Primitive progenitor cells in human acute myeloid leukemia

Studies in immunodeficient mice and in long-term bone marrow culture

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Leukemische voorlopercellen in acute myeloide leukemie

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geboren te Almelo

CONTENTS

INTRODUCTION

1.1 Acute myeloid leukemia

Acute myeloid leukemia is a malignant clonal proliferation of immature hematopoietic cells. Leukemic blasts may express abilities for maturation to a variable degree, which leads to morphological heterogeneity. Generally the transformed leukemic stem cell is committed to the granulocytic lineage. Sometimes a predominance of blast cells from the erythroid or megakaryocytic lineage may be observed. The leukemic transformation may occur at the level of a pluripotent or a less primitive hematopoietic cell. (1,2,3) This is apparent from the observation of clonal markers, e.g. unique cytogenetic abnormalities in single versus several blood cell lineages. (4)

1.2 Leukemogenesis

It is generally accepted that leukemia is the result of a multistep process. (5,6) According to this idea, the leukemia-initiating cells initially contain a single genetic lesion resulting in none or only slight differences in proliferation and cell survival as compared to their normal counterparts. Additional genetic lesions may result in full leukemic transformation. The search for the targets of oncogenic events has elucidated various mechanisms that lead to leukemia. Genetic lesions may result in the activation of genes that contribute to leukemogenesis (oncogenes) or loss of the activity of genes that prevent tumor growth (tumor suppressor genes). (7) The genes that are involved in leukemia affect specific cellular regulatory mechanisms: growth factor receptor function, e.g. G-CSF (8,9,10,11), signal transduction (e.g. the ras family (12)), transcription (e.g. myc (13)) , differentiation (e.g. RAR/PML (14)) or programmed cell death (e.g. bcl-2 (15) p53 (16,17)) Proliferation of AML cells is controlled by hematopoietic growth factors (HGF). In vitro leukemic cells from some cases exhibit spontaneous proliferative activity. This may be caused by the autocrine production of HGF or by constitutional activation of metabolic pathways, that are normally activated only when a growth factor signals via its receptor. (18) In normal hematopoiesis the proliferation of blood cells induced by HGF is accompanied by maturation and loss of clonogenic potential.

1.3 Epidemiology

The incidence of AML in the Netherlands is 2.5 cases per 100,000 individuals per year. (19) The incidence increases with age, e.g. from less than 1 per 100,000 persons per year under the age of 20 to more than 10 per 100,000 persons over the age of 70. (20)
Some environmental factors have been consistently linked to this disease: radiation exposure (21,22,23), alkylating agents (24), chronic benzene

exposure (25), and cigarette smoking. (26) Hereditary conditions may carry an increased risk of AML: e.g. Down's syndrome, Bloom's syndrome, Fanconi's anemia. (27,28,29) Infrequently families with an unexplained high risk of AML have been described. (30) Taken together these categories represent a minority of all primary AML patients. Some hematological diseases are associated with a high risk of transformation to (secondary) acute myeloid leukemia. The risk is especially high in chronic myeloid leukemia (75%) and myelodysplastic syndromes (50%), and less so (incidence 10% or less) in polycythemia vera, agnogenic myeloid metaplasia and paroxysmal nocturnal hemoglobinuria. In these diseases an additional leukemogenic event is assumed to determine the transformation of the preexistent hematological disease towards acute leukemia.

1.4 Classification of AML

The heterogeneity of AML has resulted in a classification of the disease. The first efforts to define subgroups of AML were based on a combination of morphology and cytochemistry. The French-American-British (FAB) Classification is still mainly based on these parameters. (31) The use of this system has allowed the identification of typical morphological variants of AML. Interestingly, certain morphological variants have now been linked to a specific cytogenetic abnormality with a characteristic immunophenotype. Acute promyelocytic leukemia (FAB M3) is generally characterized by a t(15;17) translocation involving the PML gene on chromosome 15 and the retinoic acid receptor- (RAR-) gene on chromosome 17. In this subtype of AML all-trans retinoic acid (ATRA) may induce maturation of the AML cells in vitro and in vivo. (32,33,34) AML M2 is associated with a translocation t(8;21) involving the AML 1 gene on chromosome 21 and the ETO gene on chromosome 8. It occurs in ±15% of de novo AML cases. In these leukemias the exposure of the leukemic blasts to G-CSF or IL-5 in serum free culture results in morphological maturation towards neutrophils and eosinophils, respectively. (35) Acute myelomonocytic leukemia with eosinophilia (AML M4eo) has also been associated with a chromosomal aberration: inversion 16, which gives rise to a new CBFβ /MYH11 fusion transcript. (36) The identification of the latter three classes of AML with a relatively favourable prognosis mark the beginning of a trend to ultimately classify AML by the DNA-defect. Ideally this shoud lead to distinction of biological subgroups of leukemias with predictable clinicopathological characteristics and specific lesions that may serve as targets for the design of novel therapeutic strategies.

1.5 Clinical presentation and treatment of AML

The accumulation of leukemic blasts in the bone marrow of the patient results in interference with normal stem cell proliferation and differentiation. The resulting anemia, leukocytopenia and thrombocytopenia generally explain the symptoms of the patients. Specific problems may result from leukemic infiltration (e.g. gums, central nervous system), hyperleukocytosis or metabolic dysregulation.

The purpose of AML therapy is to eradicate all malignant cells. Most treatment protocols start with remission induction efforts with a combination of cytostatic drugs (generally an anthracyclin and cytarabin). These protocols may also include consolidation therapy at varying intensities, depending on the subtype of AML and the condition and age of the patient. Treatment-related toxicity and resistant disease (primary refractory AML or relapse) interfere with achievement of long term survival in a majority of patients. Long term survival, probably cure is achieved today in 20-40% of patients in major studies. (37-53)

2 STEM CELLS

2.1 Normal hematopoietic stem cells

All mature blood cells develop from a pool of stem cells. These stem cells differentiate into progenitor cells which proliferate and mature into blood cells of the individual lineages. Although large numbers of relatively short-lived blood cells are produced every day, hematopoietic stem cells are rare. They are functionally defined by their extensive self-renewal capacity and the ability to contribute to blood cell formation of multiple lineages for prolonged periods of time. The parameter of choice to measure the abilities of hematopoietic cell subsets is long-term in vivo repopulation of the hematopoietic system. It has been estimated that the frequency of the murine stem cell is in the order of 1 or 2 per 100,000 bone marrow cells. (54) Characterization of stem cells has required the development of purification strategies for normal stem cells and a number of assays that allow te characterization of a defined subset of hematopoietic cells.

2.2 Concept of AML stem cells

The concept of tumor stem cells postulates that some cells in a tumor have the capacity to maintain long-term growth of the tumor. (55) The progeny that is derived from these malignant stem cells lacks these abilities for self-renewal . Following maturation hematopoietic cells normally become non-dividing. In acute myeloid leukemia there is a maturation arrest of the malignant cells which results in an accumulation of immature blasts. In the studies of Lotem and Sachs a mouse leukemia clone was induced to morphological maturation in vitro by a preparation containing several cytokines. Morphological maturation was associated with loss of the potential to transfer leukemia in vivo. (56) When these experiments were repeated with a related leukemic cell clone that was maturation incompetent, survival was not prolonged upon cytokine stimulation, suggesting that maturation induction of the leukemic clone was responsible for the therapeutic effect of the cytokines. (57) The progression of the AML cells along the maturation route may have resulted in the loss of clonogenic potential. Thus the use of cytokines may indeed induce maturation in leukemic cells with long-term potential and the activation of normal differentiation pathways may be therapeutic in AML. In man all-trans retinoic acid (ATRA) stimulates cellular differentiation in AML M3, resulting in complete remission. Study of the characteristics and functional abilities of AML cells with long-term abilities may shed light on pathophysiologic mechanisms of therapeutic failure or success.

3 ASSAYS FOR NORMAL HEMATOPOIETIC PROGENITOR CELLS

3.1 Colony forming cells

The establishment of in vitro assays allowed studies of hematopoietic progenitor cells and their regulation. (58,59) In vitro these progenitor cells may be seeded in a semisolid matrix (e.g. agar, methylcellulose) to form a progeny of colonies of cells. Colony assays have been developed for several subsets of progenitor cells.In such an assay a human or murine multipotential progenitor cells common to granulocytes, erythrocytes, monocytes, and megakaryocytes (CFU-GEMM) has been defined. (60,61,62) Committed progenitor cells of a single lineage may be assessed in the following assays: erythroid burst forming units (BFU-E), erythroid colony-forming units (CFU-E), granulocyte colony forming units (CFU-G), monocyte colony forming units (CFU-M), granulocyte/monocyte colony forming units (CFU-GM) or megakaryocyte colony forming units (CFU-Mega). (63-67) The CFU-Blast is a subset of in vitro colony-forming cells with the capabilities for self-renewal and production of progeny of several lineages, and in part defined by stroma adherency. (67) A cell producing very large colonies (>0.5mm in diameter, containing at least 50,000 cells) and strictly dependent on a combination of hematopoietic growth factors has been described as high proliferative potential colony-forming cell (HPP-CFC). (68,69,70) The HPP-CFC has later been divided into several subtypes. These subtypes respond to different (combinations of) growth factors and have been termed in order of increasing maturity: Pro-HPP-CFC-1, HPP-CFC-1, HPP-CFC-2, HPP-CFC-3 and the low proliferative potential colony-forming cell (LPP-CFC). However, results of in vivo transplantations could not be predicted using the HPP-CFC assay.(71) In the murine system a macroscopic spleen colony test was among the first assays to be developed for the analysis of hematopoietic precursor cells. (72) Spleen nodules, representing colonies of hematopoietic cells, on day 7 (colony forming unit-spleen (CFU-S) day 7) or 12 (CFU-S day 12) after transplantation were shown to be representative of cells with the ability to initiate at leat short term repopulation of the mouse bone marrow. These cells are in the same range of maturity as CFU-Blast or HPP-CFC. However, it has subsequently been shown (e.g. using exposure to 5-FU or cell sorting) that the large majority of colony forming cells can be separated from cells with long-term abilities. (73,74,75,76) This has led to reappraisal consistent with the notion that in vitro colony forming cells in normal bone marrrow mainly represent cells with short term, transient in vivo repopulating ability.

3.2 Long-term culture of human hematopoietic cells

To detect cells with long-term abilities long-term in vitro stromal cell culture systems were developed. Hematopoietic cells in the bone marrow are sustained by a complex network of other cells. For instance, erythroid islands in the marrow consist of a central macrophage surrounded by erythroblasts. (77) When clumps of murine marrow are cultured in vitro, a confluent stromal layer will develop, and hematopoiesis may be maintained for many weeks. Dexter et al. designed culture conditions that allowed the development and maintenance of a hemopoietic microenvironment in vitro. (78) These cultures sustain a network of multilayered stromal cells, including adipocytes, fibroblastic cells, barrier cells, macrophages, endothelial cells and extracellular matrix. (79,80,81) Primitive hematopoietic cells are localized beneath or in the stromal layer. On examination with a phase-contrast microscope they are phase-dark, in contrast to cells situated above the stromal layer which are phase-bright. (82) Cells with the ability to initiate long-term growth in vitro are often described as long-term culture initiating cells (LTC-IC). Colonies of primitive cells consist of flattened polygonal cells. Such primitive clones of at least 5 cells are termed cobblestone areas (CA). Upon proliferation and maturation the progeny of these primitive cells moves towards the surface of the stromal layer and may be released into the medium. (83) The interactions with the components of the stromal layer appear complex and may involve the production of hematopoietic growth factors and other cytokines. In addition, the contact between the hematopoietic cell and the stromal layer may in part regulate hematopoietic cell function, although it has recently been shown with the use of a transwell system that direct contact between stroma and hematopoietic cell is not required for long-term culture. (84,85)

3.3 The cobblestone area forming cell assay

Ploemacher et al. (86) developed a quantitative assay that enumerates the numbers of a series of primitive hematopoietic cell subsets in vitro. In this assay, stroma is grown in a 96-wells plate. This stromal layer is seeded with hematopoietic cells, at limiting dilution. Between 5 days to 9 weeks after seeding the wells may be examined for cobblestone areas, using a phase-contrast microscope. The frequency of the cobblestone area forming cells (CAFC) in a given sample is calculated with the use of Poisson statistics, based on the proportion of negative wells. It has been shown that the time of appearance of a CA after seeding is related to the primitiveness of the CAFC. In murine hematopoiesis it was demonstrated that the numbers of day 28-35 CAFC of a graft predicted long-term repopulating ability, whereas earlier appearing CAFC were associated with transient repopulating ability. (73,86-89) For investigation of human hematopoiesis the murine

FMBD-1 cell has been used as a stromal layer. Unstimulated FMBD-1 cells express m-RNA for many cytokines e.g. TGF-1β, M-CSF, IL-6 and SCF. To stimulate growth of the human cells the medium contains many components: e.g. IL-3, G-CSF, fetal calf serum (12.5%), horse serum (12.5%) and hydrocortisone. It was shown that immature human hematopoietic cells with long-term abilities in this assay are characterized as CD34 positive, 5-fluorouracil resistant, HLA-DRlow, Rhodamine-123dull, and predominantly produce CA at 5-8 weeks following seeding. Hematopoietic cells initiating CA at earlier intervals after the onset of culture were highly sensitive to 5-FU, and expressed Rhodamine123 and HLA-DR at varying intensities. (73)

4 ASSAYS FOR AML PROGENITOR CELLS

4.1 Clonogenic cells in AML

AML colony forming cells (CFU-AML) may express various phenotypes and display considerable heterogeneity among patients. (90,91,92) Generally CFU-AML express more immature immunophenotypes as compared to the unseparated AML cell population. This is e.g. apparent from the profiles of expression of CD34, CD38, CDw65 and CD15. Cellular differentiation of CFU-AML in vitro can be induced. (93,94,95,96) CFU-AML recovered from methylcellulose cultures may be replated and some CFU-AML may produce secondary colonies. (93) The capacities for (limited) differentiation and self renewal are consistent with the identity of CFU-AML as progenitor cells. As became evident from thymidine suicide studies CFU-AML are an actively proliferating cell population. (97)

4.2 Immunodeficient mouse models for human AML

4.2.1 SCID mice

In 1983 Bosma reported the discovery of a mouse with severe combined immunodeficiency (SCID) in a CB17/Icr inbred strain. (98) The defect was shown to be the result of a new recessive mutation, mapping to mouse chromosome 16 near the λ–light chain locus. (99) The involved gene has not yet been cloned. The phenotype of the SCID mouse consists of many abnormalities. B and T cells are nearly absent. The minimal numbers of B and T cell progenitors show a normal rearrangement of the immunoglobulin μ-gene or the T cell receptor β–chain. SCID mice are prone to develop thymomas. There is a striking defect in the repair of double-strand DNA-breaks, rendering the mice extraordinarily sensitive to irradiation. (100) It has been suggested that the SCID gene defines a doublestrand DNA break repair activity that is also required for efficient joining of V(D)J coding ends of the B

and T cell receptor. (101) NK cells are present, and may induce graft rejection. (102,103) Granulocytic, erythroid and megakaryocytic cell differentiation and proliferation appear to be normal. (104)

4.2.2 SCID mice support long term growth of normal human hematopoiesis

The question whether human hematopoiesis can be established in immunodeficient mice has been the subject of active research. The group of John Dick showed that SCID mice, that had been conditioned with total body irradiation, accept an intravenous graft of human bone marrow. After one month 0.5-5% of human hematopoietic cells can be demonstrated in the bone marrow of the SCID mouse. Additional treatment with human cytokines (PIXY, a GM-CSF/IL-3 fusion peptide, and stem cell factor) results in much higher levels of human cells. Myeloid, erythroid and B cell lineages and progenitor cells (CFU-GM, CFU-GEMM) were detected. (105) It has been shown that the immunophenotype of the human hematopoietic cell engrafting immunodeficient mice is CD34+/CD38-. Engraftment may be achieved with as few as 100 cells. In contrast, high numbers of CD34 negative cells failed to proliferate in these mice. (106) Cells with the ability to initiate long-term culture may expand in numbers after transplantation in immunodeficient mice. (107) These data indicate that the cell engrafting the bone marrow of immunodeficient mice is a primitive hematopoietic cell that may maintain hematopoieisis of several blood lineages for at least a few months.

4.2.3. SCID mouse model for AML

Human AML cells may produce solid tumors when inoculated subcutaneously into mice that had been previously immunosuppressed by thymectomy and total body irradiation. Such AML tumors grow to a maximum size and then regress. At the time of regression the AML cells often show morphological signs of maturation. Sporadically a tumor may be maintained or even be retransplanted into a secondary recipient. Dissemination of leukemia in the recipient has not been observed. (108,109) The finding that normal human hematopoiesis may be sustained in immunodeficient mice for several months suggested the possibility of similar establishment of human AML in these animals. However, initial experiments with the purpose to engraft primary AML in immunodeficient mice failed. Experimental conditions of transplantation of AML into SCID mice were the subject of subsequent study. (110-118) Pretransplant conditioning, graft size, route of transplantation and HGF support have been shown to influence engraftment and subsequent proliferation. Recipients have been prepared for transplantation with TBI or chemotherapeutic agents. Additional conditioning with

asialoGM1 antisera, with the purpose to eliminate mouse NK cells, resulted in some improvement. (119) Diverse routes of administration of the cells have been employed, i.e., subcutaneous, intraperitoneal, intravenous routes and injections into fragments of human fetal bone. Engraftment ensued following any of these routes, but the optimal way of administration remained questionable. In the first series of experiments in which reproducible growth of human AML in SCID mice was accomplished human hematopoietic growth factors (hHGF) were administered as support following transplantation. It was shown that hHGF (the investigators used a construct of interleukin 3 and granulocyte macrophage colony-stimulating factor (PIXY) and stem cell factor) enhanced the outgrowth of AML, although engraftment in the absence of hHGF was also seen. (115) A variant of the SCID mouse model is the SCID-hu mouse. This model was developed by implanting human fetal bone fragments into SCID mice. After injection into such bone fragments human hematopoietic cells proliferate in a human microenvironment. Multilineage hematopoietic activity is maintained in these structures, without exogenous growth factor treatment. (120) In this system primary AML could be maintained, allowing retransplantation into secondary recipients. (121) More recently a new mouse strain has gained interest as a recipient for human hematopoietic cells: the non-obese diabetic (NOD)/SCID mouse. In addition to the SCID defect, this mouse has a deficiency of NK cells and a macrophage maturation defect. Transplantation experiments of human hematopoietic cells show more reproducible engraftment and higher levels of engrafted human hematopoietic cells. (122,123) This particular mouse strain will be used more frequently in the foreseeable future.

5. Introduction to the experimental work

The experimental work of this thesis consists of two parts. The first part relates to exploration of conditions to facilitate engraftment of primary human AML in SCID mice. In Chapter 2 the role of total body irradiation, the transplantation route and hematopoietic growth factor support with interleukin-3 are investigated. Chapter 3 describes experiments to reduce the graft size required for engraftment of human AML cells in the SCID mouse bone marrow. Despite the fact that SCID mice are nearly devoid of T and B cells there is a substantial residual graft resistance that needs to be overcome, which may be mediated by macrophages or NK cells. The effect of macrophage depletion of the recipient on the required graft size is investigated.

