x10^4$)
controls			13	299 ± 90
i.p. osmotic pump	50	7	6	30 ± 5
	100	7	12	12 ± 2
i.p. bolus injections	50	7	7	34 ± 55
	75	7	5	0.3 ± 0.1
	75	4	8	12 ± 12
	150	4	8	0.2 ± 0.1

Mean number ± SD of leukemic cells of bone marrow fractions of 3 rats. Frequency was quantified one day after finishing the treatment, i.e. day 10 after inoculation of LT12 cells.

Effect of in vivo administration of DT-mGM-CSF on normal hemopoietic progenitors

To determine toxicity on hemopoietic progenitors in the bone marrow, rats were sacrificed one day after the treatment with 75 μg/kg/day administered by i.p. bolus injections for 7 days (Table 4). Except in the case of CFU-E, DT-mGM-CSF treatment for 7 days did not lead to a reduction in the number of committed progenitors, i.e. CFU-GM and BFU-E, or in the number of the more immature progenitors, CFU-S day 8, CFU-S day 12 and CAFC week 2 or in the number of CAFC week 6, which reflect the most primitive hemopoietic progenitor cell type. The number of CFU-E was significantly reduced by 70% accompanied by a reduction of the red blood cells in the marrow. Histopathologic analysis of the bone marrow in femora revealed an active myelopoiesis with large numbers of myeloid cells (Fig 3), which could possibly explain the relative reduction in erythroid cells from the marrow.

Table 4
Effect of DT-mGM-CSF treatment on normal bone marrow progenitor cell subsets and on leukemic LT12 cells

cell type	cells/femur ($x10^3$)		% of control
	controls	treated	
LT12*	3000 ± 900	3 ± 1	0.1
CFU-E	520 ± 78	130 ± 89	27.5
BFU-E	30 ± 17	40 ± 5	134
CFU-GM	440 ± 168	680 ± 62	155
CFU-S day 8	6.3 ± 2.0	5.4 ± 3.4	86
CFU-S day 12	1.4 ± 1.5	1.6 ± 1.1	114
CAFC week 2	26 ± 5	62 ± 10	238
CAFC week 6	3.4 ± 1.1	2.9 ± 0.5	85

Mean number ± SD of progenitors of the pooled bone marrow fractions of 3 rats are presented. Treatment was for 7 days and the rats were sacrificed on day 8. * as derived from Table 3

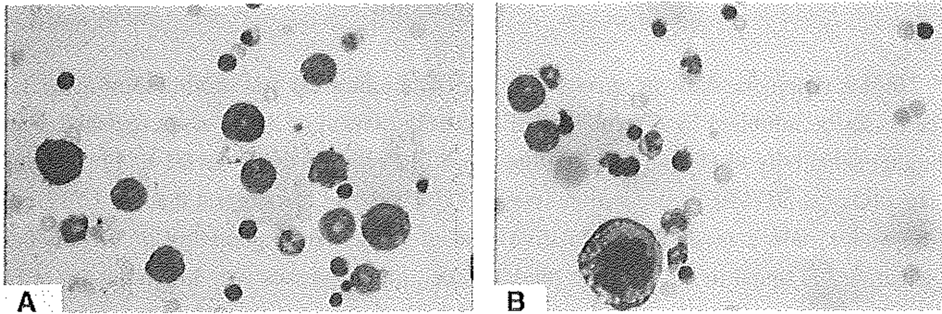

Figure 3
May-Grünwald-Giemsa stained cytospin preparations of rat bone marrow cells after treatment with DT-mGM-CSF (75 µg/kg/day) (A) and normal bone marrow (B).
The BM of treated rats contained a significant myelopoiesis and a reduced number of erythrocytes. The small cells resemble lymphocytes (Magnification 630x)

Blood cellularity and chemistry

Besides the effect of DT-mGM-CSF treatment on hemopoietic cells in the bone marrow, we also studied the blood cellularity in the peripheral blood (Table 5). The number of white blood cells (WBC) slowly increased up to 2.5 fold at the end of the treatment period. Differential cell counts were assessed by microscopic evaluation of May-Grünwald-Giemsa stained smears of peripheral blood from the treated rats and revealed an increase in neutrophil numbers. The number of red blood cells (RBC) was not affected by treatment but a decrease was observed in animals which were bled before. The effect on the number of platelets suggests an effect on the thrombopoiesis. Furthermore, blood chemistry revealed a progressive increase in the ALAT, ASAT and AP levels (Table 5), indicative of liver cell damage. After cessation of treatment the levels returned to normal.

Table 5
Effect of DT-mGM-CSF treatment on peripheral blood cellularity and chemistry

parameter	day of treatment					
	day 0	day 2	day 4	day 7	day 10	day 12
WBC	12.8 ± 3.5	12.2 ± 0.9	17.5 ± 2.4	29.5 ± 10.5	32.5 ± 11.7	24.8 ± 8.3
RBC	7.4 ± 0.2	8.5 ± 0.5	9.6 ± 1.4	8.5 ± 0.8	8.5 ± 0.6	5.5 ± 1.3
platelets	657 ± 35	553 ± 50	742 ± 155	1063 ± 345	56 ± 51	635 ± 492
ALAT	31 ± 4	106 ± 31	194 ± 33	291 ± 42	229 ± 112	ND
ASAT	57 ± 0.6	660 ± 79	1270 ± 47	2096 ± 230	1814 ± 886	ND
AP	136 ± 22	851 ± 65	855 ± 168	1775 ± 1161	544 ± 432	ND
Albumin	27 ± 0.7	23 ± 1.1	25 ± 0.2	24 ± 0.7	22 ± 0.8	ND

Data are presented as mean ± SD of three rats. Blood samples were collected during the treatment with DT-mGM-CSF. Administration was daily from day 0 to day 7 at a dose of 75 µg/kg/day i.p. injected. White blood cells (WBC) and platelet counts are x10^9/L. Red blood cell (RBC) counts are x10^{12}/L. ALAT, ASAT and AP are in IU/L, albumin is in mg/ml.

