CHARACTERIZATION OF HUMAN ACUTE MYELOID LEUKEMIA PROGENITOR CELLS: GROWTH FACTOR RESPONSIVENESS AND MEMBRANE PHENOTYPES.

(Karakterizering van menselijke acute myeloïde leukemie voorloper cellen: groei factor reactiviteit en membraan phenotypen).

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CONTENTS

ABBREVIATIONS

AET	- 2-aminoethylthiouronium bromide hydrobromide
ALL	- acute lymphoid leukemia
AML	- acute myeloid leukemia = ANLL
AML-CFU	- acute myeloid leukemia-colony forming unit
ANLL	- acute nonlymphocytic leukemia = AML
AUL	- acute undifferentiated leukemia
BFU-e	- burst forming unit-erythroid
BSA	- bovine serum albumin
cAMP	- cyclic adenosine monophosphate
CD	- cluster of differentiation
CFU-e	- colony forming unit-erythroid
CFU-Eo	- colony forming unit-eosinophil
CFU-G	- colony forming unit-granulocyte
CFU-GEMM	- colony forming unit-granulocyte/erythroid/monocyte/ megakaryocyte
CFU-GM	- colony forming unit-granulocyte/monocyte
CFU-M	- colony forming unit-monocyte
CFU-MK	- colony forming unit-megakaryocyte
CLL	- chronic lymphocytic leukemia
CM	- conditioned medium
CML	- chronic myeloid leukemia
CSF	- colony stimulating factor
DAG	- diacylglycerol
DMSO	- dimethyl sulfoxide
DNA	- deoxyribonucleic acid
EGF	- epidermal growth factor
Epo	- erythropoietin
FAB	- French-American-British Cooperative Group
FACS	- fluorescence activated cell sorting
FBL	- fucose binding lectin
FCS	- fetal calf serum
FITC	- fluorescein isothiocyanate
GAM	- goat-anti-mouse
G-CSF	- granulocyte-colony stimulating factor
GM-CSF	- granulocyte macrophage-colony stimulating factor
H-1	- hemopoietin-1
HGF	- hematopoietic growth factor
HPCM	- human placental conditioned medium
^{3}H-TdR	- tritiated thymidine
Ig	- immunoglobulin
IL	- interleukin
IMDM	- Iscove's modified Dulbecco's medium
IP_3	- inositol triphosphate
LIF	- leukemia inhibitory factor
LPS	- lipopolysaccharide
M-CSF	- macrophage-colony stimulating factor
MDS	- myelodysplastic syndrome
MGG	- May-Grünwald-Giemsa
MoAb/MCA	- monoclonal antibody
Multi-CSF	- multilineage-colony stimulating factor (=IL-3)
mRNA	- messenger ribonucleic acid
NBM	- normal bone marrow
PB	- peripheral blood
PBS	- phosphate buffered saline
PDGF	- platelet derived growth factor
PGE	- prostaglandin E
PHA	- phytohemagglutinin
PHA-LCM	- phytohemagglutinin leukocyte conditioned medium

PHA l.f.	- phytohemagglutinin leukocyte feeder
PIP_2	- phosphatidyl inositol biphosphate
PK-A	- protein kinase-A
PK-C	- protein kinase-C
RIA	- radioimmunoassay
TdT	- terminal deoxynucleotidyl transferase
TGF-β	- transforming growth factor-β
TNF	- tumor necrosis factor
UEA	- Ulex europaeus agglutinin

Monoclonal antibodies

Code:	Reactivity with normal hematopoietic cells:
CD2	- T cells, most of the NK cells.
CD7	- T cells, NK cells.
CD11b	- Monocytes, macrophages, granulocytes, NK cells.
CD13	- Most of the cells of the granulocytic/monocytic lineages; part of CFU-GM.
CD14	- All cells of the monocytic lineage but no CFUs.
CD15	- All cells of the granulocytic lineage but no CFUs.
CD19	- B cells.
CD33	- Cells of the granulocytic/monocytic lineages (except granulocytes); CFUs (except CFU-blast).
CD34	- Blasts; CFUs.
CD_w65 (Vim-2)	- All cells of the granulocytic/monocytic lineages but no CFUs.