The second part describes experiments aimed at a characterization of leukemic stem cells. Investigation of long-term growth abilities of AML cell subsets was performed using two independent assays for long-term growth:

the CAFC assay and SCID mouse transplantations. Normal bone marrow cells with long-term abilities express the immunological cell surface marker CD34. In Chapter 4 the relation between growth characteristics and CD34 expression of AML cell subsets is investigated. In view of the heterogeneity of AML it is difficult to identify a common denominator of primitive leukemic cells. We hypothesized that most leukemic stem cells, similar to normal stem cells, might be more primitive than colony forming cells and be out of cell cycle. Experiments to test this hypothesis are presented in Chapter 5. Chapter 6 is concerned with the investigation of a possible new therapeutic agent, a potent growth factor-toxin conjugate. We asked the question as to whether primitive AML cells would be killed by the hematopoietic growth factor (GM-CSF) coupled to a Diphteria toxin.

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CHAPTER 2

CONDITIONS FOR ENGRAFTMENT OF HUMAN ACUTE MYELOID LEUKEMIA IN SCID MICE

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ABSTRACT

Transplantation of human AML into severe combined immunodeficient (SCID) mice provides a useful experimental model but graft failure has been reported. We investigated the influence of a number of factors on the outgrowth of AML in the SCID mouse bone marrow (BM). The transplantation route and the dose of total body irradiation (TBI) were examined using the cell line HL-60 as a model for AML. The role of graft size and recombinant human IL-3 (IL-3) were investigated with patient samples of AML cells.

Intravenous transplantation was demonstrated to be superior to intraperitoneal transplantation. Pretransplant conditioning resulted in a dose dependent increase of AML growth in the SCID mouse. Cell dose titrations ranging from $3x10^7$-$3.6x10^5$ AML cells iv per mouse revealed a minimum of $1.1x10^6$ to be required for reproducible engraftment. Earlier and more extensive infiltration by human AML cells was seen following injection of greater cell numbers.

IL-3 given posttransplantation to SCID mouse recipients, promoted AML growth in three cases, whereas a fourth AML cell specimen also grew without support of IL-3. In vitro growth factor responsiveness of AML cells to IL-3 did not predict IL-3 dependence of AML growth in vivo.

INTRODUCTION

Human acute myeloid leukemia (AML) transplanted into severe combined immunodeficient (SCID) mice allows for the study of growth and interventions of growth of human AML in an in vivo model. However, mainly unexplained graft failures have been observed. The experimental conditions of transplantation of AML into SCID mice have varied greatly (1-9). Pretransplant conditioning, graft size, route of transplantation and human hemopoietic growth factor (hHGF) support might influence engraftment and subsequent proliferation. Conditioning with total body irradiation (TBI) has been used in doses ranging from 2 Gy (6,7) to 4 Gy (1,2,4). Recipients have also been prepared for transplantation of xenogeneic hemopoietic cell grafts with chemotherapeutic agents (8,9). Diverse routes of administration of the cells have been employed, i.e., subcutaneous, intraperitoneal (ip), intravenous (iv) routes and injections into fragments of human fetal bone (1-9). Engraftment ensued following any of these routes, but the optimal way of administration remains unknown. Dose titrations of AML cell numbers grafted have been examined, but repopulation has been evaluated after one month only (2). The support with hHGFs following transplantation may enhance the outgrowth of AML (2), although engraftment in the absence of

hHGF,s appears possible (1,3,6,8). Recombinant human interleukin-3 (IL-3) induces leukemic colonies in vitro and activates DNA synthesis in 80% of leukemic cell samples obtained from patients with AML (10). We investigated the conditions mentioned above to establish their influence on engraftment and proliferation of human AML in SCID mice.

MATERIALS AND METHODS

Cell suspensions and culture conditions

Bone marrow (BM) and peripheral blood (PB) samples were obtained following appropriate informed consent from four unselected patients presenting with AML diagnosed according to the criteria of the French-American-British Committee (FAB)(11). Patient A and B were diagnosed as AML M1 and had normal cytogenetics, patient C was diagnosed as AML M5 and had normal cytogenetics and finally patient D suffered from AML M4 and complex cytogenetic abnormalities were identified in the AML cells (43 XY, 2q+, -4, -7, 10q-, -17). Mononuclear cells were isolated by Isopaque-Ficoll centrifugation (1.077 g/cm^2; Nycomed, Oslo, Norway). Subsequently E-rosette forming cells were depleted. The cells were cryopreserved in 10 % dimethylsulfoxide (BDH, Poole, UK), 20% inactivated fetal calf serum (FCS) and Iscove's modified Dulbecco's medium (IMDM). After thawing by stepwise dilution (12), viability assessed by trypan blue staining was always > 90%. To determine autonomous proliferation of AML cells the uptake of tritiated thymidine by leukemic cells in serum-free and cytokine-free cultures was measured. Autonomous growth was defined as mean tritium thymidine uptake of unstimulated cultures > 7.5x10^2 dpm and expressed as decayes per minute divided by 100 as described (13). For assessing IL-3 responsiveness the uptake of tritiated thymidine in serum-free culture in the presence of 5ng/ml of IL-3 was measured and compared to the values of cultures without IL-3. A positive IL-3 response was defined as a significantly greater tritium thymidine uptake in the presence of rh-IL-3 than in unstimulated control cultures (14).

HL-60 cells were obtained from suspension cultures in IMDM medium and 10% FCS at a density of maximally 2 x 10^6 cells/mL. M-O7e cells were cultured similarly using RPMI 1640 medium (Gibco, Paisley, Scotland, UK) supplemented with 5ng/mL IL-3.

Mice and transplantation of AML

Female specific pathogen free CB17 scid/scid mice were obtained from Harlan CPB, Austerlitz, The Netherlands. They were housed under patho-

gen free conditions in a laminar air flow unit and supplied with sterile food and acidified drinking water containing 100mg/L ciprofloxacine (Bayer AG, Leverkusen, Germany). Before transplantation the mouse plasma Ig level was determined in an ELISA using a sheep anti-mouse Ig specific for mouse IgG and IgM (Boehringer Mannheim Biochemica, Penzberg, Germany). Only mice with Ig plasma levels <40µg/ml were used for transplantation. Transplantation was performed at the age of 6-8 weeks. Total body irradiation was delivered by a ^{137}Cs source adapted for the irradiation of mice. Leukemic cells suspended in 300µl Hanks, balanced salt solution (HBSS) were injected intravenously (iv) into the lateral tail vein or intraperitoneally (ip) on the day after TBI.

Human hematopoietic growth factor supply
Recombinant human IL-3 (IL-3), Escherichia coli derived, was obtained from Sandoz Pharmaceutical Company, (Basel, Switzerland) and was administered ip in 300µL (calcium and magnesium free) HBSS and 0.1% bovine serum albumin. As an alternative method of IL-3 supply in vivo SCID mice received 10^7 cells from an IL-3 producing cell line ip. This mouse fibroblast cell line termed cIL103 had been transfected with a bovine papilloma virus based vector containing the human IL-3 gene. It was maintained in RPMI 1640 (Gibco, Paisley, Scotland, UK) containing 10% FCS. IL-3 is present at high concentrations in the supernatant. The cIL103 cel line was obtained from Dr. R. W. van Leen, Gist-Brocades, Delft, The Netherlands.

Tissue collections and analysis
SCID mice were killed by CO2 inhalation and cervical dislocation in accordance with institutional animal research regulations. Cell suspensions were prepared from PB, BM, spleen and abdominal tumors. To determine the percentages of human myeloid cells samples obtained from BM, PB, spleen or tumors were incubated with fluorescein isothiocyanate (FITC) conjugated monoclonal antibody (MoAb) to CD45 (human common leukocyte antigen) and a phycoerythrin (PE) conjugated MoAb to CD33 from Beckton Dickinson (San Jose, CA, USA). To reduce nonspecific binding cells were stained in the presence of human IgG1kappa 10µg/mL (Sigma, St. Louis, MO, USA) and 1% rabbit serum in phosfate buffered saline for 30 minutes on ice. Mouse IgG1 FITC and mouse IgG1 PE conjugated antibody (Beckton Dickinson, San Jose, CA, USA) and samples from non-transplanted SCID mice were used as control. Fluorescence was measured using a FACSCAN flow cytometer and Lysis II software (Beckton Dickinson, San Jose, CA, USA). Erythrocytes and dead cells were excluded from analysis by gating on forward and orthogonal light scatter. Only cells that stained with CD33 and CD45 simultaneously were counted as human myeloid cells (15).

IL-3 level assay

Plasma samples of the SCID mice were assayed for IL-3 levels by using a commercially available kit for ELISA (Medgenix, Fleurus, Belgium). The detection limit was 0.050 ng/ml. IL-3 levels were also determined in an in vitro bioassay employing the IL-3 responsive cell line M-O7e. $5x10^4$ M-O7e cells were cultured for three days in 100µL RPMI in 96 wells microtiter plates with or without plasma samples obtained from the SCID mice and tritiated thymidine stimulation was measured. Radioactivity was determined with a 1205 betaplate scintillation counter (Beckman, Fullerton, CA, USA). All experiments were performed at three dilutions in triplicate. In each experiment standard dilutions of IL-3 were used as references. Samples were also incubated with a polyclonal rabbit anti-IL-3 antibody, generated at our laboratory, to exclude cell proliferation not mediated by IL-3. The detection limit of the bioassay was 0.1 ng/ml.

RESULTS

In vitro growth of AML cell samples

Autonomous growth was present in AML case D only. Cells from AML cases A, B and C did not show autonomous proliferation in vitro. All four samples from AML cases A thru D showed positive IL-3 responses in vitro (Table I). Influence of total body irradiation and transplantation route on HL-60 engraftment in bone marrow of SCID-mice.

To investigate the dose of TBI and to compare iv and ip transplantation, groups of SCID mice were irradiated with 0, 2, 3 or 4 Gy of TBI and transplanted iv or ip with $5x10^7$ HL-60 cells (Table II).

Table I
3H-Thymidine uptake in serum-free culture with and without IL-3 support

AML	FAB	cytogenetics	No IL-3 dpmx10^{-2} ±SD	IL-3 dpmx10^{-2} ±SD	IL-3 responder
A	M1	normal	1.0(±0.2)	46.8(±0.6)	+
B	M1	normal	3.9(±0.4)	111.0(±5.4)	+
C	M5	normal	2.5(±0.8)	25.8(±3.5)	+
D	M4	complex	16.8*(±0.3)	52.2(±3.4)	+

*Table I. 3H-Thymidine incorporation by AML cells expressed as decayes per minute/100 + or - one standard deviation (SD) was determined under serum-free conditions with or without IL-3. + : postive test * : spontaneous tritium thymidine uptake (autonomous growth). FAB: AML subtype in the FAB classification. Cytogenetics complex: 43XY, 2q+, -4, -7, 10q-, -17.*

Table II
Engraftment of HL60 in SCID mice. Transplantation route, dose of TBI

dose of TBI (Gy)	IV route %	IV route day	IP route %	IP route day
0	0	42	0	31
0	0	42	0	43
0	7	42	2	43
0			5	43
2	10	31	0	35
2	23	32	3	37
2	46	32	0	37
2			0	37
3	28	31	0	31
3	61	31	1	35
3	64	31	0	35
3			0	35
4	+		+	
4	+		+	
4	+		+	
4			+	

Table II
24 hours after 0, 2, 3 or 4 Gy of TBI SCID mice were injected intravenously (iv) or intraperitoneally (ip) with 5x10⁷ human myeloid HL-60 cells.
+: toxic death from sepsis. Each percentage represents one SCID mouse.

Significant bone marrow infiltration was apparent only after TBI and iv transplantation. For intravenous transplantation a dose effect relationship between TBI dose and percentage of HL60 cell engraftment in the mouse bone marrow (Spearman Rank test, $r=0.82$, $p<0.05$) was found. However, a TBI dose of 4 Gy resulted in death from sepsis with E. coli and/or enterococcus. Mice transplanted ip showed local intraperitoneal tumor growth, without significant BM infiltration. Similar results were obtained for the human myeloid cell line M-O7e (data not shown). In subsequent experiments we delivered 3.5 Gy of TBI and performed transplantation iv.

AML cell dose titration
Groups of mice were injected iv with graded numbers of AML cells (from patient B). Injection of 30x106 AML cells resulted in extensive BM infiltration by human cells, which became apparent at an earlier time (day 36) than following lower cell doses (Table III). Recipients of 0.36x106 cells or less remained free from overt disease and did not show AML cell repopulation as late as day 81.

Table III *Cell dose titration of AML*

Cell dose ($x10^6$ cells)	Unique animal number	Day of evaluation	% human cells BM
30	# 1	35	91
	# 2	35	8
	# 3	36	79
	# 4	36	35
10	# 5	20	0
	# 6	20	1
	# 7	36	28
	# 8	36	60
	# 9	36	6
3.3	#10	36	3
	#11	36	9
1.1	#12	36	0
	#13	36	0
	#14	47	2
	#15	61	19
	#16	67	3
0.36	#17	81	1
	#18	81	0
	#19	81	0

Table III
SCID mice received 3.5 Gy of TBI and received graded doses of AML cells derived from patient B without IL-3 support.

Human IL-3 administration

To investigate the role of growth factor support cells of one AML patient (case A) were transplanted into SCID mice. After transplantation graded doses of IL-3 were injected ip three times a week. In one group rIL-3 support was provided by injecting the IL-3 producing cell line (cIL103) in the peritoneal cavity.

Significant growth of human cells was absent in animals receiving IL-3 at a dose of 15 µg/mouse or no IL-3 at all. In recipients of 30-60µg doses of IL-3, repopulation of the bone marrow by up to 33% of human cells could be achieved, but the level of infiltration showed great variability (Table IV). Serum IL-3 levels determined 24 hours following IL-3 injection were below detection level (0.1 ng/ml in the bio-assay). Reproducibly high percentages of human AML cells were observed in mice cotransplanted with the IL-3 producing cell line. These mice also showed the highest IL-3 plasma levels. IL-3 was detected early after transplantation and its plasma concentration increa-

sed exponentially during 30-50 days to values of 20-300 ng/ml (figure I). cIL103 transplanted ip into SCID mice behaved as a malignant tumor resulting in metastatic growth and ascites and finally death of the recipients between days 40 and 50.

Figure 1 IL-3 levels after transplantation of cIL-103

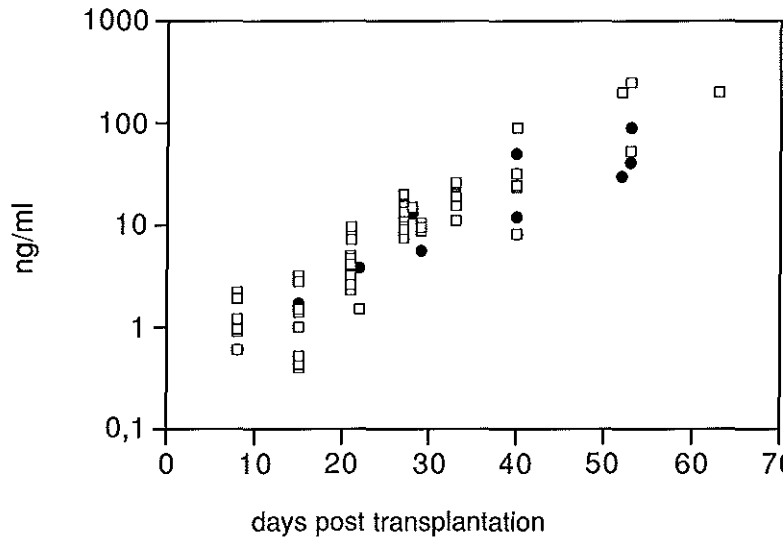

Figure 1
SCID mice were transplanted ip on day 0 with a cell dose of 10^7 of cIL103 cells after TBI. Plasma IL-3 levels were determined by ELISA (●) or in a bioassay (□) in a total of 69 samples.

Growth of AML in the SCID mouse and relation to IL-3 support
AML cells derived from patients A, B, C and D were injected iv into SCID mice (cell dose 30.10^6, 3.5 Gy TBI) and some of the mice were cotransplanted with the IL-3 producing cell line cIL103. The cells from all cases engrafted and repopulated in the SCID mice which also received cIL103 cells (Table V). Between days 29-40, the mice developed clinical signs of leukemia: especially anemia, rough fur and lethargic behaviour. FACS analysis of SCID mice BM showed extensive replacement by AML cells. The AML cells from patient B, which required IL-3 for growth in vitro, were also able to proliferate in recipients which did not receive exogenous IL-3. In contrast the cells from AML D showed autonomous proliferation in vitro, but were dependent on IL-3 in vivo.

TABLE IV
Stimulation of AML engraftment in SCID mice with graded doses of IL-3 and an IL-3 producing cell line

IL-3 dose	Unique animal number	Day of evaluation	% human cells BM
none	#20	29	0
	#21	29	1
	#22	46	0
	#23	47	0
15µg	#24	46	1
	#25	48	0
	#26	48	1
30µg	#27	29	22
	#28	46	1
	#29	48	0
60µg	#30	24	8
	#31	46	33
	#32	48	7
cIL-103	#33	29	44
	#34	29	30
	#35	29	68
	#36	29	61
	#37	29	20
	#38	29	43

Table IV
Groups of mice (3.5 Gy of TBI) were transplanted iv with 3.10⁷ AML cells (case A). They were injected intraperitoneally three times a week with increasing doses of IL-3. The IL-3 producing cell line cIL-103 was transplanted intraperitoneally in the final cohort of mice in order to stimulate AML engraftment.

TABLE V
Human AML growth with or without IL-3

AML, in vitro growth	Unique animal number	+ or - cIL103	Day of evaluation	% human cells BM
A,	#33*	+	29	44
IL-3	#34*	+	29	30
dependent	#35*	+	29	68
	#36*	+	29	61
	#37*	+	29	20
	#38*	+	29	43
	#20*	-	29	1
	#21*	-	29	1
	#22*	-	46	0
	#23*	-	47	0
B,	#39	+	40	42
IL-3	#40	+	40	57
dependent	#41	+	40	50
	# 1*	-	35	91
	# 2*	-	35	8
	# 3*	-	36	79
	# 4*	-	36	35
	#42	-	40	79
	#43	-	40	89
C,	#44	+	40	64
IL-3	#45	+	46	15
dependent	#46	-	43	3
D,	#47	+	33	15
IL-3	#48	+	33	15
responsive	#49	+	33	18
and	#50	-	33	2
autonomous	#51	-	33	2

Table V
*Growth of human AML in SCID mice with or without IL-3 support. AML cells from four different patient were transplanted iv into SCID mice (graft size 30.10⁶ AML cells, 3.5 Gy of TBI). In vitro three out of four AMLs were IL-3 dependent (AML A, B and C), one also showed autonomous growth (AML D). Groups of SCID mice were cotransplanted with cIL-103, an IL-3 producing cell-line. *data from earlier experiments, reported in Table III and IV.*

DISCUSSION

Conditions required for optimizing engraftment of AML in SCID mice were investigated: the role of pretransplant conditioning, of route of transplantation, of the graft size of AML and stimulation of AML growth by IL-3 administered in vivo after transplantation.

Results of experiments with the cell line HL-60 demonstrated that iv transplantation allowed for engraftment and extensive AML growth in BM in contrast to ip transplantation. For intravenous transplantation, a dose effect relationship between dose of TBI and growth of human AML cells in the mouse BM is apparent. The effect of TBI may be mediated by immunosuppression or by the creation of space (16). As NK-cell and LAK-cell function are retained in SCID mice, immunosuppression directed against these cells may facilitate engraftment further. Obviously there should be sufficient space in the hemopoietic tissue for the incoming graft, since transplantation of feasable numbers of syngeneic myeloid cell lines is impossible without creating space (17), similar to syngeneic bone marrow (18).