Discussion

In this report we show *in vitro* and *in vivo* data indicating that proliferation of rat LT12 cells can be inhibited by DT-mGM-CSF. Seventy percent of AML cases revealed a much higher sensitivity to DT-huGM-CSF than the LT12 cell line to DT-mGM-CSF, based on *in vitro* ID_{50} in ^{3}H-TdR incorporation assays (34). Therefore, we suggest that the LT12 model represents only a moderately sensitive case of AML. An explanation of this moderate sensitivity is found in the fact that the LT12 cell line expresses very low numbers of GM-CSFR, which are not detectable by the available binding techniques. In addition, dose effect titration experiments using the AML/SCID leukemia model indicated that efficient elimination of AML cells was achieved at dose levels as low as 6.25 µg/kg/day (8). However, a comparative evaluation of the systemic toxicity, the bone marrow specific toxicity and the antileukemic efficacy of DT-mGM-CSF treatment *in vivo* would enable us to derive a therapeutic ratio.

We compared the continuous administration scheme by means of osmotic pumps placed i.p. for 7 days, with the i.p. bolus injections. Both administration routes were effective but using i.p. bolus injection administration we received a higher leukemia cell kill. In leukemic rats treated with 75 µg/kg/day for 7 days by i.p. injections a substantial antileukemic effect of 3 log leukemic cell kill in the bone marrow was found, but at the expense of 50% toxicity related lethality. At lower concentrations more animals survived but also a reduced antileukemic effect of the toxin was observed. Thus, the therapeutic window is narrow in this rat leukemia model.

The marginal reduction in the number of CFU-C could be explained by the sensitivity of the CFU-GM and mature myeloid cells at continuous exposure to high concentrations of DT-mGM-CSF. This effect of DT-mGM-CSF on CFU-C was also reported by others (35). The sensitivity of myeloid cells is not unexpected because mature granulocytes and macrophages express in the order of 1500 to 2000 GM-CSFR/cell (27, 36, 37) and the committed progenitor CFU-GM 500 receptors/cell (37). In contrast, the most primitive hemopoietic cells have a low expression or even lack GM-CSFR expression (27, 37-39).

AML cells express equal numbers of GM-CSFR as do the CFU-GM (36). Therefore, the difference in sensitivity between the AML cells and the committed CFU-GM is not explained by differences in receptor number. Other factors could be responsible for the difference in sensitivity, for instance variations in the efficiency of internalization and processing of the recombinant toxin between normal hemopoietic cells and leukemic cells.

At the lower concentrations no effect on the development of CFU-C was observed. *In vivo*, we observed a leukocytosis in treated rats, which might have been caused by the stimulating effect by the GM-CSF part of the toxin fusion protein, although, we can not exclude that excessive myelopoiesis was induced by inflammation or tissue necrosis induced by the toxin part of the fusion protein.

The sites at risk for side effects of DT-mGM-CSF are, besides the hemopoietic system, the vasculature and the central nervous system (12, 13). In this study, histopathologic analysis showed no evidence of cell destruction and therefore the cause of deaths was not elucidated. At lower concentrations no tissue abnormalities were found. Although the elevation of liver enzymes is an indication that, to some degree, liver damage had occurred, only minor morphologic changes were found, unlikely to be the cause of death.

The fact that non-hemopoietic malignancies also express GM-CSFR suggests that possibly more malignancies could benefit from the cytotoxic action of this agent. It has recently been observed that GM-CSF toxins are highly cytotoxic to gastrointestinal cancer (40).

Considering the fact that, in general, primary AML samples are more sensitive for DT-GM-CSF (8, 34) than the rat LT12 cells studied in this model and that other DT based growth factor toxin fusion proteins, such as DAB_{486}-IL-2 (41, 42) and DAB_{486}-EGF (43) have been shown to be safe, tolerated and clinically active in patients, DT-GM-CSF might be a potential new therapeutic agent for the treatment of AML.

Acknowledgements

We thank W.P. van Schalkwijk for performing the serum chemistry studies and Dr. C. Wauters and Dr. L.M. Budel from the Department of Pathology, Erasmus University, Rotterdam, for performing the histologic examination. We thank Behring Werke A.G., Germany for providing the mGM-CSF and Sandoz BV, Basel, Switzerland for providing huGM-CSF.

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Chapter 7

General Discussion

7.1 Application of DT-huGM-CSF

The AML/SCID mouse leukemia model and L-CAFC assays for immature human AML progenitors showed a potential role for DT-huGM-CSF to specifically eliminate leukemic cells with short-term as well as long term leukemia initiating capacity by *in vitro* as well as *in vivo* exposure.

The main target for GM-CSF toxins is presumably the hemopoietic system. In Chapter 4 and 6, respectively, it was demonstrated that *in vitro* incubation and *in vivo* treatment with doses having a clear antileukemic effect, spare the hemopoietic system in the bone marrow. It is still unclear why normal cells with similar absolute numbers of receptors are less sensitive to DT-GM-CSF than AML cells. Internalization and toxin processing studies have to be performed to address this question. However, the lack of adequate procedures to isolate high quantities of purified AML cells of interest so far hamper these studies.

To achieve an appreciable antileukemic effect in the BNML/LT12 leukemia model dose levels of DT-mGM-CSF needed to be escalated (Chapter 6). However, severe systemic toxicity in rats was encountered at these doses. The relative insensitivity is probably due to a low number of GM-CSFR on the LT12 cells. The accumulation of toxins in certain organs such as the liver and kidney caused organ damage and high levels of toxin may damage tissues with a low expression of GM-CSFR. Thus, the therapeutic window for DT-mGM-CSF for the LT12 cell line in the rat model is narrow. Side effects could be diminished using lower concentrations of DT-mGM-CSF. This, however, reduced the antileukemic effect in the BNML/LT12 model. The observation that most human AML samples are up to 100 fold more sensitive to DT-huGM-CSF than rat LT12 cells, based on *in vitro* ID_{50} experiments, is encouraging. Considering the data from our experiments and from other DT based GF-toxins (1-3), DT-huGM-CSF might be a potential new therapeutic agent for treatment of patients with AML.