CHAPTER 1

Introduction

1.1. *Leukemia*

Hematopoiesis or blood cell formation takes place primarily in the bone marrow. A small population of immature multipotential bone marrow cells is able to 1) undergo selfrenewal, i.e. produce new stem cells, or 2) differentiate to committed progenitor cells. These unipotent precursors have the ability to proliferate and mature along their differentiation lines giving rise to functional blood cells (Figure 1). The end cells will be eliminated from the circulation after a certain period and need to be replaced by new mature cells. The continuous process of blood cell formation is regulated by cellular interactions with bone marrow stroma cells and by soluble regulatory molecules, the hematopoietic growth factors (HGFs) (1,2).

Leukemia or blood cancer results from a neoplastic transformation of hematopoietic cells and is evident as an accumulation of cells at a certain stage of development. Leukemia cells accumulate in the bone marrow and blood and then eventually replace the normal hematopoietic cells. Because the accumulation may occur at various maturation stages in the different lineages, distinct forms of leukemia have been identified (3-6). Chronic lymphoid leukemias (CLL) or chronic myeloid leukemias (CML) are characterized by an expansion of mature blood cells. In the acute leukemias (acute lymphoid leukemia (ALL) or acute myeloid leukemia (AML)) maturation has been arrested at early stages of maturation. Accumulation of a certain cell type does not imply that transformation has occurred in the cells of that particular maturation stage. For instance, CML is identified by an expansion of granulocytic cells in marrow and blood, whereas transformation has taken place in pluripotent stem cells (7). Thus transformation may occur in primitive precursor cells, whereas the defect may be expressed at later stages of maturation.

1.2. *Acute Myeloid Leukemia (AML)*

Acute myeloid leukemia (AML) (or acute nonlymphocytic leukemia (ANNL)), like most other leukemias is a clonal disease. This implies that the leukemia originates from a single transformed progenitor (8-12). In certain cases, transformation apparently occurs in a pluripotent stem cell, whereas in other cases a committed precursor is probably the target of the transformation (9,11,12). Consequently, among clinical AML, a great variability is apparent as regards the cytogenetic (8), morphologic and