Transplantation of doses of $30x10^6$ cells may lead to rapid outgrowth of AML. However, it should be noted that at days 40-80 AML cells were also detected in mice injected with low cell doses. This demonstrated the long term viability and proliferative potential of AML cells in the SCID mouse. It also indicates that the abscence of detectable AML before day 40 is not necessarily due to graft failure. The minimum cell dose necessary for reproducible engraftment was $1.1x10^6$ AML cells. A minimal cell dose of $2x10^5$ cells was observed by others (2). This may indicate that the minimum required cell dose varies for different cases of AML.

Data on the use of hHGFs to stimulate normal human hematopoiesis in the SCID mice are scarce (19,20,21), but generally administration of hHGFs resulted in stimulation of growth of human hematopoiesis. Mast-cell growth factor (c-kit ligand) and PIXY321 were shown to stimulate growth of AML in SCID mice (2). Among 17 cases, in 16 instances AML engraftment was apparent. However the quantitative level of engraftment varied considerably and 14% of the mice showed less than 10% human cells engrafted. Here we show that reproducible growth of AML, supported by the cotransplanted IL-3 producing cell line, in all four cases overgrew the mouse bone marrow. This resulted in clinical signs and symptoms of acute leukemia. Lack of growth of AML A, C and D without IL-3 supply apparently demonstrates IL-3 dependence in vivo.

Our results indicate that the required rh-IL3 dose is relatively high: i.e. more than 60µg ip three times per week. Intraperitoneal cotransplantation of cIL103 results in earlier and more extensive growth of AML. This growth advantage is probably caused by the high and continuous IL-3 plasma levels

achieved in these mice (figure I). The use of cIL103 as a source of IL-3 in vivo is relatively easy and reduces the need for purified IL-3. However, the cell line in vivo shows the characteristics of a malignant tumor. SCID mice die from the tumor after about 50 days, ultimately interfering with the experiments on AML growth. As human AML growth in vitro can be stimulated by other hematopoietic growth factors one may assume that AML growth in the SCID mouse supported by IL-3 can be augmented by GM-CSF, G-CSF, stem cell factor or other cytokines stimulating AML proliferation in culture.

In vitro IL-3 dependency of AML cells (as assessed with activation of DNA-synthesis in vitro) was not predictive for the dependency on IL-3 in vivo. The AML cells of patient B were IL-3 dependent in vitro but did not require IL-3 in vivo. Perhaps these AML cells supply their own cytokines when engrafted into mouse bone marrow stroma or they may grow truly autonomously. Similar results, in vivo growth without hHGF support and in vitro hHGF dependency, were found for the human myeloid cell line M-O7e. In contrast, the AML cells of patient D grew autonomously in vitro but clearly needed IL-3 in vivo. The reasons for the discrepancy between in vitro and in vivo responsiveness to IL-3 remain to be investigated.

Apparently proliferation of cells from the majority of acute myeloid leukemias can be achieved and maintained in SCID mice for a more extended period of time than in culture. This expands the possibilities to study in particular long term growth of AML.

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CHAPTER 3

FACILITATED ENGRAFTMENT OF HUMAN HEMATOPOIETIC CELLS IN SEVERE COMBINED IMMUNODEFICIENT MICE FOLLOWING A SINGLE INJECTION OF CL_2MDP-LIPOSOMES

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ABSTRACT

Transplantation of normal and malignant human hematopoietic cells into severe combined immunodeficient (SCID) mice allows for evaluation of long term growth abilities of these cells and provides a preclinical model for therapeutic interventions. However, large numbers of cells are required for successful engraftment in preirradiated mice due to residual graft resistance, that may be mediated by cells from the mononuclear phagocytic system. Intravenous (iv) injection of liposomes containing dichloromethylene diphosphonate (Cl_2MDP) may eliminate mouse macrophages in spleen and liver. In this study outgrowth of acute myeloid leukemia (AML) cells and umbilical cord blood (UCB) cells in SCID mice conditioned with a single iv injection of Cl_2MDP-liposomes in addition to sublethal total body irradiation (TBI) was compared to outgrowth of these cells in SCID mice that had received TBI alone.

In four cases of AML, a 2-10 fold increase in outgrowth of AML cells was observed. Administration of 10^7 UCB cells reproducibly engrafted SCID mice that had been conditioned with Cl_2MDP-liposomes and TBI, whereas human cells were not detected in mice conditioned with TBI alone. As few as $2x10^4$ purified CD34+ UCB cells engrafted in all mice treated with Cl_2MDP-liposomes. Unexpected graft failures were not observed among SCID mice treated with macrophage depletion. Histological examination of the spleen showed that TBI and Cl_2MDP-liposomes iv resulted in a transient elimination of all macrophage subsets in the spleen, whereas TBI had a minor effect. Cl_2MDP-liposomes were easy to use and their application was not associated with appreciable side-effects. Cl_2MDP-liposome pretreatment in combination with TBI allows for reproducible outgrowth of high numbers of human hematopoietic cells in SCID mice.

INTRODUCTION

Severe combined immunodeficient (SCID) mice may be used as a model for the in vivo analysis of proliferation of human hematopoietic cells. (1-5) In hematological diseases such as acute myeloid leukemia (6,7), chronic myeloid leukemia (8), acute lymphoblastic leukemia (9,10,11) and lymphoma (12) SCID mice provide a useful model for analysis of long-term growth and therapeutic interventions in vivo.(13,14,15) Conditioning of SCID mice using sublethal TBI and intravenous transplantation allows for outgrowth of these cells in the SCID mouse bone marrow. (1,16)

However, even following sublethal irradiation of SCID mice large numbers

of human hematopoietic cells are required for reproducible outgrowth and graft failures are frequently observed. (1-3,5-15) These problems restrict the practical possibilities for analysis of subsets of hematopoietic cells. As SCID mice are T and B cell deficient, likely explanations for residual graft resistance may be that transplanted human hematopoietic cells are cleared by either recipient mononuclear phagocytes or by recipient NK cells. (17,18) Macrophage depletion with the purpose of facilitating engraftment of allogeneic and xenogeneic bone marrow grafts has been investigated before, using less specific means such as silica or carageenan. In irradiated mice, carrageenan and silica abrogated or weakened resistance to parental, allogeneic and rat marrow grafts. (19-22) However, both carrageenan and silica are highly toxic agents. Furthermore, they do not completely eliminate macrophages and exert undesired effects on non-phagocytic cells. (19,21,23)These disadvantages clearly compromise their applicability in bone marrow transplantation in general and in the SCID mouse model in particular. In vivo macrophage depletion has been achieved with liposomes which contain clodronate (Cl_2MDP). Such liposomes are ingested by macrophages. After intracellular disruption of the liposomes clodronate effectively kills these cells. (24) Which population of macrophages is eliminated depends on the route of administration of Cl_2MDP-liposomes. (25) Intravenous injection of the liposomes mainly eliminates phagocytic cells in liver and spleen, the candidate effector cells in graft resistance, because of their direct contact with circulating hematopoietic cells. Fraser et al. (26) showed that intravenous injection of Cl_2MDP-liposomes into unirradiated SCID mice prolonged the time needed for clearance of intravenously injected human peripheral blood lymphocytes. Similarly, application of these liposomes to SCID mice with an established human thymus/liver graft resulted in increased numbers of circulating human lymphocytes. To determine the effect of Cl_2MDP-liposomes on engraftment of normal and leukemic human hematopoietic cells in irradiated SCID mice we compared transplantations with or without additional Cl_2MDP liposome treatment.

MATERIALS AND METHODS

Acute myeloid leukemia cells and umbilical cord blood cells

Peripheral blood (PB) samples were obtained following informed consent from four patients presenting with AML, diagnosed according to the criteria of the French-American-British Committee (FAB). (27) AML cells and umbilical cord blood samples (UCB) were isolated by Isopaque-Ficoll centrifugation (1.077g/cm^2; Nycomed, Oslo, Norway) and then cryopreserved. (28) After thawing the viability of AML and UCB cells as assessed by trypan blue exclusion was always>70%.

Preparation of CD34+ umbilical cord blood fractions
UCB cells were incubated with an IgG2a antibody against CD34 (MoAb 561) which was noncovalently linked to rat-anti-mouse IgG2a-conjugated immunomagnetic beads (Dynabeads, Dynal, Oslo, Norway). CD34+ cells were eluted from the beads using a polyclonal antibody preparation directed against the Fab fragment of the CD34 antibody (Detachabead, Dynal). (29)

Immunodeficient mice and transplantation of AML and umbilical cord blood (UCB)
Female specific pathogen-free CB17 scid/scid mice (5-9 weeks of age) were purchased (Harlan CPB, Austerlitz, The Netherlands). Non obese diabetic SCID (NOD/SCID), specifically NOD/Lt-SCID/Sz mice (11 weeks of age), were obtained from Jackson Laboratories, Bar Harbour, MA. Housing, total body irradiation (TBI) and transplant procedures have been described. (16) AML #1 and AML#2 engrafted in SCID mice without support of human hematopoietic growth factors and AML#3 and #4 were IL-3 dependent in SCID mice. The latter SCID recipients received 60μg of human IL-3 (Gist Brocades, Delft, The Netherlands) in 200μL HBSS and 1%BSA (Sigma) intraperitoneally, five days a week as described. (16) Mice transplanted with UCB did not receive growth factor treatment.

Liposome preparation
Cl_2MDP-liposomes were prepared as reported. (30) Briefly, 75mg phosphatidyl choline (Lipoid GmbH, Ludwigshafen, Germany) and 11mg cholesterol (Sigma, St Louis, MO) were dissolved in chloroform in a round bottom flask. After low vacuum rotary evaporation at 37°C the lipids were dispersed by gentle rotation in 10ml PBS in which 2.5mg clodronate (a gift of Boehringer Mannheim, GmbH Mannheim, Germany) was dissolved. The resulting liposomes were washed twice at 100,000g for 30 minutes to remove free, non-entrapped diphosphonate. The liposomes were then resuspended in 4mL phosphate buffered saline (PBS) (liposome stock solution). SCID mice were injected in a lateral tail vein with 0.2mL liposome stock solution on the day before transplantation of the hematopoietic cells.

Tissue collections
SCID mice were killed by CO_2 inhalation followed by cervical dislocation in accordance with institutional animal research regulations. Cells or tissues from cohorts of mice transplanted with the same graft were evaluated, between days 22 and 47 after transplantation using flow cytometry and cytology. (31) Spleens of selected mice were extirpated and cryostat sections were prepared for pathologic examination.

Flow Cytometry

To determine the percentage of human hematopoietic cells in the SCID mouse, bone marrow samples from mice that had been transplanted with UCB grafts were incubated with mouse monoclonal antibodies to human CD33 , CD34, CD45 and CD38. The initial leukemias and BM samples from mice that had been transplanted with leukemic grafts were stained or double-stained with the following mouse monoclonal antibodies): CD34-FITC, CD34-PE, CD38-PE, CD34-FITC/IgG1-PE, CD34-FITC/CD38-PE, CD34-FITC/HLA-DR-PE, CD34-FITC/c-kit-PE, CD34-FITC/CD33-PE and finally CD45-FITC/CD33-PE. Mouse IgG1-FITC and mouse IgG1-PE conjugated antibodies and samples from non-transplanted SCID mice were used as controls. c-kit-PE was purchased from Immunotech, Marseille, France, all other antibodies from Becton Dickinson, San Jose, CA, USA. The phenotypes of cells recovered from the SCID mouse bone marrow was compared to those of the grafts. Fluorescence was measured using a FACSCAN flow cytometer and Lysis II software (Becton Dickinson, Immunocytometry Systems). Erythrocytes and dead cells were excluded from analysis by gating on forward and orthogonal light scatter. Cells recovered from SCID mouse bone marrow (BM) with positive staining for two antibodies specific for human hematopoietic cells were considered to be graft derived. (32)

Immunohistochemistry of spleen sections

Freshly obtained SCID mouse spleens were embedded in Tissue-tek II (Miles Laboratories, Inc., Naperville, MI), frozen and stored at -70°C. Cryostat sections of 5µm were prepared. Tissue fixation and immunoperoxidase staining of cryostat sections were performed essentially as described by de Jong et al. (33) For fixation a hexazotized pararosaniline solution (0.5mL, 4%) was added to NaNO2 (0.5mL, 4%), diluted in 165mL sterile water and applied to dry tissue sections (2 minutes) followed by washing in PBS. (33) MAb binding was detected using a modified protocol involving NiSO4-supplemented DAB and counterstaining of the nuclei with nuclear fast red. (34) Sections were incubated with a panel of monoclonal antibodies to determine the effect of TBI and TBI with macrophage depletion by Cl_2MDP on macrophage subsets in the spleen as representatives of phagocytic cells in direct contact with the blood stream. The antibodies used were F4/80 (35,36) (identifying especially red pulp macrophages), ER-HR3 (37,38) (major subset of red pulp macrophages), ER-TR9 (39,40) (marginal zone macrophages), MOMA-1(41) (marginal metallophilic macrophages), Monts-4 (42) (marginal metallophilic and white pulp macrophages) and N418 (anti-CD11c) (43,44) (dendritic cells). To confirm results with other antibodies in addition BM8 (45) (red pulp macrophages), SER-4 (46) (marginal metallophilic macrophages) and ER-BMDM1 (47) (dendritic cells) were applied.

RESULTS

Pretreatment with Cl_2MDP-liposomes enhances engraftment of AML
We examined the effect of additional Cl_2MDP liposome treatment on the engraftment abilities of AML cells (from case #1-4) in SCID mice treated with TBI and liposomes versus TBI alone (controls). Liposome treatment in addition to TBI resulted in an increase in the percentages of leukemic cells in the mouse bone marrow (BM) by 2-10 fold (Table 1). Leukemic tumor load per mouse increased 3-12-fold (Table 1). Graft failure was defined as less than 0.5% of AML cells in the SCID mouse BM. Among the 25 mice transplanted with AML cells following liposome treatment no graft failures were observed, whereas six graft failures were seen in controlrecipients ($p=0.02$, Fisher's Exact Test) (Table 1). The immunophenotypes of the leukemic cells recovered from the SCID mice (as assessed by flow cytometry) were identical to those of the original grafts.

Comparison of growth of AML in SCID mice and in NOD/SCID mice
The outgrowth of graded cell doses of AML #2 in SCID mice pretreated with Cl_2MDP liposomes was comparable with that in NOD/SCID mice.(Table 2).

Macrophage depletion enhances outgrowth of UCB cells in SCID mice
Enhancement of engraftment of normal human hematopoietic cells was assessed in cell dose titration experiments of UCB cells. Transplantation of $10x10^6$ UCB cells after TBI did not allow for reproducible engraftment (Table 3). By comparison grafts of only $1x10^6$ UCB cells engrafted in 5 of 6 SCID mice prepared with additional macrophage depletion. Thus an approximately 10-fold reduction of the minimal cell numbers required for engraftment was seen as a consequence of additional conditioning with Cl2MDP liposomes. (Table 3). Cell dose titrations with CD34+ selected UCB cells were performed in mice conditioned with TBI and macrophage depletion. As few as $10x10^3$ CD34+ UCB cells reproducibly engrafted SCID mice (data not shown).

Extensive depletion of all spleen macrophage subsets by the combination of Cl_2MDP liposomes and TBI, limited effect of TBI alone
To evaluate the effect of Cl_2MDP liposomes on SCID mouse macrophages, which are possibly involved in scavenging of transplanted human hematopoietic cells, cryostat spleen sections were incubated with a panel of antibodies identifying distinct mononuclear phagocyte subpopulations. TBI at 3.5 Gy without administration of Cl_2MDP-liposomes had negligible effects on macrophage subpopulations in the spleen at day 4 after conditioning. Red pulp, white pulp and marginal zone macrophage populations identified

Table I Growth of human AML cells in SCID mice: effect of pretreatment with Cl_2MDP liposomes

	AML#1		AML#1		AML#2		AML#3		AML#4	
Cell numbers transplanted ($x10^6$)	1		10		10		20		30	
conditioning	MD +TBI	TBI	MD +TBI	TBI	MD +TBI	TBI	MD +TBI	TBI	MD +TBI	TBI
% of AML cells in SCID BM (mean ±SD)	76±18	17±16	91±5	41±43	19±18	7±6	63±17	25±22	10±13	1±1
AML tumor load* ($x10^6$) (mean±SD)	24±8.5	2.0±2.1	58±12	23±27	11±11	3±3	44±10	8±7	2±1	0.3±0.4
graft failures /transplanted SCID mice	0/8	1/7	0/5	0/5	0/5	1/5	0/4	1/3	0/3	3/5

Table I
Irradiated groups of SCID mice (3.5Gy) were transplanted with cells from 4 cases of AML with or without Cl_2MDP pretreatment and evaluated on the same day, between days 35 and 47.
**The tumor load was determined by counting the number of nucleated cells obtained by flushing of two mouse femora, equivalent to 13.5% of the total mouse bone marrow (50) as well as the percentages of human hematopoietic cells determined by flow cytometry.*
Abbreviations: BM: bone marrow, SD: standard deviation. MD: macrophage depletion by pretreatment with Cl2MDP liposomes, TBI: total body irradiation

by immunophenotypic analysis had not changed significantly (Table 4). In contrast, dendritic cells (identified by the monoclonal antibodies N418 and ER-BMDM1) had disappeared from the red pulp and from the white pulp as a consequence of the sublethal dose of TBI alone. Combined treatment with TBI and Cl_2MDP liposomes depleted all identifiable macrophages from the white pulp, the red pulp and the marginal zone (Table 4). At day 45 after transplantation, all mononuclear phagocyte subsets had reappeared except ER-TR9 positive marginal zone macrophages. (Table 4).

Table II
Cell dose titration of AML #2 in SCID mice with and without Cl_2MDP liposome pretreatment and in NOD/SCID mice

Conditioning	MD+TBI	TBI	TBI
Animals	SCID mice	SCID mice	NOD/SCID mice
Cell numbers transplanted ($x10^6$)			
30		77	
		93	
10	81	19	45
	56	38	36
	69	66	
3.3	83	3	34
	7	1	34
	32	3	33
	31		
1	1	0	7
	4	0	2
	5	0	13
	2		
0.3	0.2		5
			4

Table II
NOD/SCID mice were transplanted in the same experiment as the SCID mice. All mice were evaluated on days 28 and 29. Data represent percentage of infiltration of human cells in the bone marrow of individual mice. Abbreviations: BM: bone marrow, MD: macrophage depletion by pretreatment with Cl2MDP liposomes, TBI: total body irradiation

Toxicity of Cl_2MDP-liposomes

The injection of 0.2mL of Cl_2MDP-liposomes on the day before TBI and transplantation did not result in appreciable side effects during the 45 day observation interval. Among the 56 mice conditioned with TBI alone 6 animals died before evaluation (11%). In comparison 6 deaths were noted among 91 mice conditioned with TBI and additional macrophage depletion (8%).