The observation that both immature and mature human AML cells are eliminated by *in vitro* incubation with DT-huGM-CSF in combination with the limited toxicity to normal hemopoietic progenitors, provides a solid basis for *ex vivo* purging of AML cells from autologous stem cell grafts. *Ex vivo* treatment of the transplants with DT-huGM-CSF would minimize the risk of a possible graft contamination with AML cells. However, considering the fact that relapses are also caused by the residual malignant cells in the host that have survived the conditioning regimen makes the benefit of the purging approach for AML questionable (4, 5).

Future studies with DT-huGM-CSF should be focussed on possible improvements of the efficacy in clinical applications. Studies should address to what extent the development of neutralizing antibodies affects the potency of DT-huGM-CSF. The co-administration of immunosuppressive drugs as 15-Deoxyspergualin might reduce the production of antibodies (6). An alternative strategy is chemically coupling of polyethylene glycol to the GF-toxin, so

that it is less immunogenic (7). In addition, studies on the pharmacokinetics in animals and man (8) are important for a better interpretation of the animal studies. Also the impact of soluble GM-CSFR (9) which might act as antagonists, should be explored. A combined therapy of DT-huGM-CSF with other GF-toxins or cytostatic drugs might also improve the efficacy to kill AML cells.

7.2 Other GF-toxins for the treatment of AML

Thus far, few studies have reported dose-dependent cytotoxicity to myeloid leukemic cells using GF-toxins targeted to growth factor receptors (10-12). However, the earlier studies were either performed *in vitro* using leukemic cell lines or in the case of the *in vivo* study (13) with a leukemic HL60 cell line, and it is highly questionable to what extent it represents the leukemic stem cell. *In vitro* analysis of cytotoxic efficacy of GF-toxins on primary AML cells in the current studies showed the perspectives of the different GF-toxins. Besides GM-CSF- (Chapters 3-6) and IL-6- (Chapter 2) also G-CSF- and M-CSF-toxins were evaluated for their potential to eliminate leukemic cells (Table 1). G-CSF-PE and M-CSF-PE were both cytotoxic to LT12 cells expressing the relevant human cytokine receptors. However, primary AML cells which express the G-CSFR and c-fms were not sensitive for these toxins. Actually, AML cell proliferation was stimulated in the ^{3}H-TdR uptake assay. With regard to the varying results obtained in this study, some issues should be discussed.

Table 1
Toxic activity of GF-toxins on LT12 cells and on primary AML cells

GF-toxin [a]	LT12 cells [b] range ID_{50} ng/ml	primary AML[c] range ID_{50} ng/ml	reference [d]
IL-6-PE4E	1-5	40 - 500 (6/19)	Chapter 2
DT-huGM-CSF	1-5	0.1 - 16 (18/22)	Chapters 3-5, (12, 13)
huGM-CSF-PE	1-5	100-1000 (6/16)	UD, (13)
DT-mGM-CSF	10-15	ND	Chapter 6
mGM-CSF-PE	not toxic	ND	UD
DT-huG-CSF	not toxic	ND	UD, (10)
huG-CSF-PE	1-5	Stim	UD, (11, 14)
DT-huM-CSF	not toxic	ND	UD
huM-CSF-PE	0.01-0.05	Stim	UD

[a] except in IL-6-PE4E, PE stands for PE40 and DT stands for DT_{388}. [b] LT12 cells were transfected with the human receptors IL-6R, GM-CSFR α unit, G-CSFR and c-fms for evaluation of IL-6-PE4E, huGM-CSF toxins, huG-CSF toxins and huM-CSF toxins, respectively. ID_{50} is the concentration of the toxin required to induce 50% reduction in ^{3}H-TdR incorporation. [c] ND = not determined; Stim = an increase in the ^{3}H-TdR incorporation due to the GF-toxins. In parenthesis the number of sensitive samples out of the total number of AML samples tested. Highest dose tested was 1000 ng/ml. [d] UD = unpublished data

7.2.1 Are enough receptors bound and internalized?

Most studies performed with GF-toxins showed that it was crucial to have several thousands of binding sites/cell for detecting the cytotoxic activity of the fusion proteins. The absolute number of cytokine receptors on normal hemopoietic cells as well as leukemic cells is low (15, 16), which might hamper the potential of this approach. However, there are a number of reports, showing toxicity to cells expressing a low number of receptors. For instance, a glioma tumor cell line expressing less than 100 huIL-4 receptors per cell is sensitive for huIL-4-PE (17), and murine sarcoma cells expressing about 500 to 1400 mIL-4 binding sites per cell are sensitive for mIL-4-PE (18). In studies with IL-6-PE, cells with 200-600 IL-6R/cell could be killed (19), although high concentrations IL-6-PE were needed. In addition, our data show that human AML cells, in general expressing 200 to 1200 GM-CSFR (20, 21) are sensitive for DT-huGM-CSF (Chapters 3-5).

Receptor binding and DNA synthesis studies have revealed that minimal numbers of receptors per cell are required to induce proliferation of AML blasts (20, 22). Consequently, exposure to GF-toxins might result in a biphasic response in which stimulation precedes inhibition in a dose dependent manner. Higher receptor occupancy may be necessary for intoxication. The biphasic response was observed in our studies with the IL-6-PE and huGM-CSF-PE (data not shown) and also in studies with DAB-IL-2 (23). In these studies, cells targeted by the fusion proteins showed an initial stimulation followed by a rapid inhibition of protein synthesis. These observations suggest that until intoxication mediated by the enzymatic domain dominate, the ligand moiety of the fusion protein may induce proliferation. This proliferative action prompted concern about the stimulation of AML growth by GF-toxins *in vivo*. However, it is evident that exogenous GFs do not affect the rate and duration of complete remission in the great majority of cases (24).

It is unclear whether proliferation stimuli influence the efficacy of GF-toxins. We observed that by replacing of an essential amino acid in huGM-CSF, which was described by Lopez et al (25) as being necessary for a proliferative response, an increased toxicity for AML cells was induced (data not shown). It will be very interesting to further investigate the effect of depleting sequences in GFs, necessary for their normal activities as regards the cytotoxicity of the GF-toxin.