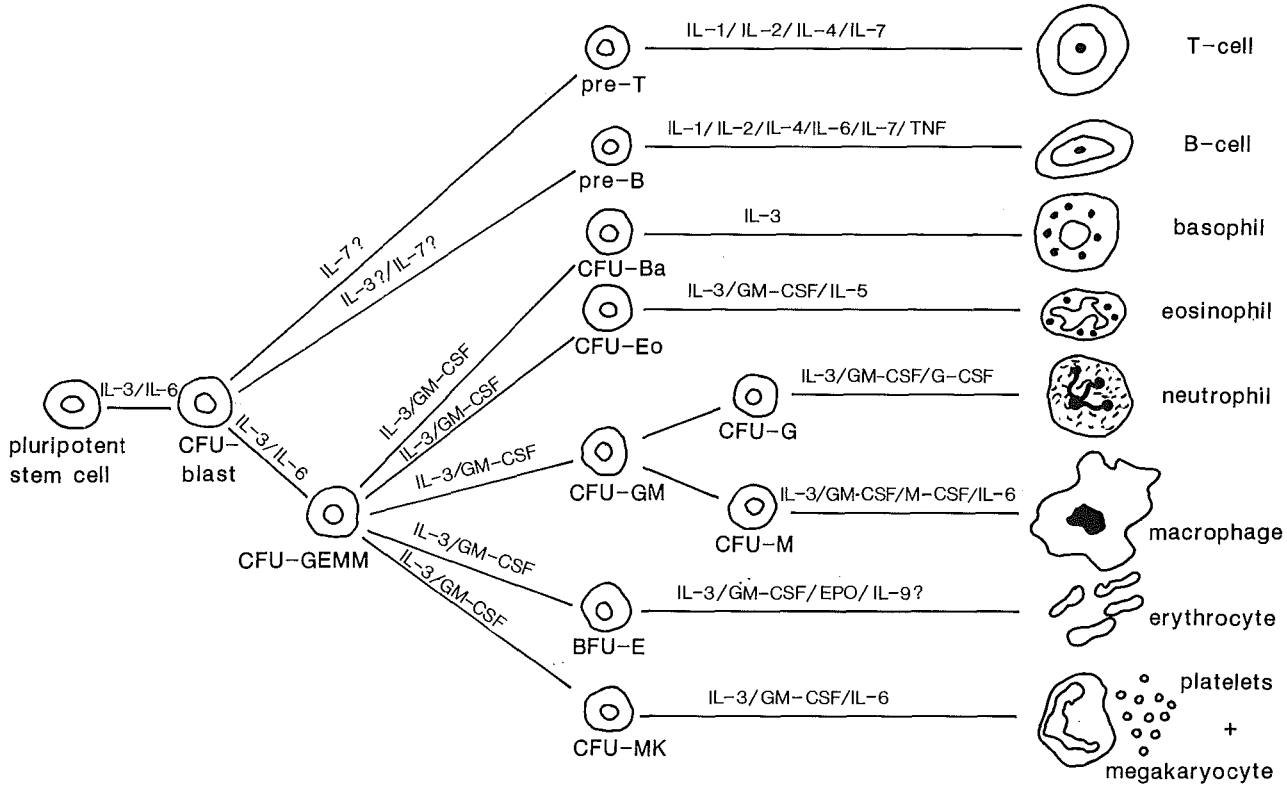

Figure 1: Scheme of normal hematopoiesis. Interaction of hematopoietic growth factors with normal marrow target cells. CFU = colony forming unit; BFU = burst forming unit; IL = interleukin; CSF = colony stimulating factor; TNF = tumor necrosis factor.

cytochemic (5,6) and immunophenotypic features of the leukemia (3,4). Morphologically and cytochemically distinct types of AML are classified according to the criteria of the French-American-British (FAB) group (see Table 1) (5,6). AML cells often exhibit abnormal karyotypes (8). Certain cytogenetic abnormalities appear nonrandomly and are specific for certain subtypes of AML. For instance, the translocation t(8;21) is typical of a subgroup of leukemias usually found among FAB-M2 cases (13). The translocation t(15;17) is typical of FAB-M3 leukemias (14). Leukemias with an inversion or deletion of the long arm of chromosome 16 are related to the subgroup of FAB-M4 with eosinophilia (15,16).

Analysis of membrane surface and intracellular antigen expression allows a further dissection of the heterogeneity of leukemias (Table 1) (3,14,17). AML cells are characterized by the expression of membrane antigens specific for myelomonocytic cells. Monoclonal antibodies (MoAbs) that react with a particular differentiation antigen have received a uniform code: CD (= cluster of differentiation) (4,18). Most of the cells of the monocytic and granulocytic lineages express CD13 and/or CD33 antigens (19). Granulocytic cells often express CD15 (20), whereas the monocytic cells usually carry the CD14 marker on their surface (21). CD34 is a membrane antigen characteristic of immature myeloid and lymphoid cells and is found on AML cells in approximately 50% of the cases (22,23). In certain CD34 positive AML cases cells may also express terminal deoxynucleotidyl transferase (TdT), CD2 or CD7, which were initially thought to be specific for lymphoid cells (4,17). In fact, an increasing number of reports have presented AML and ALL cases that express both myeloid and lymphoid (B or T cell) features. Probably, in those instances transformation has taken place in pluripotent hematopoietic progenitor cells (17). Detailed information about the expression of immunological markers in leukemias and their corresponding CD codes has been summarized recently (4,18).