Table III Cell dose titration of 2 umbilical cord blood samples in SCID mice with and without Cl_2MDP pretreatment

	UCB #1		UCB #2	
Conditioning	MD+TBI	TBI	MD+TBI	TBI
Cell numbers transplanted ($x10^6$)				
30	71		75	
			56	
10	24	1	11	0
	6	0	18	0
	11	0	12	1
	6	0	6	0
3.3	9	0	3	0
	3	0	0	0
	9	0	1	0
			0	
1	1		1	
	1		3	
			11	
			0	

Table III
Irradiated SCID mice were transplanted with graded doses of umbilical cord blood cells with or without macrophage depletion. Data represent percentage of infiltration of human cells in the bone marrow of individual mice. All mice were evaluated on days 41 and 42.
Abbreviations: ND: not done, UCB: umbilical cord blood graft MD: macrophage depletion by pretreatment with Cl2MDP liposomes, TBI: total body irradiation

Table IV Macrophage populations in the spleen of SCID mice after TBI or TBI and macrophage depletion.

Antibodies	spleen region	Control	Day 4		Day 45	
			TBI	MD +TBI	TBI	MD +TBI
F4/80	wp	2+	4+	0*	1+	2+
	rp	4+	4+	0*	4+	4+
ER-HR3	wp	1+	1+	0	1+	2+
	rp	3+	3+	0	3+	3+
ER-TR9	mz	2+	1+	0	0	0
MOMA-1	pwp	3+	2+	0	3+	2+
	rp	2+	3+	0	1+	1+
Monts-4	wp	3+	3+	0	3+	2+
	rp	1+dim	2+	0	1+	<1+
N418	wp	4+	0	1+	3+	4+
	mz	2+	1+	0	2+	3+
	rp	1+	0	0	1+	1+

Table IV To determine the effects of TBI and macrophage depletion by Cl2MDP liposomes on spleen macrophage populations SCID mice were evaluated on days 4 and 45 after TBI, and compared to untreated SCID mouse controls. The data obtained with these antibodies were similar to data obtained with the use of the independent antibodies BM-8, SER-4, and ER-BMDM1 (data not shown). 2 or 3 mice per group were evaluated.

*Abbreviations: TBI total body irradiation, MD: macrophage depletion by pretreatment with Cl_2MDP liposomes, wp: white pulp; rp: red pulp; pwp: peripheral white pulp; mz: marginal zone. The number of macrophages staining with a specific antibody within these anatomically defined regions were scored semiquantitatively: 4+: >50% positivity-confluent; 3+: 50%; 2+: 10% - 50%; 1+: scarce. *= only remnants*

DISCUSSION

Macrophage depletion by intravenous administration of Cl_2MDP-liposomes prior to sublethal TBI enhances the engraftment of human hematopoietic cells in the SCID mouse bone marrow. This treatment is easy to apply and without significant toxicity. Outgrowth of primary human AML increased 2-10 fold and engtraftment of umbilical cord blood cells was achieved with at least 10 fold smaller grafts (Tables 1 and 3). Probabilities of graft failure were significantly reduced (Table 1). Thus, conditioning with TBI and Cl_2MDP-liposomes in combination permits the establishment of

relatively small human hematopoietic cell grafts, e.g. $1x10^6$ unseparated UCB cells or $1x10^4$ CD34 positive UCB cells and may facilitate the use of the SCID mouse model for the study of normal and malignant human hematopoietic cells.

The observations suggest a functional role of murine phagocytic cells in the clearance of human hematopoietic cells that engraft SCID mouse bone marrow, similar to the delayed clearance of human lymphocytes from the SCID mouse circulation as observed by Fraser et al. (26) Other more indirect immunological mechanisms cannot be excluded. One of these mechanism relates to the modulation of NK cell function by macrophages. Depletion of macrophages in the liver (Kupffer cells) with Cl_2MDP-liposomes was associated with a parallel decrease of the number of NK cells. (48) Furthermore Kupffer cell conditioned media appeared to enhance NK cell viability and function in vitro. (48) In the study by Fraser et al. (26) complete depletion of macrophages in the red pulp of the spleen was observed while many white pulp macrophages remained. Our data show that white pulp macrophages are eliminated by Cl_2MDP-liposomes in sublethally irradiated mice.

NOD/SCID mice have multiple immunological defects. In addition to B and T cell deficiency, NK function is absent and these mice may also have a macrophage maturation defect. (49) It has been shown that the tumor load in the spleen of SCID/NOD mice transplanted with human CEM T lymphoblasts was increased 4-fold as compared to similarly transplanted SCID mice. (49) Our results indicate that the outgrowth of AML and UCB grafts in irradiated SCID mice conditioned with additional Cl_2MDP-liposomes may be as effective as that in sublethally irradiated NOD/SCID mice.

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CHAPTER 4

LONG TERM LEUKEMIA INITIATING CAPACITY OF A CD34 NEGATIVE SUBPOPULATION OF ACUTE MYELOID LEUKEMIA

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ABSTRACT

Acute myeloid leukemia (AML) proliferation in vivo is maintained by a small fraction of progenitor cells. These cells have been assumed to express an immature phenotype and to produce most colony forming units (AML-CFU). For one case of AML (FAB M1, normal cytogenetics) we examined the capacity of the CD34+ (25% of unseparated AML cells) and CD34- fractions to initiate leukemia in severe combined immunodeficient (SCID) mice. In addition, the production of AML-CFU and nucleated cells (NC) of these subsets was investigated in long term bone marrow culture (LTBMC). The frequencies of cobblestone area forming cells (CAFC) were also estimated; early appearing cobblestone areas (CAs) being indicative of relatively mature progenitors and late CAs representing the progeny of primitive progenitors. In mice transplanted with CD34- (98% pure) or CD34+ (98% pure) grafts, similar AML cell growth was seen throughout an observation period of 106 days. The capacity to establish long term growth from the CD34- cells was confirmed by renewed outgrowth after retransplantation. In vitro, the CD34- fraction contained both immature and mature CAFCs and produced high numbers of AML-CFU and NC in LTBMC. The CD34+ fraction produced only small numbers of AML-CFU, NC and mature CAFCs. Therefore, the expression of CD34 and the content of AML-CFU were not associated with long term growth of AML. However, similar frequencies of primitive CAFCs were observed in both fractions. Thus, both CD34- and CD34+ subsets of this AML sample contained immature progenitors with the capacity to initiate long term AML growth as characterized in vivo (in SCID mice) as well as in vitro (in CAFC assay), indicating asynchrony between functional and immunophenotypical maturation of AML progenitor cell compartments.

INTRODUCTION

The malignant cell population of acute myeloid leukemia (AML) is assumed to be maintained in vivo by a small fraction of clonogenic cells (1). Analysis of this fraction so far has been hampered by lack of assays for long term growth of AML. Long term growth of normal human bone marrow can be demonstrated in vitro, for example by long term bone marrow cultures (LTBMC). Clonogenic AML cells have mainly been studied as colony forming units of AML (AML-CFU) in short term colony assays. Although the immunophenotype of AML-CFU displayed considerable heterogeneity among patients and may be aberrant (2-7) clonogenic capacity is generally regarded to be associated with an immature phenotype. The appearance of CD38 and the loss of CD34 expression have been associated with the loss

of clonogenic capacity (8,9).
Recently, CD34 and CD38 expression of in vivo leukemia initiating cells has been investigated in mice with Severe Combined Immunodeficiency (SCID). In one case leukemia was shown to be initiated in mice injected with the CD34+/CD38- AML cell fraction (10). AML was not established from the AML fraction depleted of the CD34+/CD38- cells. The data suggest that the heterogeneous marker expression of AML allows for isolation of fractions containing high numbers of progenitors, similar to normal hematopoietic cells.
To examine long term growth of AML cells, we used transplantation in SCID mice as well as the cobblestone area forming cell (CAFC) assay. This assay allows for calculation of clonogenic cell frequencies in fractions of hematopoietic cells. The late appearing cobblestone areas (CAs) of normal hematopoiesis are considered stem cell progeny with long term engraftment potential, whereas the early appearing CAs are assumed to be derived from more mature progenitors (11).
The capacity to initiate leukemia in vivo in SCID mice, the expression of CD34 and in vitro clonogenic growth were investigated in this study. For this purpose, CD34+ and CD34- cells of one AML case were transplanted into SCID mice and investigated by long term bone marrow cultures (LTBMC), CAFC assay as well as by short term colony cultures.

MATERIALS AND METHODS

AML cells
Peripheral blood (PB) samples were obtained following informed consent from a patient presenting with AML M1 diagnosed according to the criteria of the French-American-British Committee (FAB) (12). Cytogenetics showed a normal karyogram. Mononuclear cells were isolated by Isopaque-Ficoll centrifugation. Subsequently T cells were depleted by E-rosetting (13). The cells were cryopreserved in 10 % dimethylsulfoxide, 20% heat-inactivated fetal calf serum (FCS) and Iscoves modified Dulbecco,s medium (IMDM). After thawing by stepwise dilution (14), viability assessed by trypan blue staining was always > 90%.

Flow Cytometry
The samples were incubated with PE-conjugated mouse monoclonal antibodies against CD13, CD33, CD38, CD34 or HLA-DR and fluorescein isothiocyanate (FITC) conjugated antibodies against CD15, CD34 or CD45. Mouse IgG1-FITC and mouse IgG1-PE conjugated antibodies and samples from non-transplanted SCID mice were used as controls. The antibodies were obtained from Beckton Dickinson, San Jose, CA, USA. Fluorescence

was measured using a FACSCAN flow cytometer and Lysis II software (Beckton Dickinson, Immunocytometry Systems). Erythrocytes and dead cells were excluded from analysis by gating on forward and orthogonal light scatter. Cells recovered from SCID mouse bone marrow (BM) staining simultaneously for CD33 and CD45 were counted as human myeloid cells (15).
In some cases human cells were separated from the mouse BM using fluoresence activated cell sorting. Cells were recovered from the SCID mouse BM under sterile conditions and incubated with CD38-PE (Beckton-Dickinson). Sorting was performed using a FACS Vantage (Beckton-Dickinson Immunocytometry Systems). The setting of the sorting window was as shown in Fig 2B.

Preparation of CD34+ and CD34- fractions
AML cells were incubated with an IgG2A antibody against CD34 (MoAb 561 (16)) which was noncovalently linked to rat-anti-mouse IgG2a-conjugated immunomagnetic beads (Dynabeads, Dynal, Oslo, Norway). CD34+ cells were eluted from the beads using a polyclonal antibody preparation directed against the Fab fragment of the CD34 antibody (Detachabead, Dynal) (17). The eluted cell population was devoid of CD34 antibodies and was 98% CD34+ as determined by FACS analysis after restaining with CD34-PE (HPCA-2, Beckton Dickinson, San Jose, CA, USA) (Fig 1b). Non-bound cells were further depleted by a similar second incubation with the CD34 immunomagnetic beads. In the final CD34- fraction, 2% of the cells weakly stained with CD34-PE (Fig 1c) in a FACScan reanalsis.

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, and supplied with sterile food and acidified drinking water with 100mg/L ciprofloxacine (Bayer AG, Leverkusen, Germany). Before transplantation, 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 40µg/ml were excluded. 3.5 Gray of TBI was delivered by a ^{137}Cs source (Gammacell, Atomic Energy of Canada, Ottawa, Canada) adapted for the irradiation of mice. Leukemic cells, suspended in 300µl Hanks balanced salt solution (HBSS) (Gibco, Breda, The Netherlands), were injected intravenously (iv) into the lateral tail vein. This particular AML was known to proliferate in SCID mice without administration of human hematopoietic growth factors (18).

Tissue collections
SCID mice were sacrificed by CO2 inhalation followed by cervical dislocation in accordance with institutional animal research regulations. Cell suspensions were prepared from the BM and used for flow cytometry and the preparation of cytospin slides. Cytospin slides were stained with May-Grünwald Giemsa and morphology was microscopically evaluated.

Colony culture assay
Cells (5x 10^4) were plated in 35-mm dishes (Beckton Dickinson, San Jose, CA, USA) in 1mLα-MEM (Gibco, Gaithersburg, MD, USA) containing 0.8% methylcellulose, 5% fetal calf serum (FCS), 1% bovine serum albumen (BSA), glutamine (2mM/L), insulin (10µg/mL), linoleic acid, and cholesterol (1.5x 10^{-5}M/L), sodium selenite (1x 10^7M/L, nucleosides (each at 1mg/mL), 2-mercaptoethanol (1x 10^4M/L), iron saturated human transferrin (0.6mg/mL), penicillin (100U/mL) and streptomycin 850µG/mL). Assays were performed either without hematopoietic growth factors, or with granulocyte-macrophage stimulating-factor (GM-CSF) (Behringwerke AG, Marburg, Germany, 5ng/mL), interleukin-3 (IL-3) (Sandoz BV, Basel, Switzerland, 10ng/mL) , GM-CSF and IL-3 or with stem-cell factor (SCF) (Immunex, Seattle, WA, USA, 200ng/mL), GM-CSF and IL-3.

Long Term Bone Marrow Culture
Long term bone marrow culture (LTBMC) was performed as described previously (11). Briefly, confluent layers of the FBMD-1 stromal cell line in 25cm^2 flasks were overlaid with either 10^5 unseparated AML cells or 10^5 CD34- cells or 10^6 CD34+ cells per flask. The cells were cultured in -modified Dulbecco's modified Eagle medium (DMEM, Gibco, Breda, The Netherlands) supplemented with HEPES (3.5mM) (Sigma, St Louis, MO, USA), glutamine (2mM), sodium-selenite (10^{-7}M), β-mercaptoethanol (10^{-4}M), 12.5% FCS, 12.5% horse serum (HS) and hydrocortisone 21-hemisuccinate (10^{-6}M final concentration), in the presence of IL-3 (10ng/ml) and G-CSF (20ng/ml). IL-3 (10ng/ml) and G-CSF (20ng/ml) were added weekly to the cultures. Flask cultures of each fraction and of the unfractionated AML were set up in triplicate and maintained at 33^0 C for 50 or 63 days with weekly half-medium changes and therefore removal of only half of the non-adherent (NA) cells. The output of NA cells and CFU-AML output of individual flasks was determined every two weeks.

Cobblestone-Area-Forming Cell Assay
The cobblestone area forming cell (CAFC) assay was performed as previously described (11). Briefly, confluent stromal layers of FMBD-1 cells in 96-wells plates were overlaid with isolated CD34+, CD34- or the unse-

parated AML cells in a limiting dilution setup. The medium was the same as used for LTBMC. Input values were 2854 nucleated cells (NC) per well for unseparated and CD34- cells and 28664 nc/well for CD34+ cells. 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 hematopoietic clone of at least five cells (Cobblestone area, CA) beneath the stromal layer was determined about every 14 days and CAFC frequencies were calculated using Poisson statistics as described (19,20).

RESULTS

AML growth in SCID mice

Results of SCID mouse transplantations were obtained in 4 separate experiments using different graft sizes. Transplantation of 7.5 x 10^6 of unseparated AML cells, the CD34+ or the CD34- fraction resulted in reproducible AML outgrowth as of day 35 (Table 1). The CD34- fraction, as well as unseparated AML cells and the CD34+ fraction maintained AML growth in SCID mice during the latter period. Transplantation of 1x10^6 AML cells resulted in reproducible outgrowth of AML between days 55 and 106, while earlier AML outgrowth was mainly below the level of detection (Table 1).

Graft failures were defined as a percentage of AML cells in the SCID mouse bone marrow smaller than 1%. Three graft failures occurred between days 55 and 10^6 among 42 transplanted mice: i.e. in two instances among mice transplanted with unseparated AML and in one recipient of the CD34- fraction. These three mice had each received a graft of 1x10^6 cells. The mice were not evaluated at time points later than day 106.

Table I

Time of Evaluation	Graft Size (x10^6)/mouse	% AML cells in BM (±SEM)*		
		CD34- Fraction	CD34+ Fraction	Unseparated AML
35-45	7.5	20±9	38±14	20±11
	1	0#	23±23#	6±4#
55-106	7.5	29±16	34±12	68±20#
	1	24±9	20±4	9±3

*Table I The percentages of huAML cells were determined on FACS scan analysis. BM: bone marrow. SEM: Standard Error of the Mean. * Graft failures included as 0%.*

Retransplantation of BM recovered from recipients of 30 x 10^6 CD34- cells on day 28 resulted in regrowth of AML in two secondary recipients. The secondary grafts contained 4.0 x 10^6 and 4.6 x 10^6 AML cells. Following transplantation of the latter secondary recipients bone marrow infiltration by AML cells was 1% at day 54 and 28% at day 95, respectively.
A cell dose titration ranging from 0.33 x 10^6 to 30 x10^6 of unseparated AML cells showed a dose effect relationship between cell dose and engraftment of AML (Table 2). Outgrowth of AML was not observed after transplantation of 9 x 10^4 CD34+ cells (data not shown).

Flow cytometry
Expression of CD34 and CD38 of unseparated AML cells before transplantation is shown in Fig 2A. The AML cells were positive for CD33, CD45, HLA-Dr CD13 and CD15. Expression of CD34 and CD38 was analyzed in the bone marrow of all mice described. Irrespective of the initial graft, AML cells recovered from the bone marrow of SCID mice were CD34- and stained homogeneously for CD38 (Fig 2B). The CD34+ /CD38- cells had converted to a CD34-/CD38+ phenotype following growth in the SCID mouse. Bone marrow cells containing more than 10% human cells were used for more extensive analysis with CD13, CD15, CD33, CD34, CD38, CD45 and HLA-DR. The latter analysis was performed on the bone marrow of 22 mice, that had received an unseparated graft (n=8), a CD34+ graft (n=6) or a CD34- graft (n=6). Similar to the originally grafted AML cells CD45+ cells were also positive for CD33 and HLA-DR, most cells were positive for CD13 and CD15. The light scatter properties of the AML cells had not changed: a homogeneous population of cells with light scatter properties confined to the blast region.

No. of Mice Engrafted/transplanted		
CD34- Fraction	CD34+ Fraction	Unseparated AML
4/5	6/8	5/5
0/2	1/4	2/3
4/4	6/6	2/2
11/12	9/9	7/9

Graft failures were defined as <1% AML cells in the bone marrow.
[a] values calculated on the basis of data from less than four engrafted mice.

Flow cytometry did not reveal differences between AML cells recovered from mice transplanted with the CD34- fraction, the CD34+ fraction or unseparated AML cells.

Table II
Cell dose titration of unseparated AML cells transplanted into SCID mice

Cell dose cells (x 10^6)	days after transplant	%AML cells mouse BM
30	35	8
	35	91
	35	79
	35	37
10	35	28
	35	60
	35	6
3.3	35	3
	35	9
1	44	2
	64	19
	64	3
0.3	81	1
	81	0
	81	0

Table II
AML cell proliferation in the bone marrow of SCID mice transplanted with graded doses of unseparated AML cells, in one experiment. Each percentage represents one SCID mouse.

Morphological evaluation of cytospin preparations
The bone marrow of 12 mice was analyzed for morphology, 4 transplanted with unseparated AML cells, 4 transplanted with the CD34+ fraction and 4 transplanted with the CD34- fraction. In addition the bone marrow of one secondary recipient in the retransplantation experiment was analyzed. In all cases human cells in SCID recipients with AML growth appeared as large mononuclear cells with a large nucleus containing one or two nucleoli among normal mouse BM cells. The scant cytoplasm contained a limited number of azurophilic granulae. The large cells were absent from normal mouse BM and the morphology was similar to the initial patient sample. Morphological differences were not apparent between AML cells recovered from mice transplanted with the CD34+ fraction, the CD34- fraction or unseparated AML.