To induce GF-toxin mediated cytotoxicity by a receptor expressed abundantly we evaluated c-fms, member of the family of tyrosine kinase receptors, which is expressed at least tenfold higher in absolute numbers in comparison to the cytokine receptor family. Although, LT12 cells transfected with the human c-fms were very sensitive for M-CSF-PE, no toxicity could be detected to primary AML cells expressing a similar density of c-fms (data not published). It might however be that the expression of c-fms is restricted to the majority of mature AML cells and not expressed by the relatively small subset of AML progenitors, responsible for short- and long-term proliferation.

Besides the number of receptors on the cell surface, also the affinity of GF-toxins determine the absolute number of bound GF-toxins. Competitive displacement analysis has revealed that GF-toxins tend to be 5 to 10 fold less efficient in receptor binding than the native ligand. However, IL-4-PE and also DT-G-CSF bind 200-fold less and are therefore less cytotoxic to cells (10, 26). The decrease in affinity can be explained in various ways. The first explanantion is that the fusion of toxins to the GF may alter the conformation of GF or mask critical residues involved in receptor binding. A second explanation might be that the E.coli expression and purification protocol used may not be optimal for the correct folding of GF-toxins.

The complexes of GFR and GF-toxins are clustered in clathrin coated pits and rapidly internalized into a vesicle. For most cytokine receptors internalization is induced within minutes after binding to the receptor at 37°C (27-31). However, an efficient internalization and degradation of receptor proteins requires a signal sequence in the cytoplasmic tail of the protein (32). So far, no studies have reported on the blocking of internalization due to the GF-toxin binding.

There is now a growing body of evidence to support the postulation that the extent of surface binding and the rapidity of internalization of fusion proteins does not predict the efficacy of toxins (33-37).

7.2.2 Is the toxin processing efficient?

After internalization the endocytic vesicles will convert into a lysosome in which the endocytosed molecules will be degraded. It has been shown that highly cytotoxic immunotoxins (ITs) are retained for a longer period of time inside cells and are more slowly metabolized in contrast to the less toxic ITs (33). These differences in intracellular processing may depend on the specific signals of the targeted proteins. Using GF-toxins the problem might be that GFs often target the toxin to lysosomes before the toxin can get to the cytosol.

To cleave DT and PE into cytotoxic fragments, the toxins need to be exposed to proteases. Both toxins can be processed by furin (38), a protease which has been localized primarily in the trans-Golgi apparatus (39, 40). PE is transported to the trans-Golgi network as a necessary step on its way to the cytosol (41, 42) and will therefore be exposed to significant amounts of furin. DT is however translocated from the endosome to the cytosol via membrane insertion and passage through ion-conductive channels (43). Although classic furin recognition sequences are present in DT, it also contains dibasic sequences at its cleavage site which could be recognized by additional proteases (44). Kreitman showed that in comparing ITs composed of an anti-TAC(Fv) and PE40 or DT_{388} (48) or anti transferrin receptor (13) and PE40 or DT_{388}, the PE based immunotoxins were more toxic for cells. He also presented evidence that different cell types process the PE-based and DT-based GF-toxins or ITs intracellularly with different efficiencies (13). In a comparative study of DT_{388}GM-CSF and GM-CSF-PE38KDEL it was shown that the PE molecule was more toxic

to gastrointestinal cancer cell lines and that the DT molecule was more toxic to leukemic cell lines.

Ultimately, the toxin target is protein synthesis and the susceptibility of EF-2 to be ADP-ribosylated. A typical mammalian cell should contain about $4x10^6$ molecules of EF-2. One molecule of toxin has the capacity to induce a proliferation arrest (45-47). Thus, the ADP-ribosylation of EF-2 is probably not the rate-limiting step of efficient intoxication mediated by GF-toxins.

In conclusion, the mechanism for the superior antileukemic toxicity of DT-GM-CSF in comparison to other hemopoietic GF-toxins remains unclear. Other GM-CSF toxins or DT-based cytokine fusion proteins did not have the same potential. One could speculate that AML cells process the DT-based GM-CSF intracellularly more efficiently due to the right combination of GF and toxin.

7.3 Future perspectives

The search for a new potent GF-toxin for the treatment of AML might be focussed on one hand on testing other cytokines as targeting proteins. For instance, PIXY321 (49) might enhance the occupancy of receptors per cell, because 2 receptor types could be targeted. Stem cell factor (SCF) and thrombopoietin (TPO) could also be of interest. As members of the tyrosine kinase receptor family the number of receptors expressed should be sufficient for mediating cytotoxicity. Although some cytokines may not be therapeutically used as a targeting agent for GF-toxins, they might give information about the kind of receptors expressed by the leukemic and normal stem cells. It should be noted, however, that the lack of cytotoxicity is not always due to the absence of receptors. This statement was supported by the observation that there is differential killing between leukemic and normal cells by a IL-6PE and DT-GM-CSF.

On the other hand, research could be focussed on altering the structure of the GF-toxin or using enhancers of cytotoxicity. Examples of a successful alteration was the IL-4-PE (50) which was made circular to increase the binding affinity. Enhanced toxicity was observed by adding multi drug resistance (MDR) modulators, such as Cyclosporin-A and PSC 833 to immunotoxins (51, 52). In combination with GF-toxins we maximally observed a 5-fold increase in sensitivity of AML cells to IL-6-PE (data not shown). Sensitivity of AML cells to other GF-toxins was not affected. Little data is available on the operational mechanism. One explanation is that MDR modulators might stabilize the membranes thereby inhibiting degradation of the GF-toxins (53).

A major problem to overcome is the existence of undesirable toxicities of PE- and DT-based GF-toxins. Whether the toxicity found in our rat and mouse studies is predictive for man, is unknown. Altering the toxin binding domain, as was done for PE (54), might eliminate the toxic side effects and thereby the therapeutic ratio will be widened.

Alternatively, a new exciting approach is the recent finding that eukaryotic cells can be genetically modified to express and secrete immunotoxins (55, 56). If producer cells are insensitive to GF-toxins and the production could be manipulated, this approach opens new prospects for site-directed delivery of GF-toxins by bone marrow tropic cells for the local treatment of residual leukemia. With this approach the problems of systemic toxicity can most probably be limited.