Morphological examination and immunophenotyping have revealed different subpopulations of leukemic cells within AML patients (5,6,24,25). This heterogeneity appears to be a reflection of successive stages of maturation of AML. Thus, the AML population is often characterized by a "leaking" maturation block, i.e. the maturation arrest is incomplete. Leukemic blast cells with pronounced maturation features can be distinguished from the blast cells with immature characteristics. The suggestion that AML blasts can be divided into leukemic precursors and "end" cells is further supported by studies using in vitro colony

methodology (see next paragraph) (24-26).

TABLE 1. Morphological classification of acute myeloid leukemias: comparison with immunophenotypes.

FAB	Leukemia type	Marker expression
AUL	Acute undifferentiated leukemia	HLA-DR, CD34, (TdT), (CD13), (CD33), (CD7)
AML-M1	Acute myeloid leukemia without differentiation	(HLA-DR), CD34, CD13, CD33, (CD15)
AML-M2	Acute myeloid leukemia with differentiation	(HLA-DR), (CD34), CD13, CD33, CD15, (CD11b)
AML-M3	Acute promyelocytic leukemia	CD13, CD33, CD15, (CD11b)
AML-M4	Acute myelomonocytic leukemia	HLA-DR, (CD34), CD13, CD33, (CD14), (CD11b)
AML-M5	Acute monocytic leukemia	HLA-DR, CD13, CD33, CD14, (CD11b)
AML-M6	Acute erythrocytic leukemia	(HLA-DR), (CD34), (CD33), (CD13), (GP A)
AML-M7	Acute megakaryocytic leukemia	HLA-DR, CD34, CD33, (GP IIIa)

Markers in parentheses are not always expressed.

FAB: French-American-British nomenclature; CD: Cluster of differentiation; TdT: Terminal deoxynucleotidyl transferase; HLA-DR: MHC Class-II antigen; GP A: Glycophorin A; GP IIIa: Glycoprotein IIIa.

1.3. *Immunophenotypic characterization of AML colony forming cells (AML-CFU) in relation to normal hematopoietic progenitors*

AML progenitor cells have been cultured in semisolid agar or methylcellulose colony assays that were initially applied to grow normal bone marrow precursors (27,28). These normal precursors include multipotential granulocyte-erythrocyte-macrophage-megakaryocyte colony forming units (CFU-GEMM), granulocyte-macrophage-CFU (CFU-GM), granulocyte-CFU (CFU-G), macrophage-CFU (CFU-M), eosinophil-CFU (CFU-Eo) and erythroid precursors, i.e. burst forming units (BFU-e) and CFU-e (see Figure 1) (29,30). Relatively mature CFU-GM, or "late" CFU-GM, give rise to colonies at day 7 of culture, whereas immature, or "early" CFU-GM generate colonies after 14 days. Normal marrow progenitor cells can be discriminated from the nonproliferating mature hematopoietic cells using immunological markers and cell separation procedures (e.g. fluorescence activated cell sorting (FACS) or complement mediated lysis) (30). Moreover, immunological separation also reveals differences in marker expression between CFU-GEMM, "early" (day 14)

CFU-GM and "late" (day 7) CFU-GM (24,25).

In AML, populations of cells with different maturation characteristics can be distinguished as well. Separation of immunologically distinct subsets of AML cells with FACS or complement mediated lysis and subsequent colony culture showed that, among the AML blasts, colony forming cells (AML-CFU) express the immature surface phenotypes, whereas the cells with no or limited colony forming abilities are immunophenotypically more mature (24-25). Phenotypes of AML-CFU vary among different cases with AML. AML-CFU may express surface markers characteristic of CFU-GEMM, whereas in other cases AML-CFU exhibit surface antigens typical of committed precursors ("early" (day 14) CFU-GM or "late" (day 7) CFU-GM) (24,25).