Fig 1 Preparation of CD34+ and CD34- fractions

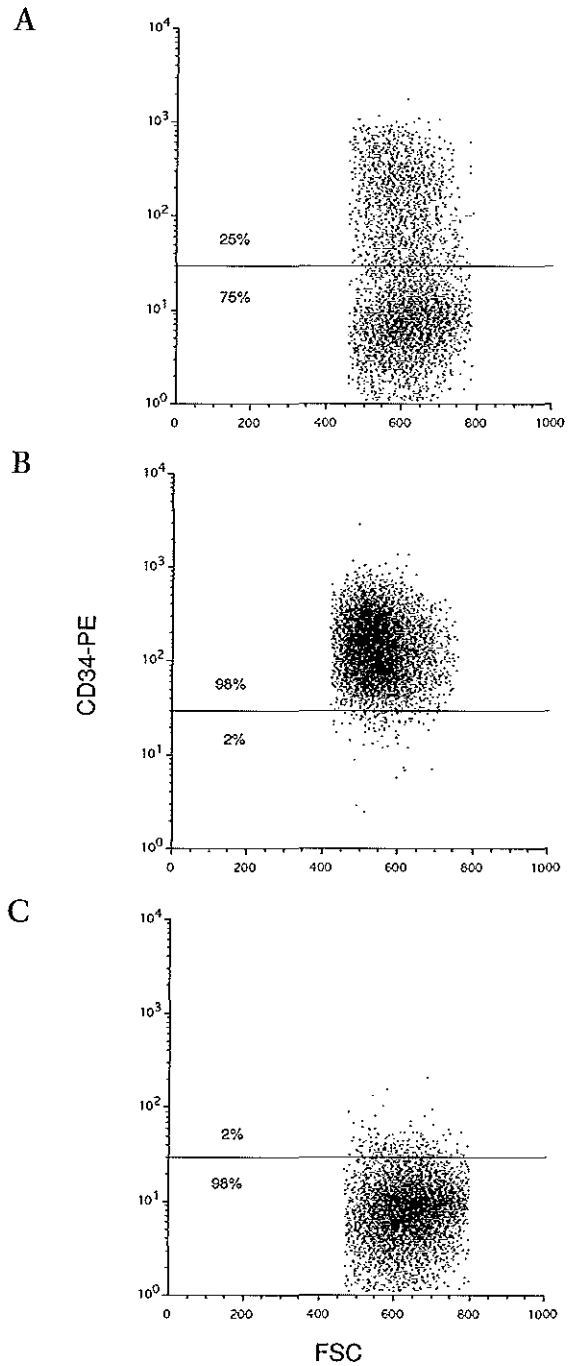


Fig 1
Dot plot of forward light scatter vs. CD34 PE fluorescense intensity of unseparated AML cells (Ia), and the CD34+ (Ib) and CD34- (Ic) fractions of AML after cell separation using Dynal beads.

Table III AML-CFU in CD34 positive and CD34 negative cell fractions

	AML-CFU per 10^5 cells					
	AML	unseparated	CD34-	fraction	C34+	fraction
Growth factor added						
none	0	0	0	0	0	0
GM-CSF	0	0	0	0	0	0
GM-CSF + IL-3	858	630	923	628	0	0
IL-3	559	526	819	631	0	0
SCF + IL-3 + GM-CSF	1014	793	1066	774	10	0

Table III AML colony (AML-CFU) formation of unseparated AML cells, the CD34- fraction or the CD34+ fraction in the presence of the indicated hematopoetic growth factors. The data were collected from two separate experiments.

Colony culture assay of AML cell fractions

Almost all AML-CFU were recovered in the CD34- fraction and minimal numbers the CD34+ fraction (Table 3). Cultures were performed with fractions obtained in two separate experiments. No colonies were formed in the absence of growth factors. Uptake of tritiated thymidine in serum free culture (21) was not activated in the absence of growth factors (data not shown).

Colony culture assay of AML cells recovered from SCID recipients

The bone marrow of the SCID mice was obtained at variable times after transplantation and inoculated in a colony assay to assess numbers of AML-CFU. AML-CFU were recovered from the SCID mice following transplantation until as late as day 106 (Table 4). AML-CFU were derived from the bone marrow of these recipients of unseparated AML cells, the CD34+ as well as the CD34- fraction. Colony formation was not apparent in cultures without growth factors.

Long Term Bone Marrow Culture (LTBMC)

CD34- cells in the LTBMC flasks produced many non-adherent cells and AML-CFU (Fig 3a and 3b). In contrast, very low numbers of AML-CFU and NC were produced by CD34+ cells. AML-CFU or nucleated cell production by the CD34- fraction or unseparated AML cells did not decline for the duration of the assay.

Fig 2

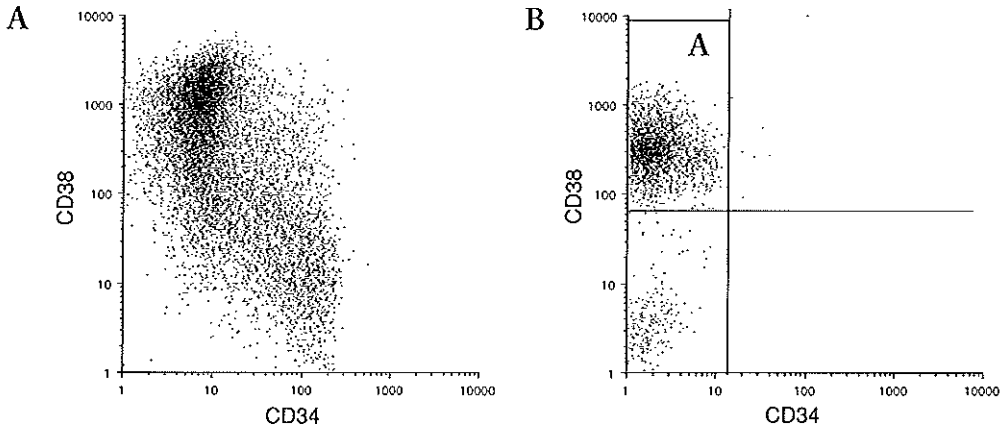


Fig 2
Dot plots of CD34-FITC fluorescence vs. CD38-PE fluorescence of unseparated AML cells (IIA) and of human AML cells recovered from SCID mouse bone marrow of a mouse that had received a CD34+ graft (IIB). Human AML cells in panel A contained more than 90% of the mouse BM as determined on CD33-FITC/CD45-PE stained sample. Panel A was used as a window for fluorescence activated cell sorting.

Table IV AML colony formation of cells recovered from SCID mice.

	TRANSPLANT (AML-CFU per 10^5 human cells)		
	AML unseparated	CD34- fraction	C34+ fraction
Days after transplantation			
0	793	774	0
35	132		
85		4	6 37 5
106	2 78	173	61 126

Table IV AML colony (AML-CFU) per 10^5 human cells formation in a short term colony assay of AML cells recovered from mice that had initially been transplanted with unseparated AML cells, the CD34- fraction or the CD34+ fraction in the presence of IL-3, GM-CSF and SCF. The number of colonies is expressed per 10^5 sorted human AML cells. Colonies were not observed in the absence of growth factors or in BM of untransplanted mice (data not shown).

Fig 3

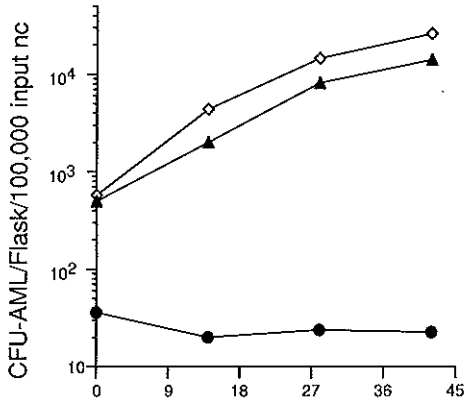


Fig 3a
Production of CFU-AML in LTBMC flasks per 10^5NC input of unseparated AML cells, the CD34+ fraction and the CD34- fraction of AML cells.

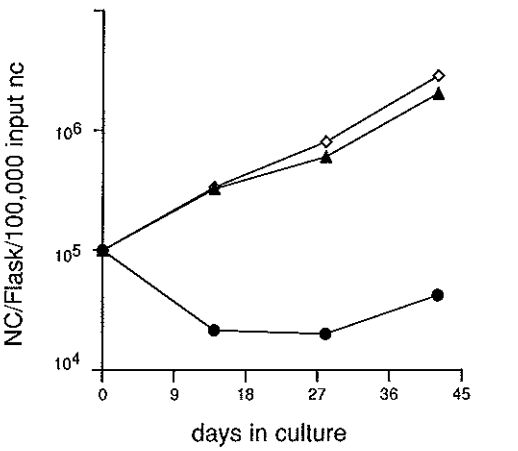
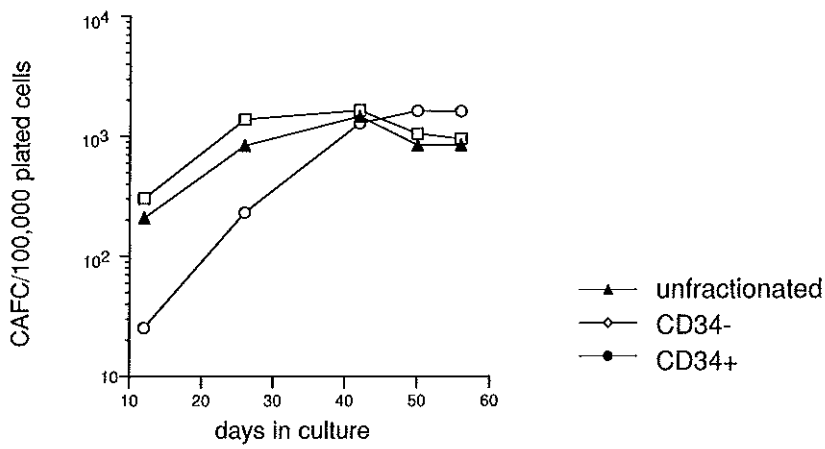


Fig 3b
Production of nucleated cells in LTBMC flask per 10^5 NC input of unseparated AML, the CD34+ fraction and the CD34- fraction of AML cells.

Fig 4
Cobblestone area forming cell frequencies of unseparated AML cells and the CD34+ and CD34- fractions.



Cobblestone Area Forming Cell assay (CAFC assay)
Frequency analysis of early and late CAs produced by the CD34- fraction, the CD34+ fraction or unseparated AML cells (Fig 4) revealed that nearly all early CAs were produced by the CD34- fraction. The CD34+ fraction produced 25 day 12 CAs/10^5 input NC (SEM 3.5), unseparated AML cells produced 303 day 12 CAs (SEM 44,7), 25% of unseparated AML cells were CD34+. Thus, 98% of early CAs of unseparated AML cells were produced by the CD34- fraction. However, late CAs appeared in similar frequencies following seeding of both fractions in culture. CAs from the CD34- and CD34+ fraction were maintained until at least day 56.

DISCUSSION

Here we present an analysis of the progenitor cell compartment of a single case of human AML employing a variety of progenitor cell assays. Data from the SCID mouse model show that the CD34- fraction of AML cells had the same capacity to initiate and maintain long term growth of AML in vivo as had the CD34+ fraction. The CD34-fraction was capable of maintaining AML growth for more than three months and was also able to initiate renewed proliferation of AML after transfer to a second recipient.
The outgrowth of AML following transplantation of CD34- grafts cannot be attributed to contaminating CD34 positive cells. The cell dose titration revealed that more than 0.3 x 10^6 of unseparated AML cells (i.e. more than 8 x 10^4 CD34+ cells) are required to obtain measurable AML growth. For extensive bone marrow infiltration (>20%) at day 35 more than 3.3 x 10^6 of unseparated AML cells (i.e. more than 83 x 10^4 CD34+ cells) are required (Table 2). Contamination of CD34- grafts by CD34+ cells was far below these levels, as the CD34- fractions were 98% pure.
The leukemic origin of the cells expanding in the SCID mice was confirmed by morphologic examination and by the leukemic immunophenotype. In addition, proliferation of human hematopoietic cells was observed in the absence of exogenous hematopoetic growth factors. Under these circumstances normal bone marrow does not expand in SCID mice (22).
Other investigators have reported one case of AML in which long term growth capacity was restricted to the CD34+/CD38- fraction (10). In the latter case the immunophenotype of the repopulating AML cells was identical to that of the fraction of normal bone marrow that contains hematopoietic stem cells. However, our data demonstrate that the expression of CD34 and long-term leukemia initiating capacity do not necessarily correlate and can be separated as independent characteristics of AML progenitors. The difference between these and previously published data is probably due to cellular heterogeneity of AML. This heterogeneity may become apparent when additional cases of AML are examined. As yet only few

cases of AML have been investigated as regards the distribution of AML cells with long term capacity over phenotypically defined AML compartments.
Cases of AML proliferating in SCID mice without exogenous growth factor supply have been described by others (10,23). AML cells recovered from the SCID mice had retained an absolute dependence on hematopoietic growth factors as regards the colony forming abilities in vitro. Therefore there was no in vivo selection of an AML subclone in the SCID mice that is independent on IL-3. Interaction with the bone marrow microenvironment or stimulation by mouse cytokines offer more likely explanations for the proliferative outgrowth of AML in SCID recipients.

In contrast to the similarities between the CD34+ and the CD34- fractions with respect to in vivo growth, clear differences were demonstrated in vitro. AML-CFU were almost entirely contained in the CD34- fraction (Table 3). Both the CD34+ and the CD34- fractions initiated long term growth. Consistently, early CA production, an index of late progenitors, was mainly seen following seeding of CD34 negative cells in the CAFC assay. Thus, the presence of AML-CFU or early CAFC in a particular fraction of AML cells does not predict for long term growth of AML. Similarly, the production of NC and AML-CFU in LTBMC was mainly observed in the CD34- fraction (Fig. 3). The latter two parameters therefore do not correlate with long term growth of AML either.
The only in vitro property shared by the CD34+ and CD34- fractions was the presence of late CAFCs (Fig 4). In normal hematopoiesis late CAFCs are generally regarded as more primitive normal hematopoietic cells. Using chimeric mouse models, late CAFCs (week 4-8) have been shown to be correlate with normal stem cells that induce stable multi-lineage chimerism in vivo. In contrast early CAFC (day 10) frequencies accurately reflect those of day 10 spleen colony-forming cells, which express transient repopulating ability in mice (24). For human hematopoiesis, it has been demonstrated that late CAFCs are predominantly contained in the CD34+, Rhodamine123dull, HLA-DRlow fraction which is in agreement with previously reported long term culture initiating cell characteristics (11). Similar to the late CAFCs of normal hematopoiesis, late CAFCs of the case of AML investigated here, are cells with long term growth capacity. This was demonstrated by the long term growth of leukemic late CAFCs in vitro in the CAFC assay and confirmed by the long term growth of both fractions containing late CAFCs in SCID mice. Since other clonogenic assays (AML-CFU, LTBMC, early CAFC formation in the CAFC assay) failed to correlate with long term growth capacity in vivo, we conclude that the late appearing CAFC is a highly immature cell observed in vitro in this case of AML.
The capacity to induce long term growth is generally accepted as the para-

meter of choice for immaturity. The CD34+ and CD34- fractions both contained these immature subsets. This is not observed in normal bone marrow and may be a phenomenon that characterizes the maturation abnormality of this case of AML. Only CD34- cells were recovered from SCID mice transplanted with the CD34+ or CD34- fractions, indicating that the CD34+ and CD34- precursors both generated CD34- progeny. It should be noted that more mature progenitor cells such as AML-CFU and early CAFCs were observed in the CD34- fraction only. This indicates that the majority of more mature progenitor cells becomes CD34 negative.
In some cases normal bone marrow cells overgrow leukemic cells in LTBMC (25,26). The CD34+ fraction of this case of AML could have contained normal CD34+ precursors. However, these normal precursors would have produced CFU-GM and NCs at 4 weeks of culture (23).
Thus, both CD34- and CD34+ subsets of this AML sample contained immature progenitors with the capacity to initiate long term AML growth as identified in vivo as well as in vitro, indicating asynchrony between functional and immunophenotypical maturation of AML progenitor cell compartments. The capacity of AML subpopulations to initiate and maintain leukemia in vivo cannot be determined on the basis of the expression of CD34 or the production of AML-CFU.

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CHAPTER 5

FLUOROURACIL SELECTIVELY SPARES AML CELLS WITH LONG-TERM GROWTH ABILITIES IN IMMUNODEFICIENT MICE AND IN CULTURE.

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ABSTRACT

A subset of leukemic cells is assumed to maintain long-term growth of acute myeloid leukemia (AML) in vivo. Characterization of these AML progenitor cells may further define growth properties of human leukemia.
In vitro incubations with 5-fluorouracil (5-FU) have been used for enrichment of normal primitive hematopoietic stem cells. By analogy to normal hematopoiesis it was hypothesized that primitive leukemic stem cells might be kinetically more inactive than colony forming cells (AML-CFU). To examine this hypothesis, conditions were established for incubation with 5-FU that eliminated all AML-CFU. These conditions selected a 5-FU resistant AML fraction that was evaluated for its capacity for long-term growth by transplantation into mice with severe combined immuno-deficiency (SCID) and long-term culture in the quantitative cobblestone area forming cell (CAFC) assay.
Transplantation of the 5-FU resistant fraction of four cases of AML into SCID mice resulted in growth of AML. Whereas no AML-CFU survived, 31-82% of primitive (week 6) CAFC were recovered from the 5-FU treated cells. Hematopoietic cells proliferating in the CAFC assay were shown to be leukemic by cytological, cytogenetic or molecular analysis. The reduction of AML growth as determined by outgrowth of AML in SCID mice was in the same order of magnitude as the primitive (week 6) CAFC reduction. This indicates that both assays measure closely related cell populations and it that the CAFC assay can be used to study long-term growth of AML. These results demonstrate a hierarchy of AML cells that includes 5-FU resistant progenitors. These cells are characterized as primitive (week 6) CAFC and as leukemia initiating cells in SCID mice.

INTRODUCTION

It is currently assumed that a subset of leukemic cells maintains long-term growth of acute myeloid leukemia (AML) in vivo. Investigation of this subset may be useful for understanding outgrowth and relapse of AML. This would require the isolation and functional characterization of the subpopulation of leukemic cells with the capacity to maintain AML. (1)
Isolation of precursors from normal bone marrow has required the combination of functional assays and purification strategies. One particular purification strategy is based on incubation with 5-fluorouracil (5-FU). 5-FU is toxic to cells in S-phase and this antimetabolite interferes with mRNA splicing in metabolically active cells. (2) 5-FU resistance is a property of murine hematopoietic cells with long-term in vivo repopulating abilities (LTRA) and long-term abilities in vitro, indicating that these cells are mainly in a

kinetically quiescent state (G0-phase). (3-7) Early human hematopoietic progenitors showed similar resistance to 5-FU. (8-10) Prolonged incubation of unseparated human hematopoietic cells with 5-FU selects for quiescent pluripotent cells with an immature phenotype. (11)

For identification of AML progenitors clonogenic assays in semi-solid media have been used. This resulted in identification of the AML colony forming unit (AML-CFU). Thymidine suicide studies showed that a high proportion of AML-CFU are synthesizing DNA. (12) It is likely that AML-CFU may be eliminated by concentrations of 5-FU, that allow for the survival of quiescent subsets of primitive leukemic cells as is the case in normal hematopoiesis.