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Summary / Samenvatting

The objective of the studies described in this thesis was to explore the utilities of growth factor toxin fusion proteins for the treatment of AML. Current therapies based on chemotherapy and bone marrow transplantation are curative in up to 50 % of patients. Hence, the persistence of chemoresistant cells after treatment which may cause a relapse, urges for the development of new cytotoxic agents with specific cytotoxicity for leukemic cells. AML cells frequently express a variety of cytokine receptors with the same density on the cell surface and affinity for their ligand as their normal counterparts. A prerequisite for the cytokine receptor targeting approach is that the cytokine receptor of choice is absent on the hemopoietic pluripotent stem cell, so that normal hemopoietic cells can regenerate during and after toxin treatment. The choice of toxins and cytokines as basic elements to produce the fusion proteins are discussed in the **Introduction (Chapter 1)**.

To evaluate the potency of GF-toxins to kill AML cells, preclinical studies were performed *in vitro* using cell lines and primary AML cells and *in vivo* in a rat leukemia model and the SCID/AML model.

In **Chapter 2** a study is described using IL-6 fused to full length Pseudomonas exotoxin (PE), from which the binding capacity was changed by 4 point mutations in the PE gene. Although IL-6 receptors (IL-6R) are expressed on the cell surface of 50% of all AML cases, IL-6 does not induce a strong proliferative signal for AML cells *in vitro*. Nevertheless, AML cells expressing IL-6R, as detected by flow cytometric analysis, were killed by IL-6PE. Cells from the *in vitro* growing cell line LT12, which was derived from the Brown Norway rat Myeloid Leukemia (BNML) model, were transfected with the human IL-6R. Although a 5 log cell kill could be induced in *in vitro* assays, we showed that IL-6PE had a marginal potential in leukemic rats for eliminating leukemic cells expressing the IL-6R. Due to the cross reactivity of huIL-6 to rat IL-6R the toxic side effects could be evaluated. A systemic dose limiting toxicity, probably due to the pleiotrophic action of IL-6, was observed. These studies showed that the GF-toxins approach was in principle feasible, but cytokines with more restricted activities should be studied for preference.

Chapter 3 describes the evaluation of the potency of huGM-CSF fused to Diphtheria toxin (DT) against primary human AML cells in *in vitro* studies. Using the tritiated thymidine incorporation (^{3}H-TdR) assay we observed that 18 out of 22 AML samples studied were sensitive to DT-huGM-CSF. Sensitivity, expressed as the concentration DT-huGM-CSF required for 50% inhibition of proliferation (ID_{50}), ranged from 0.1 to 16 ng/ml. The AML cells with the ability to proliferate could not be distinguished from the non-proliferating cells. Therefore, it was not possible to correlate the degree of sensitivity of the AMLs for DT-huGM-CSF with the level of receptor expression. Experimental evidence was found that DT-huGM-CSF was cytotoxic towards huGM-CSF-responsive cells, but not towards nonresponsive AML cells.

In **Chapters 4 and 5** a newly developed leukemia model was used, which is based on xeno-transplantation of primary human AML cells into severe combined immunodeficient

(SCID) mice. The AML/SCID mouse model showed reproducible growth of human AML for prolonged periods of time. Furthermore, an *in vitro* leukemic long-term culture system (the cobblestone area forming cell assay on stromal cell cultures; L-CAFC) has become available, that sustains malignant cell growth over periods of 2 to 4 months. The AML/SCID mouse model and the L-CAFC assay allow investigation of long-term leukemic cell growth and to evaluate the intervention with new therapeutic drugs, such as GF-toxins. **Chapter 4** describes the evaluation of the sensitivity to DT-huGM-CSF of human AML cells with a long term leukemia initiating potential. *In vitro* incubation of AML cells for 48 hours with a high concentration of DT-huGM-CSF resulted in a decrease in the number of long-term leukemia initiating cells *in vivo* as well as *in vitro* by 1 to 3 logs. In contrast to AML cells, the normal bone marrow stem cell populations treated with DT-huGM-CSF under the same conditions, were not affected. Also the bone marrow progenitor cell subpopulation giving rise to CFU-GM was found to be insensitive to DT-huGM-CSF.

These encouraging results were a logical basis to investigate the ability of DT-huGM-CSF to eliminate AML *in vivo*. In **Chapter 5** seven AMLs were transplanted into SCID mice. Transplanted mice were subsequently treated for 7 days with DT-huGM-CSF starting 3 days after transplantation. Five AML samples were huGM-CSF responsive as well as DT-huGM-CSF sensitive in *in vitro* short-term proliferation assays (^{3}H-TdR). SCID mice transplanted with four of these AML samples were completely devoid of human cells 30 days after finishing treatment, whereas untreated SCID mice contained human leukemic cells in their bone marrow, analysed by flow cytometry. At day 80 in 3 out of 4 AML cases, human cells were still undetectable in the transplanted SCID mice. Using specific human short-tandem repeat primers very low numbers of human cells were found. One huGM-CSF responsive AML sample was moderately sensitive to DT-huGM-CSF given the observation at day 40 that to some degree AML cells survived in the SCID mouse. Two AML samples, selected for their non-responsiveness to huGM-CSF, were not affected by the DT-huGM-CSF *in vivo* treatment . These *in vivo* experiments provide evidence that leukemia initiating cells were eliminated by the DT-huGM-CSF treatment.

HuGM-CSF does not cross-react with the murine GM-CSFR. Therefore, the SCID mouse model is suitable for the analysis of the antileukemic efficacy of DT-huGM-CSF but not for the analysis of its toxic side effects. In **Chapter 6** experiments are described, using the experimental rat model BNML/LT12, to evaluate the antileukemic effect of murine GM-CSF fused to DT to rat leukemic cells in comparison with toxic side effects to normal tissues. Based on the ID_{50} from ^{3}H-TdR incorporation assays, the LT12 cell line represents a moderately sensitive group of AML cells. *In vivo* treatment of leukemic BN rats with DT-mGM-CSF showed a clear antileukemic effect of a 3 log cell kill. Typically observed side effects were the toxicity to the liver and to a lesser extent the kidney.

In **Chapter 7,** DT-huGM-CSF and other GF-toxins are discussed for their potency to treat AML. Although most GF-toxins were shown to be less effective at eliminating AML cells,

the encouraging results of DT-huGM-CSF indicate that GF-toxins can have a role in the treatment of AML. DT-huGM-CSF should now be evaluated for its clinical potential.

Samenvatting

Het doel van het beschreven onderzoek is de mogelijkheden te onderzoeken van groeifactor toxine fusie eiwitten (GF-toxines) voor de behandeling van AML. De huidige therapieën zijn gebaseerd op chemotherapie en beenmergtransplantatie, die in maximaal 50% van de patiënten genezend zijn. Chemoresistente cellen zijn verantwoordelijk voor het niet aanslaan van een therapie of voor een eventueel recidief. Deze resistentie geeft aan dat er nieuwe cytotoxische middelen ontwikkeld moeten worden, die specifiek gericht zijn tegen leukemiecellen. AML cellen expresseren in de meeste gevallen tal van cytokine receptoren die een gelijke dichtheid op het celoppervlak en affiniteit hebben als de receptoren op normale cellen. Een voorwaarde voor de GF-toxine benadering is dat de beoogde cytokine receptor afwezig is op de pluripotente hemopoietische stamcel, zodat normale hemopoietische cellen kunnen worden geregenereerd tijdens en na de toxine behandeling. De keuze van de toxines en de cytokines als basiselementen voor de fusie-eiwitten worden bediscussieerd in de Introductie **(Hoofdstuk 1).**

Voor de evaluatie van de GF-toxines en hun potentie om AML cellen te elimineren, werden preklinische *in vitro* studies verricht, gebruik makend van cellijnen en primaire AML cellen en ook *in vivo* studies, met behulp van een ratten leukemie model en het SCID/AML model.

In **Hoofdstuk 2** wordt een studie beschreven van IL-6 gefuseerd met het volledige Pseudomonas exotoxine (PE) molecuul, waarvan de bindingscapaciteit veranderd is door 4 puntmutaties in het PE gen. Hoewel de IL-6 receptoren (IL-6R) in 50% van de AMLs worden geëxpresseerd door leukemiecellen, geeft IL-6 geen sterk proliferatief signaal. Desalniettemin kunnen IL-6R expresserende AML cellen geëlimineerd worden door IL-6PE. Cellen van de *in vitro* groeiende cellijn LT12, welke afkomstig is van de Brown Norway ratte Myeloide Leukemie (BNML), werden getransfecteerd met de IL-6R. Hoewel met IL-6PE een 5-log reductie verkregen werd in de IL-6R expresserende LT12 cellijn in *in vitro* experimenten, had IL-6PE maar een gering effect in leukemische ratten. Vanwege de kruisreactiviteit van humaan IL-6 met de ratte IL-6R konden ook de toxische bijwerkingen geëvalueerd worden. Met IL-6PE werd een systemische, dosis-beperkende, toxiciteit gevonden. Dit wordt waarschijnlijk veroorzaakt door de pleiotrofe werking van IL-6. Deze studies lieten zien dat behandeling met GF-toxines mogelijk is, maar dat bij de keuze van het cytokine de voorkeur uitgaat naar cytokines met beperkte activiteit.

Hoofdstuk 3 beschrijft de evaluatie van de cytotoxiciteit op primaire AML cellen door huGM-CSF gefuseerd aan Difterie toxine (DT). In *in vitro* experimenten (^{3}H-TdR) werd vastgesteld dat 18 van de 22 bestudeerde AMLs gevoelig waren voor DT-huGM-CSF. De gevoeligheid, uitgedrukt als de concentratie DT-huGM-CSF die nodig is voor 50% remming van de proliferatie (ID_{50}), varieerde van 0.1 tot 16 ng/ml. De AML cellen die de capaciteit hadden om te prolifereren konden niet onderscheiden worden van niet-prolifererende cellen.

Daarom was het niet mogelijk om een correlatie te leggen tussen de gevoeligheid van de AML monsters voor DT-huGM-CSF met de dichtheid van het aantal receptoren. Verder werden aanwijzingen gevonden dat DT-huGM-CSF cytotoxisch was voor huGM-CSF responderende en niet voor huGM-CSF ongevoelige AML cellen.

In **Hoofdstukken 4 en 5** werd een nieuw ontwikkeld leukemie model bestudeerd, dat gebaseerd is op xeno-transplantatie van primaire humane AML cellen in immunodeficiënte (SCID) muizen. Het AML/SCID muizen model laat een reproduceerbare groei van humane AML over langere periodes zien. Verder is een *in vitro* lange termijn kweek systeem beschikbaar gekomen voor kweek van leukemiecellen over een periode van 2 tot 4 maanden (de "cobblestone area forming cell assay" op stromale celkweken; L-CAFC). Het AML/SCID muize model en de L-CAFC assay kunnen gebruikt worden voor het onderzoek van de lange termijn groei van leukemie cellen en voor de evaluatie van nieuwe therapeutische agentia, zoals GF-toxines. **Hoofdstuk 4** beschrijft de evaluatie van de gevoeligheid van AML cellen, die een lange termijn groei potentie hebben, voor DT-huGM-CSF. *In vitro* incubatie van AML cellen met een hoge concentratie van DT-huGM-CSF gedurende 48 uur resulteerde in een afname van het aantal leukemie initiërende cellen in zowel SCID muizen als CAFC met 1 tot 3 log. In tegenstelling tot AML cellen waren normale beenmerg stamcellen, die onder dezelfde condities geïncubeerd werden met DT-huGM-CSF, niet gevoelig. Ook de beenmerg voorlopercel subpopulatie die de CFU-GM leveren, was ongevoelig voor DT-huGM-CSF.