We sought to identify AML cells resistant to 5-FU. In analogy to normal bone marrow such AML cells may constitute a primitive subset of AML, that is enriched for AML cells with long-term growth abilities. We selected conditions for incubation with 5-FU that eliminated all AML-CFU. The capacity of the 5-FU resistant AML fraction to initiate leukemia in vivo was investigated by transplantation into mice with a severe combined immunodeficiency (SCID). In addition, the long-term in vitro abilities were investigated in the cobblestone area forming cell assay (CAFC assay), a limiting-dilution type long-term culture system for hematopoietic progenitor cells. (7,8,13,14)

MATERIALS AND METHODS

Acute myeloid leukemia cells

Peripheral blood (PB) samples were obtained following informed consent from four patients presenting with AML diagnosed according to the criteria of the French-American-British Committee (FAB). (15) AML cases were selected for their abilities to proliferate in SCID mice and the presence of a leukemia specific marker. AML #1 had a normal karyogram. The leukemic growth characteristics in the CAFC assay and SCID mice of AML #1 have been described before. (16) AML case #2 showed 47XX,+8, del7(q22-q36) as the cytogenetic markers. AML #3 had normal cytogenetics but carried a point mutation in the G-CSF receptor (G-CSF-R) gene. (17) AML #4 had a translocation (9;22) (Table 1). Nucleated cells were isolated as a buffy coat. The cells were cryopreserved in 10 % dimethylsulfoxide, 20% heat-inactivated fetal calf serum (FCS) and Iscove's modified Dulbecco's medium (IMDM, Gibco, Breda, The Netherlands). After thawing by stepwise dilution (18), the viability as assessed by trypan blue staining ranged from 72-92%.

Incubation of AML cells with 5-Fluorouracil
5-Fluorouracil (5-FU; Sigma, St Louis, MO, USA) was dissolved in phosphate buffered saline (PBS). AML cells ($2x10^6$/L) were incubated in 175 cm^2 polystyrene tissue culture flasks (Becton Dickinson, NJ) in IMDM (Gibco) and 10% FCS with 5-FU at 0, 15, 25, 40 and 80µg/mL for 24 hours at 37°C in an atmosphere of humidified 5% CO2. Equivalent proportions of the flasks based on input values were used in the various assays (CFU-AML, CAFC, SCID mouse transplantations), without corrections for cell loss or viability in order to assess true variations in the recovery of specific subsets of cells.

Serum free colony culture assay
Cells (the equivalent of 4x 10^4 input cells) were plated in 35-mm dishes (Becton Dickinson, San Jose, CA, USA) in 1mL IMDM (Gibco) containing 0.9% methylcellulose, 1.5% bovine serum albumin (BSA, Sigma, St Louis, MO), insulin (0.025U/mL), linoleic acid (2.8µg/mL), cholesterol (7.8µg/mL), sodium selenite (0.18ng/mL), β-mercaptoethanol (3.5µl/mL), and human transferrin (0.62mg/ mL). Assays were performed in triplicate in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Behringwerke AG, Marburg, Germany, 5ng/mL), interleukin-3 (IL-3) (Sandoz BV, Basel, Switzerland, 10ng/mL) and stem-cell factor (SCF) (Immunex, Seattle, WA 200ng/mL). Colonies were scored after 14 days of incubation at 37°C in a humidified atmosfere and 5% CO_2 .

SCID mice and transplantation of AML
Female specific pathogen-free CB17 scid/scid mice (5-8 weeks of age) were purchased (Harlan CPB, Austerlitz, The Netherlands) and housed under specific pathogen-free conditions in a laminar air flow unit. The mice were supplied with sterile food and acidified drinking water with 100mg/L ciprofloxacine (Bayer AG, Leverkusen, Germany). The plasma Ig level of these mice was determined with an ELISA using a sheep anti-mouse antibody reacting with mouse IgG and IgM (Boehringer Mannheim Biochemica, Penzberg, Germany) and animals with plasma Ig levels over 40µg/ml were excluded. To enhance outgrowth of human hematopoietic cells in vivo macrophage depletion was performed with the use 0.2mL Cl_2MDP liposomes intravenously on the day before transplantation as described. (19-21) Total body irradiation (TBI) at 3.5Gy (dose rate: 0.95Gy/minute) was delivered by a ^{137}Cs source (Gammacell, Atomic Energy of Canada, Ottawa, Canada) adapted for the irradiation of mice. The graft size was the equivalent of $10x10^6$ AML input cells for AML #1 and #2, the equivalent of $20x10^6$ AML input cells for AML #3 and the equivalent of $30x10^6$ AML input cells for AML #4. The transplants, suspended in 300µL Hanks balanced salt solution (HBSS) (Gibco, Breda, The Netherlands) and 0.1%BSA

(Sigma), were injected intravenously (iv) into a lateral tail vein. The first two cases of AML had been shown to proliferate in SCID mice without support of human hematopoietic growth factors and AML#3 and #4 were IL-3 dependent. The latter SCID mice received 60µg of human IL-3 (Gist Brocades, Delft, The Netherlands) in 200µL HBSS and 1%BSA (Sigma) intraperitoneally, five days a week as described. (22)

Tissue collections
SCID mice were sacrificed by CO2 inhalation followed by cervical dislocation in accordance with institutional animal research regulations. Cell suspensions were prepared from the bone marrow (BM) of both femora and used for flow cytometry and the preparation of cytospin slides. Cytospin slides were stained with May-Grünwald Giemsa and morphology was microscopically evaluated.

Flow Cytometry
The initial leukemias and BM samples from mice that had been transplanted with leukemic grafts were stained or double-stained with the following mouse monoclonal antibodies: CD34-FITC, CD34-PE, CD38-PE, CD34-FITC/IgG1-PE, CD34-FITC/CD38-PE, CD34-FITC/HLA-DR-PE, CD34-FITC/c-kit-PE, CD34-FITC/CD33-PE and finally CD45-FITC/CD33-PE. Mouse IgG1-FITC and mouse IgG1-PE conjugated antibodies and samples from non-transplanted SCID mice were used as controls. The antibodies were obtained from Becton Dickinson, San Jose, CA, USA. Fluorescence was measured using a FACSCAN flow cytometer and Lysis II software (Becton Dickinson, Immunocytometry Systems). Erythrocytes and dead cells were excluded from analysis by gating on forward and orthogonal light scatter. To determine the percentage of human cells in the mouse bone marrow cells staining with at least two different antibodies for human hematopoietic cells were counted as human cells. (23)

Fluorescent in situ hybridization (FISH) for detection of trisomy 8
We used FISH for detection of trisomy 8 in interphase nuclei of cells of AML #2 following transplantation in SCID mice and proliferation in the CAFC assay. The DNA probe was specific for the α-satellite sequences on the centromeric region of chromosome 8 (D8Z2). (24) Following denaturation and hybridization procedures the hybridized probe was detected using fluorescein isothiocyanate (FITC) labeled avidin (Vector Laboratories, Burlingame, CA). Nuclei were counterstained with propidium iodide (Sigma, St. Louis, Mo). The slides were analysed employing a fluorescense microscope (standard 14 IV FL Zeiss) equipped with a (FITC) filter combination 09 (BP 450-490, FT510, LP520; Zeiss). (25)

Table I Characteristics of the analyzed cases of AML

AML	FAB Classification	Leukemic marker*	IL-3 dependent in SCID mice#
#1	M2	none	no
#2	M1	+8, del7(q22-36)	no
#3	M1	R-G-CSF gene mutation	yes
#4	M4	t(9;22)	yes

**Leukemic marker indicates a leukemia-specific characteristic of the AML described.*
#IL-3 dependent indicates that for extensive AML proliferation in vivo SCID mice have to be injected with human IL-3 (60μg intraperitoneally, 5 days a week).
**:>90% of the AML cells staining with a specific antibody as determined by flow*

Detection of G-CSF receptor gene mutation

AML #3 is characterized by the presence of a point mutation in the G-CSF receptor (G-CSF-R) gene resulting in an aberrant G-CSF-R isoform, unique to this case of AML. (17) PCR of the G-CSF-R gene was performed as described with the forward primer FWI16 5'-ACCCTTTGTGTTCCAC-CAGT-3' (in intron 16) and GR RV1 3'-GGTATTCGGGTACTGATTCTA-GATG-5' (nucleotide 2745-2769) followed by digestion with *Kpn*1, which selectively degrades the PCR product of the mutated G-CSF receptor gene.

Cobblestone-Area-Forming Cell Assay

The cobblestone area forming cell (CAFC) assay was performed as described.(8) Briefly, confluent stromal layers of FMBD-1 cells in 96-wells plates were overlaid with AML cells in a limiting dilution method. The cells were cultured in IMDM supplemented with 20% of a selected horse serum (HS) batch and hydrocortisone 21-hemisuccinate (10^{-6}M final concentration). IL-3 (12.5ng/mL) and G-CSF (20ng/mL) were added weekly to the cultures. Input values were the equivalent of 50.000 nucleated cells (NC) per well. Twelve dilutions two-fold apart were used for each sample with 15 replicate wells per dilution. The percentages of wells showing at least one phase-dark hematopoietic clone of at least five cells (Cobblestone area, CA) beneath the stromal layer were determined at 2 week intervals and CAFC frequencies were calculated using Poisson statistics as described.(13)
In normal hematopoiesis stroma dependent clones that are observed late in culture are indicators of primitive long-term repopulating stem cells in vivo, while early appearing clones are tentative indictators of transiently repopulating progenitors.(7,8) At intervals the content of some wells was harvested for additional investigation.

Immunophenotype* (percentage of positive cells)					
CD34	CD38	HLA-DR	c-kit	CD33	CD45
24%	50%	+	-	+	+
+	30%	+	-	-	+
25%	+	+	+	+	+
40%	+	+	-	+	+

cytometry and compared to a IgG1-FITC or IgG1PE control.
- :<10% of the AML cells staining with a specific antibody as determined by flow
Abbreviations: FAB: French-American-British, R-G-CSF: G-CSF receptor
cytometry and compared to a IgG1-FITC or IgG1PE control.

RESULTS

Elimination of AML-CFU by exposure to 5-FU

To determine a concentration of 5-FU eliminating all CFU-AML a dose titration ranging from 0-80µg/mL of 5-FU was carried out. A 24 hours incubation at a concentration of 25µg/mL of 5-FU reduced the number of AML-CFU to less than 2% as compared to the colony numbers in the absence of 5-FU in all cases of AML tested (data not shown). A higher concentration of 40 µg/mL of 5-FU eliminated all CFU-AML (Table 2). AML cell viability varied from 50% to 80% in 5-FU containing cultures, which compared to 65% to 80 % viability for control incubations without 5-FU. This indicated that most cells sensitive to 5-FU had not been killed within 24 hours. In subsequent experiments (SCID mouse transplantations, CAFC assay) AML cells were incubated at 40µg/mL of 5-FU to examine the 5-FU resistant fraction.

Outgrowth of AML in SCID mice

The 5-FU resistant fractions of AML cases #1 thru #4 were transplanted into SCID mice in individual experiments. Groups of 3-5 SCID mice were killed at 35 to 47 days after transplantation. Whereas the 5-FU resistant fractions of AML did not contain measurable numbers of AML-CFU, in all instances these fractions had the capacity to initiate leukemia in SCID mice (Table 2). The levels of AML infiltration by the 5-FU resistant fraction in the marrow of SCID recipients were less than those of control transplantations. By comparison of these values of infiltration and those in a cell dose titration of two cases of AML the 5-FU fraction appeared to be equivalent to an approximate threefold reduction of the unseparated graft (Table 3).

Table II Growth characteristics of 5-FU exposed and control AML cell

AML case	AML cell population*	% AML mouse BM ±SD# (n of mice)	AML-CFU (/10^5)
1	untreated	91±5 (5)	2461
	5-FU exposed	44±21 (5)	0
	5-FU resistant (%)#		0
2	untreated	19±18 (5)	4
	5-FU exposed	9±15 (5)	0
	5-FU resistant (%)		0
3	untreated	63±17 (4)	71
	5-FU exposed	20±6 (4)	0
	5-FU resistant (%)		0
4	untreated	10±13 (3)	271
	5-FU exposed	3±5 (3)	0
	5-FU resistant (%)		0

Human AML cells proliferate in the SCID mouse bone marrow

Cells recovered from the SCID mouse bone marrow were characterized as AML cells. Flow cytometry was performed with the same panel of monoclonal antibodies as was used for the initial immunophenotyping of the graft. It was shown that the immunophenotypes of cells recovered from the SCID mouse bone marrow were identical to the AML immunophenotypes of the respective grafts with the following exceptions: CD34 positive/CD38 negative cells from AML case #1 had converted to a CD34 negative/CD38 positive phenotype as described earlier(16) and CD34 positive cells from case #3 had converted to a CD34 negative phenotype. Cytospin slides prepared from the bone marrow of mice transplanted with AML #1 thru #4 revealed evident blast cells. The morphological identification of leukemic blasts was relatively easy due to the considerably larger size of AML blasts as compared to normal mouse BM cells. The percentages of AML blasts counted on the cytospin slides were similar to those determined by flow-cytometry (data not shown). FISH performed on bone marrow cells proliferating in SCID mice transplanted with AML #2 confirmed the trisomy 8 in the bone marrow of all 3 mice examined. More than 90% of the cells staining with an anti-centromere probe for chromosome 8 (human cells) showed 3 fluorescent dots.

Cobblestone area forming cell assay (CAFC assay)

In parallel to the SCID mouse transplantations long-term growth of the 5-

populations in SCID mice and in vitro

week 2 CAFC (/10^5±SEM)	week 6 CAFC (/10^5±SEM)
10±4	132±28
1.7±0.5	52±8
17	39
1.9±0.5	11±3.4
<0.2*	3.4±1.0
<11	31
6.2±1	5.2±1.46
0.9±0.4	3.1±1.13
15	60
38±0.4	3.8±0.6
1.6±0.4	3.1±0.6
4	82

*Table II: # AML cells were incubated for 24 hours in IMDM and 10% FCS, with or without 40µg/ml of 5-FU. The percentage of human cells proliferating in the SCID mouse bone marrow was determined by flow-cytometry # The percentage of 5-FU resistant CFU-AML, week 2 CAFC and primitive week 6 CAFC were derived from the quotient of the values of the 5-FU exposed fraction and untreated AML cells. *value below the detection limit of the assay.*
Abbreviations: BM: bone marrow, n: number of mice in experiment, SD: standard deviation, SEM: standard error of the mean, CFU-AML: colony forming unit AML, CAFC: cobblestone area forming cell.

FU resistant fractions was investigated in the quantitative long-term bone marrow culture system, i.e. the CAFC assay. Frequency analysis of hematopoietic precursor cells in vitro showed in the number of week 2 CAFC in the 5-FU-exposed fraction a reduction towards 4-17% of the unexposed AML cells among the four AML specimens examined. In contrast, more primitive (week 6) CAFC were reduced to 31-82% only.
After 6-10 weeks of culture, cells were recovered from a series of wells used for the CAFC assay and cytospin preparations were made. Morphological examination of the hematopoietic cells harvested from the CAFC assay of AML #1 revealed 92% blast cells among the hematopoietic cells proliferating in the CAFC assay, more mature myeloid cells were nearly absent. In a CAFC assay of normal peripheral blood stem cells always less than 15% myeloblasts were counted at week 6, and all morphological maturation stages of the neutrophil series were represented. FISH analysis of case #2 revealed the trisomy 8 in 50% of the 52 evaluable cells staining with the anti-centromere probe (Data not shown). Morphologically 75% of all hematopoietic cells in the CAFC assay were myeloblasts. In AML #3 cells harvested from the CAFC assay the presence of the specific mutant G-CSF-R gene was demonstrated using PCR. (Figure 1). Morphological examination of the cells proliferating in the CAFC assay showed 83% myeloblasts. AML # 4 cells were harvested from the CAFC assay, however contamination with yeast cells interfered with further investigation.

Table III Cell dose titration of AML cells transplanted into SCID mice

Cell dose (X 10^6 per mouse)	%AML cells mouse BM	
	AML #1	AML #2
30	8	75
	91	90
	79	90
	37	
10	28	88
	60	61
	6	8
3.3	3	13
	9	4
1	2*	1
	19*	1
	3*	2
0.3	1*	
	0*	
	0*	

AML cell proliferation in the bone marrow of SCID mice transplanted with graded doses of AML cells, in one experiment. Each percentage represents one SCID mouse, evaluated on day 35. evaluated between days 44-81.*

DISCUSSION

We identified a subset of AML cells devoid of AML-CFU and resistant to 5-FU. This subset initiates AML in SCID mice and contains 31-82% of the AML cells with the ability to initiate long-term growth in the CAFC assay (Table 2). The 5-FU sensitive fraction of normal hematopoietic cells produces a high number of colony forming units (CFU-C) and week 2 CAFC in culture, whereas the 5-FU resistant subpopulation contains the great majority of primitive (week 6) CAFC (8) and primitive normal hematopoietic cells. (4-6,9-11) The cell population that we have identified here is enriched for immature leukemic precursors and shares these characteristics with its normal analogue. This cell population may be identical to or closely resemble the leukemic stem cell population. These observations suggest a hierarchy of AML cells similar to that in normal hematopoiesis: a hierarchy from kinetically quiescent immature progenitors identified as primitive (week 6)

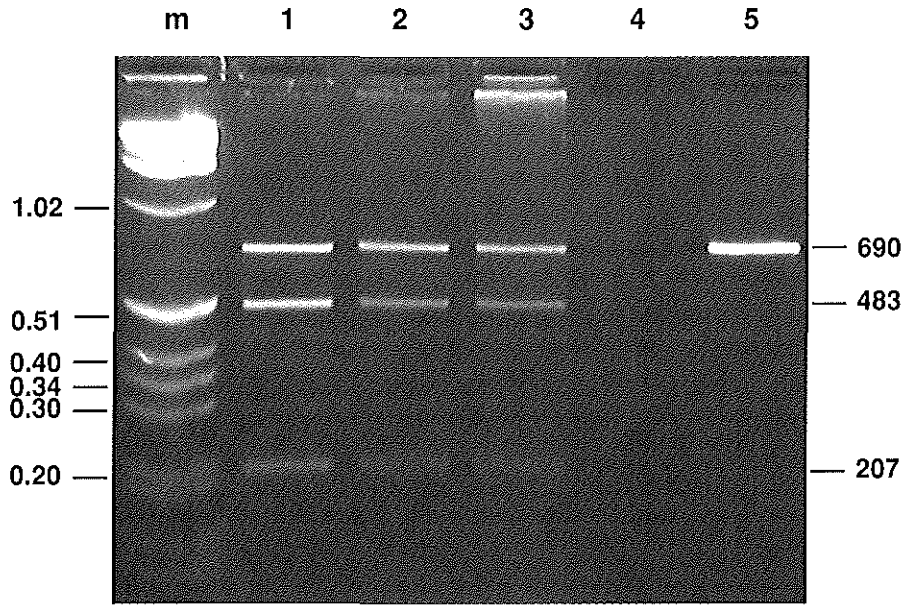

Figure 1 *Presence of a leukemia specific point mutation in the G-CSF receptor gene in cells proliferating in the CAFC assay of AML #3.*
Agarose gel electrophoresis of PCR fragments after KpnI digestion showing a thawed sample of AML#3 (lane 1), 2 samples of the CAFC assay of AML #3 harvested after 5 and 7 weeks of culture (lanes 2 and and 3 respectively). As controls a sample of the stromal cell line FMBD-1 used for stromal culture (lane 4) and a sample of AML #2 (lane 5) were included. Abbreviation M: marker lane (kb)

CAFC and the cells initiating leukemia in SCID mice towards kinetically active mature progenitors such as week 2 CAFC and AML-CFU.
The reduction of AML growth as determined by outgrowth of AML in SCID mice and by the number of week 6 leukemic CAFC was in the same order of magnitude. The hematopoietic cells proliferating in the CAFC assay could be shown to be leukemic by cytological, cytogenetic or molecular analysis, although the presence of some normal progenitors could not be fully excluded. These data indicate that both assays measure closely related cell populations and that the CAFC assay can be used for study of long-term growth of AML. The issue whether the differences observed between the percentage of 5-FU resistant week 6 CAFC and 5-FU resistant leukemia initiating cells in SCID mice are significant requires further investigation.
AML-CFU phenotypes display considerable heterogeneity among patients. (26,27) Nevertheless, a generally more immature immunophenotype is expressed by AML-CFU as compared to the unseparated AML cell population, and differentiation of AML-CFU in vitro has been observed. (26-30) AML-CFU recovered from methylcellulose cultures can be replated and produce secondary colonies.(1) The capacities for (limited) differentiation and

self renewal are consistent with the role of the AML-CFU as a progenitor cell. The data presented here show that the AML-CFU is a relatively mature progenitor and functionally distinguishable from most cells initiating long-term growth of AML. However a definitive determination of any long-term potential of AML-CFU would require isolation of this subset.
Another approach towards the isolation of primitive leukemic progenitor cells is based on the immunophenotypical similarities between normal and leukemic progenitors. A fraction of normal bone marrow enriched for repopulating stem cells has been identified as CD34+, CD33-, CD38-, HLA DR-, Thy-1low, negative for specific lineage antigens and low in rhodamine-123 uptake. (31-36) In AML a CD34+/CD38- fraction is present in approximately one third of the cases. (17) Cells initiating outgrowth of AML in immunocompromised mice were recovered exclusively from the CD34+/CD38- fraction in at least three cases of AML. (38,39) These data may not apply to each and every case of AML as we identified one case with identical abilities to initiate leukemia in SCID mice of both the CD34+ and the CD34- fractions. (16) The heterogeneity of immunophenotype among cases of AML and the apparent lack of functional significance of expression of CD34 in AML may interfere with this approach to identify leukemic progenitors by immunophenotype.
Our data indicate that selection of AML subsets based on functional characteristics such as cytokinetic activity might offer a more generally applicable approach to the investigation of leukemic progenitors.