Deze bemoedigende resultaten vormden de basis om de *in vivo* mogelijkheden van eliminatie van AML te onderzoeken. In **Hoofdstuk 5** werden 7 AML's getransplanteerd in SCID muizen. De getransplanteerde muizen werden vervolgens gedurende 7 dagen behandeld met DT-huGM-CSF, beginnend 3 dagen na transplantatie. Vijf AML's respondeerden op huGM-CSF. Deze bleken gevoelig voor DT-huGM-CSF in de *in vitro* korte termijn proliferatie experimenten (^{3}H-TdR). De SCID muizen die getransplanteerd werden met 4 van deze AML monsters bleken 30 dagen na de beëindiging van de behandeling geen humane cellen te bevatten. Dit in tegenstelling tot onbehandelde SCID muizen. Op dag 80 werden in 3 van deze 4 AML's nog steeds geen humane cellen aangetroffen in de getransplanteerde SCID muizen. Gebruikmakend van humaan specifieke repetitieve sequentie primers kon bevestigd worden dat in een enkele muis nog lage aantallen humane cellen aanwezig waren. De 2 AML's gevallen die geselecteerd waren voor het niet reageren op huGM-CSF, waren *in vivo* ook ongevoelig voor DT-huGM-CSF. Deze experimenten tonen aan dat ook *in vivo* leukemie stamcellen geëlimineerd kunnen worden.

HuGM-CSF reageert niet met muize GM-CSF receptoren. Daarom is het AML/SCID muizen model slechts geschikt voor de analyse van het antileukemisch effect van DT-huGM-CSF en niet voor de analyse van de toxische bijwerkingen. In **Hoofdstuk 6** werden experimenten beschreven die inzicht geven in de therapeutische index van DT-GM-CSF. Gebruikmakend van het BNML ratten model en DT- muize GM-CSF werd het antileukemisch effect op ratte leukemiecellen vergeleken met de toxische bijwerkingen op

normaal weefsel. Gebaseerd op de ID_{50} waarde uit ^{3}H-TdR incorporatie experimenten, kon worden geconcludeerd dat de LT12 cellijn een matig DT-GM-CSF gevoelige groep van AML patiënten vertegenwoordigt. Ondanks deze relatieve ongevoeligheid werd na *in vivo* behandeling van BN ratten met DT-mGM-CSF een 3 log afname gevonden van het aantal leukemiecellen. Karakteristieke toxische bijeffecten werden waargenomen in de lever en in mindere mate in de nier.

Tenslotte worden in **Hoofdstuk 7** DT-huGM-CSF en andere GF-toxines bediscussieerd wat betreft hun potentie om AML te elimineren. Hoewel de onderzochte GF-toxines als MCSF- en G-CSF-toxines minder effectief blijken te zijn in het elimineren van AML cellen dan DT-huGM-CSF, wijzen de veelbelovende data van DT-huGM-CSF erop dat er een rol voor GF-toxines kan zijn in de behandeling van AML. Met DT-huGM-CSF dient in de nabije toekomst een klinische studie gestart te worden.

Abbreviations

aa	:amino acids
ADP	:adenosine diphosphate
ALAT	:alanine aminotransferase
ALP	:alkaline phosphatase
AML	:acute myeloid leukemia
ASAT	:aspartate aminotransferase
bil	:total bilirubin
BFU-E	:burst forming unit-erythroid
BM	:bone marrow
BN	:Brown Norway rat
BNML	:Brown Norway acute myeloid leukemia
BSA	:bovine serum albumin
CA	:cobblestone area
CD	:cluster of differentiation
CAFC	:cobblestone area-forming cell
C-fms	:M-CSF receptor
CFU	:colony forming unit
CFU-C	:colony forming unit-culture
CFU-E	:colony forming unit-erythroid
CFU-L	:colony forming unit-leukemic
CFU-S	:colony forming unit-spleen
CL_2MDP	:dichloromethylene diphosphonate
CML	:chronic myeloid leukemia
CNS	:central nervous system
cpm	:counts per minute
CR	:complete remission
CSF	:colony stimulating factor
DNA	:deoxyribonucleic acid
DT	:diphtheria toxin
DT-GM-CSF	:diphtheria toxin fused to GM-CSF
EF2	:elongation factor 2
ELISA	:enzyme linked immunosorbent assay
FAB	:French-American-British cytomorphological classification
FACS	:fluorescence activated cell sorting
FCS	:fetal calf serum
FITC	:fluorescein isothiocyanate
FSC	:forward scatter
G-CSF	:granulocyte colony-stimulating factor
GM-CSF	:granulocyte-macrophage colony-stimulating factor
Gy	:gray
h	:human
HBSS	:Hank's balanced salt solution
HGF	:hemopoietic growth factor
HS	:horse serum

Ig	:immunoglobulin
IL-n	:interleukin-n
IMDM	:Iscove's modified Dulbecco's medium
ip	:intraperitoneally
iv	:intravenously
L-CAFC	:leukemic CAFC
LTC-IC	:longterm culture initiating cell
α–MEM	:alpha minimal essential medium
m	:murine
MC	:methylcellulose
MGG	:May-Grunwald-Giemsa
MNC	:mononuclear cells
MRA	:marrow repopulating ability
MRD	:minimal residual disease
mRNA	:messenger RNA
MTD	:maximal tolerable dose
NAD	:nicotine adenine dinucleotide
NC	:nucleated cells
ND	:not determined
No	:number
P	:probability
PB	:peripheral blood
PBS	:phosphate buffered saline
PE	:pseudomonas exotoxin
PCR	:polymerase chain reaction
PIXY	:IL-3/GM-CSF fusion protein
PLT	:platelets
PML	:promyelocytic leukemia
R	:receptor
r	:correlation coefficient
RBC	:red blood cells
SCF	:stem cell factor
SCID	:severe combined immunodeficiency
SD	:standard deviation
SEM	:standard error of the mean
SF	:serum free
SFM	:serum free medium
SPF	:specified pathogen free
SSC	:sideward scatter
TBI	:total body irradiation
TCA	:trichoroacetic acid
WBC	:white blood cells