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CHAPTER 6

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

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ABSTRACT

We studied the cell kill induced by GM-CSF fused to Diphtheria Toxin (DT-GM-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-GM-CSF for 48 hours, and AML cell kill was determined in a proliferation assay, a clonogenic assay (AML-CFU) 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-GM-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-GM-CSF. Cell kill induced by DT-GM-CSF could be prevented by coincubation with an excess of GM-CSF, demonstrating that sensitivity to DT-GM-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-GM-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-GM-CSF (100 ng/ml, 48 hours). These studies show that DT-GM-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 [1,2). 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 (1,3-5). 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 inter-

nalisation by receptor-mediated endocytosis. This is followed by processing of the DT into its active form and delivery of the NH2-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-GM-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 GM-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-GM-CSF to AML cells with long term repopulating capacity.

Applicability of DT-GM-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-GM-CSF.

Here we report on the efficacy of DT-GM-CSF for elimination of AML cells with long-term repopulating abilities in vivo, using transplantation of DT-GM-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 (20, 24). The toxicity of DT-GM-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 - 91 %.

Incubations with DT-GM-CSF
The construction and purification of DT-GM-CSF has previously been described (17). Normal bone marrow (nBM) cells from healthy donors and AML cells were exposed to DT-GM-CSF (100 ng/ml) in serum free medium (SFM) (27) at a density of 2.5 x10^6 cells/ml at 37°C in a humidified 5% CO2 atmosphere for 48 hours. Control incubations in SFM without DT-GM-CSF and the incubation with a equimolar concentration of human GM-CSF (GM-CSF; a gift from Sandoz BV, Basel, Switzerland) were performed simultaneously. For competition experiments an excess of GM-CSF (2 µg/ml) was added to the cultures containing DT-GM-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.

3H-Thymidine (3H-TdR) incorporation assay
Cells (2 x 10^4) were cultured for 72 hours in 96-well round-bottom microtiter plates in 100 µl SFM containing DT-GM-CSF (100 ng/ml). The effects of DT-GM-CSF were tested in the absence of growth factors, and in the presence of the combination of human stem cell factor (SCF 0.1 µg/ml; a gift from Amgen Biologicals, Thousand Oaks, CA, USA), human interleukin-3 (IL-3 0.025 µg/ml; a gift from Gist Brocades, Delft, The Netherlands) and human granulocyte-colony stimulating factor (G-CSF 0.1 µg/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-GM-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 diphospho-

nate (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 30 x 10^6 AML input cells for all three samples. The grafts, suspended in 300 µl Hanks balanced salt solution (HBSS) (GIBCO, Breda, The Netherlands), 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 CO2 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 (1.5x10-5mol/l, Merck, Darmstadt, Germany), cholesterol ($1.5x10^{-5}$mol/l, Merck), sodium selenite ($1x10^{-7}$mol/l, Merck), β-mercaptoethanol ($1x10^{-4}$mol/l, 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% CO2 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-GM-CSF to normal committed progenitor cells. DT-GM-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. Cultures were kept at 37°C and 5% CO2 in a humidified atmosphere. 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 (24,25), 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).

RESULTS

Antiproliferative effects of DT-GM-CSF in a proliferation assay
The response of AML samples to GM-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 GM-CSF, indicating the presence of functional GM-CSFR. AML cells from patient C showed no proliferative response to GM-CSF. All samples proliferated when incubated

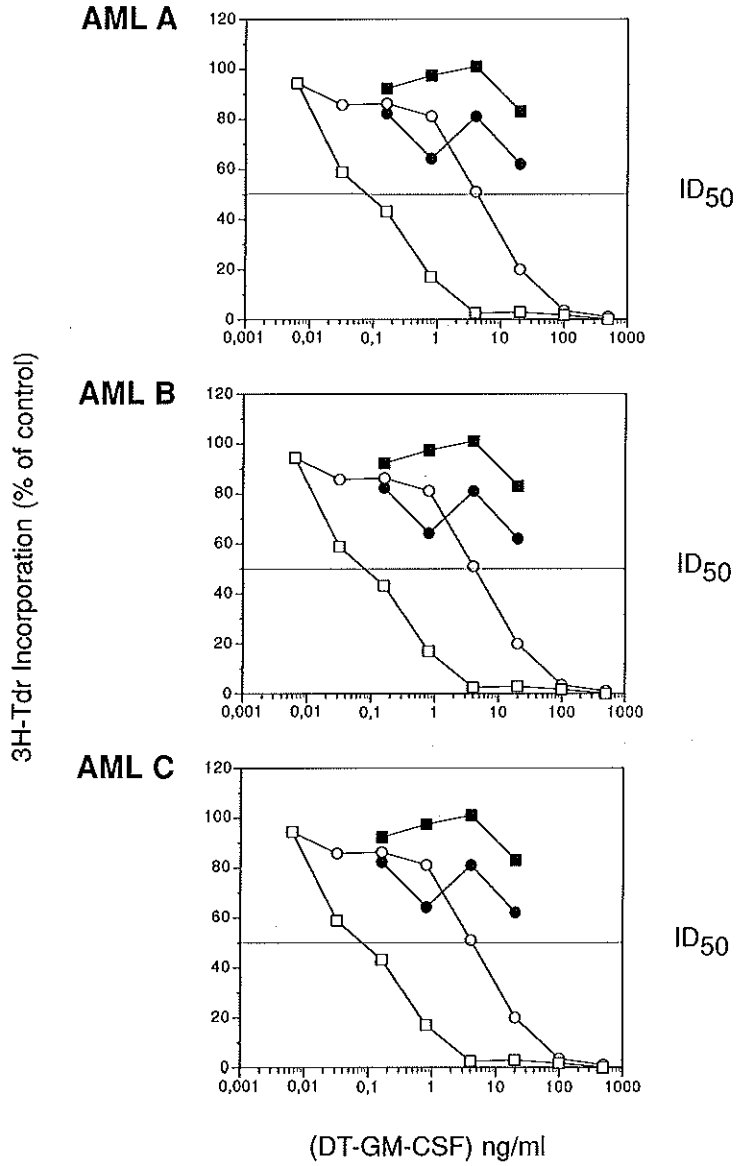

Fig 1
Proliferation inhibition of cells from AML A, B and C by varying concentrations of DT-GM-CSF as measured in the ^{3}H-TdR incorporation assay. AML cells were incubated in cytokine-free medium (□) and in SCF, IL-3 and G-CSF (○)containing medium. The effect of DT-GM-CSF was also tested in the presence of an excessof GM-CSF (2 µg/ml) simultaneously with DT-GM-CSF in cytokine free medium (■) or SCF, IL-3 and G-CSF containing medium (●). Values are given as percentages of controls

with the combination of G-CSF , IL-3 and SCF.

To determine the effect of DT-GM-CSF on unstimulated proliferation we incubated the AML cells for 72 hours at DT-GM-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 ID50, i.e. the DT-GM-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 ID50 varied from 0.04 to 5 ng/ml. AML cells from patient C were insensitive to DT-GM-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-GM-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-GM-CSF revealed that the cells surviving DT-GM-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 GM-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-GM-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.

Table I Effect of DT-GM-CSF on AML cells in the 3H-TdR uptake assay

AML	FAB	^{3}H-TdR uptake (cpm x 10^3)			ID_{50} (ng/ml)	
		ngf*	GM-CSF	SCF/IL-3 /G-CSF	autonomous growth	stimulated# growth
A	M2	1.3	8.2	33.5	0.1	5
B	M1	1.2	9.8	35.5	0.04	0.7
C	M1	1.6	2.0	31.4	>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-GM-CSF required for 50% inhibition of the autonomous proliferation or the proliferation under stimulatory conditions. * no growth factor # In the presence of SCF, IL-3 and G-CSF*

The concentration of 100 ng/ml of DT-GM-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).

Outgrowth of DT-GM-CSF exposed, GM-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-GM-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-GM-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-GM-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).

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

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

 # Mean number of percentage of human cells in SCID mouse bone marrow ± Standard deviation. Values calculated on the basis of data from five groups of transplanted 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 (data not

Fig 2

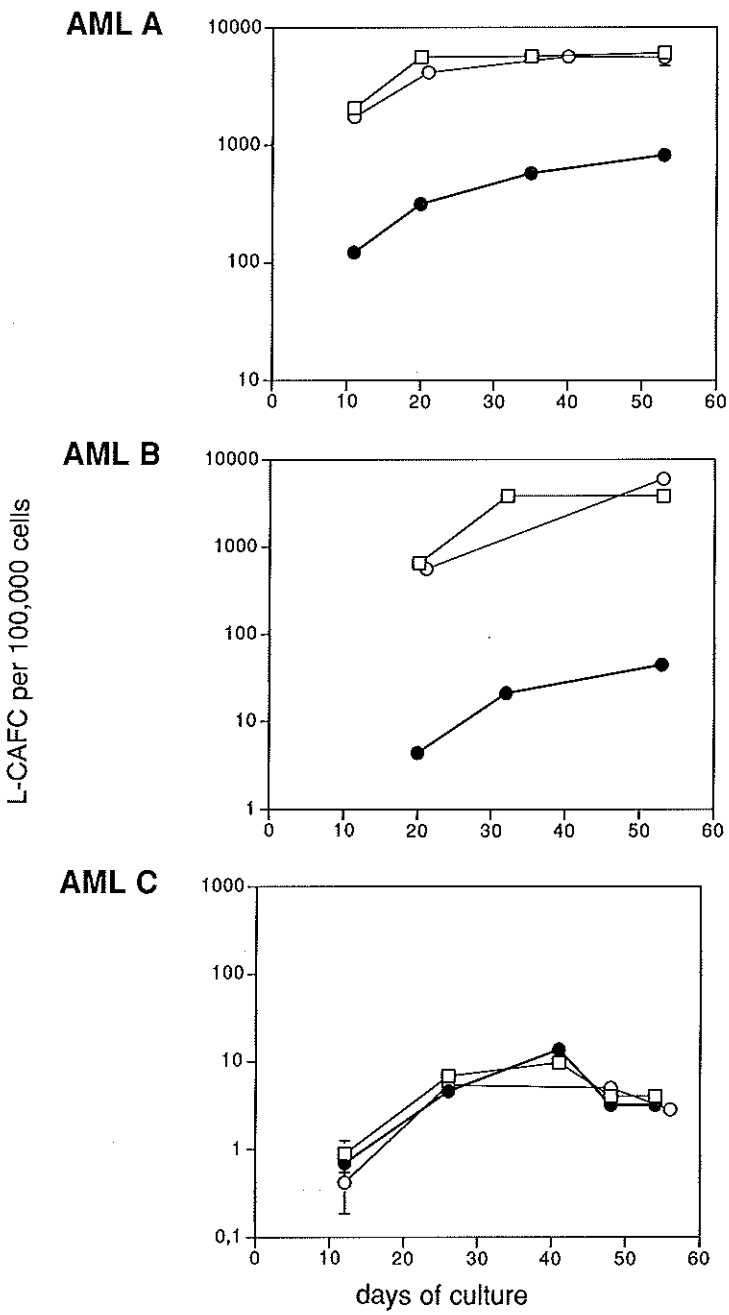

Fig 2
L-CAFC frequencies (of AML A, B and C) after incubation (48 hours) in serum free medium □, medium supplemented with DT-GM-CSF ● and medium supplemented with GM-CSF ○

shown). 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-GM-CSF exposed graft from patient A proliferated in response to GM-CSF. Renewed incubation with DT-GM-CSF reduced proliferation (data not shown). Because such AML cells could not be demonstrated in the initial graft, directly after the incubation, these data indicates that GM-CSF and DT-GM-CSF responsive cells regenerated in the mouse.

Outgrowth of DT-GM-CSF exposed AML cells in the CAFC assay
Long term growth of the DT-GM-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 (mean ±SEM) in case A and B respectively (Fig 2), 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-GM-CSF treated cell populations as compared to the control cells from patient A and B, respectively. The L-CAFC assay of the DT-GM-CSF exposed cell sample of patient C showed that week 7 L-CAFC frequencies were 3.2 ± $0.6/10^5$ as compared to 4.0 ± $0.7/10^5$ for control cells from patient C, and

Table 3 Relative effect of DT-GM-CSF exposure on normaal hemopoietic progenitor cells

	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

values calculated as the percentage of controls (±SD) from CAFC assays of normal bone marrow samples

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-GM-CSF, which is in agreement with the in vivo leukemia initiating capacity of these samples.

Coincubation of DT-GM-CSF with excess concentrations of GM-CSF
To test whether the toxicity of DT-GM-CSF is conferred by the GM-CSFR. We incubated AML cells of patient A with DT-GM-CSF in the presence of an excess amount of GM-CSF and compared the result of incubation with the effect of excess GM-CSF alone. The frequency of week 5 L-CAFC (1254 ± 295/10^5 and 1027 ± 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-GM-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-GM-CSF on AML cells as determined in 3H-TdR incorporation assay could be prevented by high concentrations of GM-CSF as well (17).

Effect of DT-GM-CSF on normal hemopoietic progenitors
To investigate the cytotoxic effect of DT-GM-CSF to normal hemopoietic progenitor cells, five samples of nBM cells were exposed to 100 ng/ml DT-GM-CSF for 48 hours in liquid culture. The relative effect on committed progenitors were determined in clonogenic assays by enumerating the number of CFU-GM and BFU-E's, and compare this with non-exposed nBM cells. 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 progenitor-subsets were unchanged after the in vitro DT-GM-CSF exposure. These data indicate that all hemopoietic progenitor cells, including the most primitive ones (week 6 CAFC), escape DT-GM-CSF induced cell kill.

DISCUSSION

Transplantation of AML cells into SCID mice may be used to determine in vivo growth of primitive AML cells (19, 21, 24,31). In this model we evaluated the sensitivity of human AML progenitor cells to in vitro treatment with DT fused to human GM-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-GM-CSF. In one of the cases (case B) AML growth in SCID mice was completely prevented by DT-GM-

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-GM-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 GM-CSF blocked the toxic action of DT-GM-CSF, indicating that leukemic stem cell reduction was mediated by the specific binding of DT-GM-CSF to the GM-CSFR, which is consistent with the specificity observed in experiments with murine GM-CSF fused to DT (12) and GM-CSF coupled to saporine (32).
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-GM-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-GM-CSF. Based on the lack of toxicity induced by the DT-GM-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-GM-CSF.
High affinity complexes of the GM-CSFR have been identified on normal endothelial cells (33). 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 (34-36), 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-GM-CSF in preclinical animal models, as we described earlier (37).
DT-GM-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-GM-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 (24) 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-GM-CSF. The optimal conditions for the use of DT-GM-CSF and its maximal efficacy have yet to be determined.
In conclusion, these results show that DT-GM-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-GM-CSF as a potential therapeutic agent in the treatment of AML.

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GENERAL DISCUSSION AND SUMMARY

AML stem cells may be defined as the subset of leukemic cells that express abilities to initiate and maintain long-term growth of AML. Until recently, human AML growth has mainly been studied in culture for limited periods of time (e.g. 1-2 weeks). Transplantation of human AML cells in various strains of mice resulted in transient and local growth. Thus, the available in vitro and in vivo systems, did not allow for study of long-term growth of AML cells. More recently, transplantation of primary human AML cells into newly developed strains of immunodeficient mice (severe combined immunodeficient (SCID) and non obese diabetic (NOD)/SCID mice has resulted in reproducible growth of AML for prolonged periods of time (see Introduction). Furthermore long-term bone marrow culture systems (e.g. the cobblestone area-forming cell assay) have become available to sustain normal and malignant hematopoiesis over periods of 2 to 4 months. These models of growth of human leukemias have provided assays for investigating long-term growth of AML and furnished insight into the characteristics of more primitive AML stem cells.

Frequency of AML stem cells

The leukemic stem cell is a rare cell. Limiting dilution experiments of AML in immunodeficient mice have shown that the frequency of the cells with the ability to initiate AML in immunodeficient mice ranges between 2 and 1,000 per 10^5 AML cells. (1,2) The frequencies of cells with the ability to produce leukemic cobblestone areas (CA) after an interval of 5-8 weeks in long term bone marrow culture vary between 4 and 2,400 per 10^5 AML cells (Chapters 4, 5 and 6). These so called late cobblestone area-forming cells are assumed to represent primitive progenitors. Thus, the frequencies of cells initiating long-term growth of leukemia in vivo and cobblestone areas in culture beyond 4 weeks are similar. This suggests that they probably represent identical or closely related cell populations. Nevertheless, these quantitative estimations are only approximations. Due to residual graft resistance of the mouse recipients and seeding efficiency of hematopoietic cells in culture these values may be underestimations. The variations observed in the frequencies of the AML stem cells among individual cases are most likely exponents of the heterogeneity of clinical AML.