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Curriculum vitae

De auteur van dit proefschrift werd geboren op 18 januari 1964 te Almelo. Het middelbare onderwijs werd genoten aan het Christelijk Lyceum te Almelo waar in 1982 het H.A.V.O. -diploma werd behaald. In datzelfde jaar werd aangevangen met het Hoger Laboratorium Onderwijs te Hengelo (Ov) en in het jaar daarop vervolgt in Delft. In 1986 werd de opleiding cum laude afgesloten in de richting Zoölogie-Histologie. Van 1987 tot 1992 was hij werkzaam als analist bij de afdeling experimentele hematologie onder leiding van J.W.M. Visser en J.G.J. Bauman van het Radiobiologisch Instituut van T.N.O. te Rijswijk. In 1989 werd aangevangen met de verkorte opleiding Biologie aan de Rijksuniversiteit Utrecht. Moleculaire celbiologie (Dr. J. Boonstra en Dr. A.F.M. Cremers) werd gekozen als hoofdvak. Het doctoraal examen werd behaald in 1992. Van maart 1993 tot maart 1997 werd het beschreven promotie-onderzoek verricht aan het Instituut van Hematologie van de Erasmus Universiteit Rotterdam, onder leiding van Prof. Dr. A. Hagenbeek.

Per 1 januari 1998 zal de auteur werkzaam zijn als "Visiting Fellow" in het Laboratory of Molecular Biology, National Cancer Institute of the National Institutes of Health at Bethesda, MD, U.S.A (Prof. Dr. I. Pastan).

The author of this thesis was born on 18 January, 1964 in Almelo, The Netherlands. In July 1982 he graduated from Grammar school at the Christelijk Lyceum in Almelo, The Netherlands. In August 1982 he started his trainee as a technician at the higher tecnnical school (HLO) in Hengelo and Delft. In 1986 he graduated with credit from the HLO with major qualifications in Zoology and Histology. After his military service he was appointed as a technician for 4 years at the Radiobiological Institute (RBI) TNO in Rijswijk. He joined the research group "Experimental Hematology and Flow Cytometry" of Dr. J.W.M. Visser and Dr. J.G.J. Bauman. During these years he went to the University of Utrecht to study Biology. Under supervision of Dr. J. Boonstra and Dr. A.F.M. Cremers from the Department of Molecular Cell Biology. Graduation as a biologist followed in November 1992. From March 1993 to March 1997 he was appointed as a PhD student on a grant from the Dutch Cancer Society at the Institute of Hematology, Department of Developmental Therapeutics, Erasmus University Rotterdam, The Netherlands. The results of studies conducted during these years are presented in this thesis.

From January 1998 the author will be appointed as a "Visiting Fellow" with the Laboratory of Molecular Biology, National Cancer Institute of the National Institutes of Health at Bethesda, MD, U.S.A (Prof. Dr. I. Pastan).

Dankwoord

Nu ik aan het begin sta van een nieuwe fase in mijn leven is het goed om nog even terug te kijken op de afgelopen jaren. Het zijn jaren waarin ik vol enthousiasme heb mogen werken in een omgeving die zowel gezellig als stimulerend is geweest. In de eerste plaats is mijn dank verschuldigd aan Anton Martens, die het vertrouwen in mij had dat ik deze klus kon klaren en mijn promotor Ton Hagenbeek die de grote lijnen van het project in de gaten hield. Ivo Touw, die al het werk met een kritisch oog bekeek. David FitzGerald en Robert Kreitman, die bereid waren om mij de "ins and outs" van het eiwitzuiveren te leren. De 3 weken dat ik bij hun in het lab mocht verblijven waren zeer leerzaam en bepaalden op korte termijn de richting en voortgang van ons project, en wat nu blijkt, ook het verloop van mijn leven op lange termijn.

Deze klus was zeker niet te klaren zonder de hulp van Elwin Rombouts die mij met woord en daad behulpzaam was. Ik beschouw het daarom ook als een kroon op onze samenwerking dat jij mij terzijde wilt staan bij de laatste beproeving. Evenals Hazel Gowing, die gelijk met mij mocht beginnen aan een onderzoek binnen de Hagenbeek-groep waardoor we als lotgenoten gekoppeld werden. Het laatste jaar werd ons toxine-groepje uitgebreid met Martijn Schrama en Nathalie Philipsen. Beiden hebben zich enorm ingespannen voor het toxine-werk en hebben een onvergetelijke indruk op mij achtergelaten. De samenwerking met Wim Terpstra en Arie Prins was ook plezierig en ondanks het "wankele" model is het een mooie serie experimenten geworden.

De rest van de "Hagenbeekjes": Carla, Erik, Esther, Ger, Gerry, Irene, Karin, Koos, Paul en "oud-Hagenbeekjes": Jan B en Jan H, John, Jolanda, Kees, Steven en Ted worden bedankt voor de vele gezellige uurtjes naast elkaar aan de flow-kasten of op het mol-bio lab. Door mijn zitplaats tussen de "Ploemachertjes" werd ik ook in deze groep opgenomen. Vooral de kamergenoten Dimitri, het duo Gerry/Paul, Paula, Robin, en voorheen ook Jan H en de vele studenten maakten het vaak (te?) gezellig. Hoewel de laboratoria voor velen als te groot wordt ervaren, heeft de afwezigheid van de spreekwoordelijke "drempel" wel geleid tot een betere samenwerking zowel op wetenschappelijk als op sociaal niveau. Iedereen van de 13e wordt daarom bedankt voor de altijd aanwezige kennis, adviezen, bereidheid tot samenwerking of belangstelling.

Hoewel het voor vrienden (die nog over zijn na een promotie-onderzoek) en de familie iets totaal onbegrijpelijks was waar ik mij mee bezighield, wil ik jullie allen danken voor het begrip, de steun en de belangstelling tijdens de afgelopen jaren. Maar mijn grootste dank en bewondering gaat toch wel uit naar mijn maatje Herma, die mij de gelegenheid gaf om de klus te klaren en toch ondanks haar eigen werk het huishouden en het gezin wist te runnen. Dit was niet mogelijk zonder onze "Gina" die altijd aanwezig was tijdens onze afwezigheid in het gezin. Ik verheug mij enorm op het Amerikaanse avontuur, dat we samen met de kids gaan beginnen.