Are these cells capable of maintaining AML growth indefinitely? This question remains unresolved. In one case leukemic growth could be maintained in SCID mice for as long as 100 days (Chapter 4). Leukemia could be reinitiated after transplantation of AML into secondary mouse reci-

pients. Human AML cells recovered from the recipient bone marrow contained AML progenitor cells (colony forming unit-AML (CFU-AML)) and these progenitor cells could also be maintained in the mouse for a minimum of 100 days. (Chapter 4 and references 1, 2, 3). Thus, the data show that human AML cells with proliferative abilities may survive without exhaustion in the murine bone marrow environment for extended periods of time. Additional experiments would be needed to define any time restriction of AML growth in these animals.

Immunophenotype of AML stem cells

Normal primitive hematopoietic cells may be enriched according to immunophenotype as they express CD34 brightly, but do not show CD38 or lineage-associated antigens. These $CD34^{++}/CD38^{-}$ cells are present at a frequency of 0.01% in normal bone marrow. (4) Flow-cytometric analysis of more than 90 AML samples disclosed that CD34 positive cells can be identified among the leukemic cell population in 83% of cases. $CD34^{++}/CD38^{-}$ AML cells are demonstrable in 35% of cases. This phenotypically distinct subset of cells is present at variable frequencies. (5) These data suggest that primitive AML cells might express the normal $CD34^{++}/CD38^{-}$ phenotype in many instances.

The characteristics of subsets of cells can be conveniently examined following cell separation, e.g. using immunomagnetic beads or fluorescence activated cell sorting. In chapter 4 data are presented of a case of AML that contains a $CD34^{+}/CD38^{-}$ fraction. The leukemic cells were separated into CD34 positive and CD34 negative fractions. AML growth could be initiated either by the CD34 positive or CD34 negative AML cell subsets. Following transplantations into SCID mouse recipients, both cell fractions infiltrated the mouse bone marrow to a similar degree. The leukemia derived from the CD34 negative AML cells could be maintained for at least 100 days. Leukemic cells recovered from mice that had received CD34 negative grafts contained AML progenitor cells (CFU-AML). Further, leukemic cells recovered from these primary mouse recipients initiated leukemia in secondary recipients. Finally, in vitro investigations showed that the CD34 negative fraction contained considerable numbers of cells with the ability to produce Cobblestone Areas (CA) after 8 weeks of culture. Taken together, the data indicate that the CD34 negative fraction of the leukemia expressed the abilities to initiate and maintain AML growth in vivo (>100 days) and in vitro (>8 weeks). The CD34 positive fraction of this leukemia had similar leukemia initiating abilities. Thus, in this case the primitive AML progenitors were inhomogeneous and expressed a diverse CD34 positive or a CD34 negative immunophenotype, which is in contrast to normal hematopoiesis. How do these data compare to the results previously reported by other

investigators? The phenotype of AML cells with the ability to engraft immunodeficient mice were always $CD34^+/CD38^-$ in another study. (1) In a third study analysis of the abilities of subsets defined by expression of CD34 and Thy-1 showed that the phenotype of AML cells with long-term abilities was CD34+/Thy1- in 5 of 6 cases evaluated.(6) In one case $CD34^+$ cells failed to initiate leukemia and only $CD34^-$ cells showed the ability to engraft. Thus, the $CD34^+/CD38^-$ subset of AML generally contains AML cells with long term potential. However, such AML cells occur in a minority of cases. (5) AML stem cells may express variable immunophenotypes. Apparently, expression of the CD34 antigen is not strictly associated with long-term abilities of AML.

AML progenitors more immature than CFU-AML

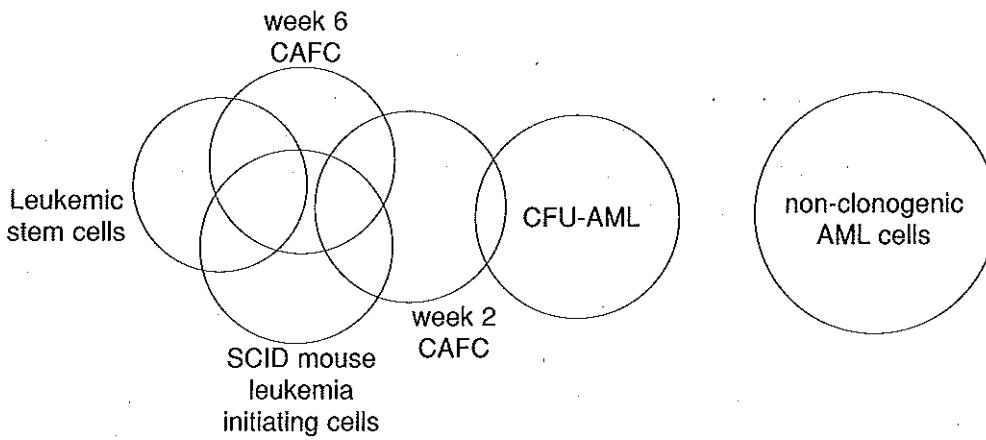
The AML progenitor cell identified as CFU-AML actively synthesizes DNA. This has been shown in thymidine suicide studies.(7) In normal bone marrow, the antimetabolite 5-fluorouracil (5-FU) preferentially eliminates hematopoietic cells that are in S-phase or have active mRNA synthesis. (8) 5-FU resistance is a property of murine hematopoietic cells with long-term in vivo repopulating abilities (LTRA) and long-term abilities in vitro, indicating that these cells are mainly in a kinetically quiescent state (G0-phase). (9-13) Most early human hematopoietic progenitors show similar resistance to 5-FU.(14,15,16) Prolonged incubation of unseparated human hematopoietic cells with 5-FU in the presence of cytokines selects for pluripotent cells in G0 phase with an immature phenotype.(17) Incubation with 5-FU may be used as a strategy to select for stem cells.

Chapter 5 summarizes experiments designed with the purpose to investigate whether the AML cell population contains AML cells more immature than the AML-CFU. Exposure of AML cells to 5-FU in vitro eliminated up to 3 log of the AML-CFU subset. However most AML cells initiating leukemia in SCID mice survived the 5-FU treatment. Similarly, a major fraction of primitive leukemic CAFC (i.e. 31-82% as assessed at week 6 in culture) retained their viability after exposure to 5-FU. Thus, apparently, the AML cell population contains a 5-FU resistant fraction of cells with long-term abilities in vivo and in vitro, a condition similar to normal hematopoiesis. Because growth of AML may be initiated with AML cell populations depleted of AML-CFU, AML-CFU are not representative of leukemic stem cells. As the week 2 CAFC have a sensitivity to 5-FU that is greater than that of week 6 CAFC and less than that of AML-CFU (Chapter 5), the maturation stage of week 2 CAFC apppears intermediate between CFU-AML and week 6 primitive CAFC.

Hierarchy of the AML cell population

The data discussed above show that AML cells constitute a hierarchically ordered cell population. This hierarchy resembles that of normal hematopoiesis in many but not all respects. SCID mouse leukemia initiating cells and week 6 CAFC measure closely related AML cell populations. Immature precursors in AML may be CD34 negative, which is in contrast to normal hematopoiesis. This appears indicative of a maturation asynchrony in certain cases of leukemia. The question at which maturation stage more exactly the AML cell loses its long-term potential requires more detailed study.

Figure 1. AML cell maturation model.



The sizes of the populations and the overlaps are arbitrary

Possible application of assays for primitive AML progenitors to the development of AML therapy.

Several investigators have correlated the in vitro sensitivity of CFU-AML to cytostatic drugs with the clinical efficacy of these drugs. However, to date these tests have unsatisfactory predictive value.(18) The deleterious effects of individual cytostatic drugs on transient and long-term growth of normal bone marrow could be predicted using results of the CAFC assay.(19, 20) In chapter 5 experiments in AML with 5-FU showed minimal cell kill of primitive AML cell progenitors and considerable killing of more mature AML cell subsets. Thus, the quantification of cytotoxic effects specifically targeted at primitive versus less primitive subsets of AML is feasible. Primitive

AML cells as an index of the repopulative potential of the neoplasm, may be the target of choice for the development of AML therapy.
A potential novel therapeutic agent was evaluated in chapter 6. Diphtheria toxin was coupled to the hematopoietic growth factor GM-CSF. The resulting fusion toxin (DT-GM-CSF) was incubated with AML cell samples and normal bone marrow. Leukemic stem cells as measured in SCID mice and in the CAFC assay were reduced by DT-GM-CSF (in 2 of the 3 cases investigated). Probably, leukemic stem cells specifically bind DT-GM-CSF via the GM-CSF receptor. Normal stem cells however, survived the incubation. The fact that application of DT-GM-CSF resulted in selective AML cell kill, supports the possibility of the clinical application of this agent. The observation that primitive leukemic stem cells express receptors for GM-CSF in itself may hold interesting possibilities for the use of hematopoietic growth factors for modulation of AML growth. The assessment of antileukemic effects specifically directed at the more primitive leukemic stem cells may hold a broader promise for the preclinical evaluation and comparison of antileukemic therapies. The SCID mouse and CAFC assays for immature AML precursors may thus be used in treatment development and perhaps as predictive assays for therapeutic effects of cytotoxic agents.

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SAMENVATTING

Acute myeloïde leukemie (AML) is een kwaadaardige ziekte van de bloedvormende cellen in het beenmerg. Zij tonen een onvermogen om uit te rijpen tot normale bloedcellen. AML cellen vallen op het eerste gezicht op door hun uiterlijke uniformiteit. Desondanks is het al geruime tijd bekend dat slechts een klein deel van alle AML cellen het vermogen bezit om nieuwe leukemische cellen aan te maken. De meeste leukemiecellen zijn niet in staat om de ziekte in stand te houden. Tot voor kort werden leukemische voorlopercellen meestal in koloniekweken onderzocht. In dit celkweek systeem ontstaan na ±2 weken kolonies van leukemische cellen. Elke kolonie stamt af van een enkele voorlopercel: de colony forming unit AML (AML-CFU). Bij analyse van de eigenschappen van voorlopercellen van normaal beenmerg is gebleken dat dergelijke kolonie-vormende cellen niet representatief zijn voor stamcellen, de beenmergcellen die het vermogen hebben om de bloedcellen langdurig in stand te houden.
In het hier beschreven onderzoek zijn twee nieuwe methoden gebruikt om de groei van AML gedurende een veel langere periode aan te houden: transplantatie van humane AML cellen in muizen met een severe combined immunodeficiency (SCID-muizen) en AML celkweek in zogenaamde lange termijn beenmergkweken (de cobblestonearea-forming cell (CAFC) assay). SCID muizen hebben een aangeboren afwijking van het immuunsysteem, de T- en B-cel functies ontbreken. Daarom zijn zij ontvankelijk voor menselijke transplantaten, zoals beenmerg. Het CAFC assay is een kweeksysteem met stromale cellen waarop menselijk beenmerg langere tijd in kweek gehouden kan worden.
In hoofdstuk 1 wordt een korte inleiding gegeven over acute leukemie, hematopoietische stamcellen en de testen die in gebruik zijn voor het meten van de diverse voorlopercellen van normaal beenmerg. Dan volgt een beschrijving van de tot dusverre bekende eigenschappen van de leukemische voorlopercel (AML-CFU), en de ervaring met SCID-muizen voor het evalueren van normale en leukemische cellen.
In de hoofdstukken 2 en 3 worden experimenten beschreven ter definiëring van optimale condities voor groei van humane AML in SCID-muizen. Onderzocht zijn de effecten van totale lichaamsbestraling van de muis, de grootte en toedieningsweg van het transplantaat en de bevordering van de uitgroei van de humane AML cellen met behulp van een hematopoietische groeifactor (interleukine-3) (hoofdstuk 2). In hoofdstuk 3 wordt de vraag gesteld of het blokkeren van de functie van macrofagen van de SCID muis de uitgroei van menselijke beenmergcellen zou kunnen vergemakkelijken. Macrofagen werden geëlimineerd door toediening van met clodronaat beladen liposomen. Uit de resultaten bleek dat een gecombineerde voorbehan-

deling van de SCID-muis met totale lichaamsbestraling en clodronaat liposomen, gevolgd door de intraveneuze transplantatie van AML, de groei van AML in de muis bevordert. Ondersteuning met de groeifactor interleukine-3 had ook een gunstig effect en was noodzakelijk in 3 van de 4 onderzochte gevallen.

In hoofdstuk 4 is de betekenis van expressie van de membraan merker CD34 op leukemische cellen onderzocht. Deze merker wordt aangetroffen op normale beenmergcellen met het vermogen tot lange termijn groei. Het vermogen om lange termijn groei van leukemie te onderhouden van CD34 positieve en van CD34 negatieve leukemiecellen werd vergeleken. Daartoe werden afzonderlijke fracties van CD34 positieve en CD34 negatieve cellen van Een leukemie in lange termijn beenmergkweek (CAFC assay) en SCID muizen onderzocht. De CD34 negatieve cellen bleken in muizen leukemie te veroorzaken en bij passage in een volgende generatie muizen opnieuw leukemie te initiëren. Ook in het CAFC assay produceerden CD34 negatieve cellen grote aantallen leukemische klonen gedurende tenminste 8 weken. Dit betekent dat het ontbreken van expressie van CD34 niet altijd aangeeft dat een leukemische cel zijn primitieve stamcel eigenschappen verliest.

In hoofdstuk 5 wordt de vraag gesteld of de cellen die uitgroeien in de SCID-muis en in lange termijn beenmergkweek inderdaad vroegere leukemiecellen zijn dan de tot dan toe bekende voorlopercel AML-CFU. Hiertoe werden leukemische cellen blootgesteld aan zodanige concentraties van het cytostaticum fluorouracil (5-FU), dat alle AML-CFU werden gedood. De overlevende cellen waren in staat om zowel in de SCID-muis als in lange termijn kweek groei van leukemie te initiëren. Hieruit blijkt dat leukemische stamcellen niet identiek zijn aan AML-CFU. Het gegeven dat leukemische stamcellen dergelijke condities overleven is een aanwijzing dat ze geen actieve stofwisseling of actieve celdeling vertonen.

Leukemiecellen hebben receptoren voor hematopoietische groeifactoren. Deze receptoren heben een hoge affiniteit voor de corresponderende groeifactor en een selectieve weefseldistributie. Het is mogelijk om toxinen aan groeifactoren te koppelen. Als een leukemiecel een dergelijk groeifactor/toxine bindt en internaliseert, wordt deze cel specifiek geëlimineerd. In hoofdstuk 6 worden de effecten van een toxine bestaande uit de groeifactor GM-CSF en difterietoxine (DT-GM-CSF) op normale en leukemische stamcellen onderzocht. Het blijkt dat normale stamcellen een 48 uur durende expositie aan hoge concentraties van het toxine zonder schade doorstaan. Daarentegen leidt dezelfde blootstelling aan DT-GM-CSF wel tot verlies van leukemische stamcellen: zowel de lange termijn groei van leukemie in SCID-muizen als die in het CAFC assay werd aanzienlijk verminderd. De effecten van DT-GM-CSF bleken specifiek via de GM-CSF receptor verlopen. Receptoren voor GM-CSF komen dus op de leukemische

stamcel voor, in tegenstelling tot de normale stamcel. Dit verschil tussen leukemische en normale stamcellen biedt potentieel therapeutische perspectieven voor een gerichte benadering van de leukemiepopulatie met ontzien van de normale stamcel.
Tenslotte wordt in hoofdstuk 7 een overzicht gegeven van de huidige inzichten in de eigenschappen van de stamcel van AML. Het is gebleken dat voor onderzoek van leukemische stamcellen thans betere systemen beschikbaar zijn dan voorheen. In immunodeficiënte muizen en in het CAFC assay kan een AML celpopulatie onderzocht worden die de 'echte' leukemische stamcel beter benadert.

ABBREVIATIONS

AML	:acute myeloid leukemia
ATRA	:all-trans retinoic acid
BM	:bone marrow
BSA	:bovine serum albumin
CA	:cobblestone area
CD	:cluster of differentiation
CAFC	:cobblestone area-forming cell
CFU	:colony forming unit
Cl_2MDP	:dichloromethylene diphosphonate
CR	:complete remission
CSF	:colony stimulating factor
del	:deletion
DMSO	:dimethylsulfoxide
DNA	:deoxyribonucleic acid
dpm	:decays per minute
DT	:diphteria toxin
DT-GM-CSF	:diphtheria toxin fused to GM-CSF
ELISA	:enzyme linked immunosorbent assay
FAB	:French-American-British cytomorphological classification
FACS	:fluorescence activated cell sorting
FCS	:fetal calf serum
FITC	:fluorescein isothiocyanate
FISH	:fluorescent in situ hybridization
FSC	:forward scatter
5-FU	:5-fluorouracil
G-CSF	:granulocyte colony-stimulating factor
GM-CSF	:granulocyte-macrophage colony-stimulating factor
GM-CSF-R	:granulocyte-macrophage colony-stimulating factor receptor
Gy	:Gray
h	:human
HBSS	:Hank's balanced salt solution
HGF(s)	:hematopoietic growth factors
HPP	:high proliferative potential
HS	:horse serum
Ig	:immunoglobulin
IL-3	:interleukin-3
IMDM	:Iscove's modified Dulbecco's medium
ip	:intraperitoneally
iv	:intravenously
LAK	:lymphokine activated killer

L-CAFC	:leukemic CAFC
LTBMC	:long-term bone marrow culture
LTC-IC	:long-term culture initiating cell
LTRA	:long-term repopulating ability
α-MEM	:alpha minimal essential medium
MGG	:May-Grünwald-Giemsa
MNC	:mononuclear cells
MoAb	:monoclonal antibody
mRNA	:messenger RNA
NA	:non-adherent
NC	:nucleated cells
ND	:not determined
NK	:natural killer
No	:number
NOD	:non-obese diabetic
PB	:peripheral blood
PBS	:phosphate buffered saline
PE	:phycoerythrin
PCR	:polymerase chain reaction
PIXY	:an IL-3/GM-CSF construct
PML	:promyelocytic leukemia
RAR	:retinoid acid receptor
R-G-CSF	:G-CSF receptor
RNA	:ribonucleic acid
SCF	:stem cell factor
SCID	:severe combined immunodeficiency
SD	:standard deviation
SEM	:standard error of the mean
SF	:serum-free
SFM	:serum-free medium
SSC	:sideward scatter
TBI	:total body irradiation
UCB	:umbilical cord blood

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Submitted for publication

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 26 februari 1960 geboren te Almelo. Na het behalen van het diploma gymnasium β (1978) studeerde hij geneeskunde aan de Rijksuniversiteit te Utrecht (artsexamen in 1985). Nadien was hij als arts-assistent Interne Geneeskunde werkzaam in het Sint Antonius Ziekenhuis te Nieuwegein, met als opleider Dr O.J.A.Th. Meeuwissen. Registratie als internist volgde in 1992. Aansluitend was hij werkzaam binnen de afdeling hematologie van het Academisch Ziekenhuis Dijkzigt te Rotterdam (Hoofd:Prof. dr. B. Löwenberg). Hier verkreeg hij de aantekening hematologie (1993) en werd begonnen met het in dit proefschrift beschreven onderzoek. Een klinisch research fellowship van de Nederlandse Kanker Bestrijding (NKB/KWF) gaf de mogelijkheden voor een langere periode van wetenschappelijk onderzoek (1994-1996). Dit fellowship bestond uit een aantal stages, welke vervuld werden binnen het Instituut voor Hematologie, Erasmus Universiteit te Rotterdam en het Hammersmith Hospital, Royal Postgraduate Medical School, te Londen.

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