One of the goals of this thesis was to further characterize surface membrane phenotypes of AML-CFUs and to search for AML-CFU surface features different from those of normal marrow CFUs. Possible discrepancies between normal marrow CFU and AML-CFU may be of use in the diagnosis of AML, for instance for the detection of minimal numbers of AML cells among normal marrow cells in patients in remission (following chemotherapy or bone marrow transplantation). A question that has been addressed in this thesis is whether it is possible to discriminate between AML-CFU and the majority of normal marrow precursors on the basis of membrane antigen density (fluorescence intensity), with combinations of surface markers employing fluorescence activated cell sorting and colony culture (Chapters 2 and 3).

1.4. *In vitro growth characteristics of AML*

Normal bone marrow progenitors require hematopoietic growth factors (HGFs), in particular colony stimulating factors (CSFs) to survive, proliferate and produce mature progeny **in vitro**. In the original culture methods crude sources of these stimuli were supplied. For instance, the cultures were carried out in the presence of peripheral blood feeder leukocytes that released the HGFs into the culture medium (27,28). Culture supernatants of peripheral blood cells, human placenta or cell lines containing mixtures of HGFs have also been used (31-33). When the colony cultures are applied to study the proliferation of human AML cells **in vitro**, it appears that in most cases the leukemic progenitor cells have retained their dependence on

HGFs. The cells usually do not form colonies in the absence of HGFs (26,28,31,34,35). Colonies obtained from AML cells differ from colonies generated from normal bone marrow progenitors in several aspects:

- Leukemic colonies vary widely in size. Predominantly small clusters (less than 20 cells) or somewhat larger clusters (less than 50 cells) are produced. Colonies containing 50 cells or more are less frequently generated in cultures of AML cells than in colony cultures of normal bone marrow cells. In other cases of AML no colonies or clusters are formed at all (26,34,35).
- Generally, only cells of the myelo/monocytic lineage are produced in AML colonies/ clusters (26,34,35). However, AML colony/cluster cells do not mature towards functional granulocytes. Thus, mainly blasts are formed (26,34,35).
- Although the colony cells are morphologically immature they express membrane markers characteristic of neutrophils, consistent with the ability of AML progenitors to produce partially "maturing" progeny (incomplete maturation) (36,37).

Since AML colony formation in agar or methylcellulose is frequently impaired or even absent, attempts have been made to develop more efficient AML culture methods. Incubation in agar cultures, after a 15-hour preincubation of AML cells in suspension with phytohemagglutinin (PHA), significantly increases AML colony numbers (38,39). In a subsequent modification, AML cells have been cultured in suspension on top of an agar underlayer containing feeder leukocytes (the PHA leukocyte feeder assay) (40). Using these culture methods proliferation of AML progenitors occurs in 80 to 90 percent of the cases. The fact that the cells under those conditions may form aggregates suggests that cell-cell interactions provide essential signals for AML growth **in vitro**. A major disadvantage of the assays in which a liquid phase is applied is, that a quantitative analysis of colony formation is difficult due to the agglutination in suspension. As a consequence, in the studies presented in this thesis, responsiveness of AML cells to HGFs has been investigated in tritiated thymidine incorporation experiments (suspension cultures) as well as in semisolid colony cultures.

1.5. *Human hematopoietic growth factors*

The genes and complementary DNAs (cDNAs) of a number of growth factors have been cloned, expressed in bacteria, yeast or mammalian cells and the activities defined in cultures of normal bone marrow cells. The human hematopoietic growth factors and their target cells are summarized in Tables 2 and 3 and in Figure 1. Six of these growth factors have a dominant role in controling hematopoiesis, i.e. interleukin-3 (IL-3), granulocyte-macrophage-colony stimulating factor (GM-CSF), G-CSF, M-CSF, erythropoietin (Epo) and IL-5 (Table 2) (2,41). The latter four HGFs appear to be lineage specific and stimulate cells of the neutrophilic (G-CSF) (42,43), macrophage (M-CSF) (44), erythrocytic (Epo) (45,46) or eosinophilic (IL-5) lineages (47). The lineage restriction of G-CSF, however, is still somewhat controversial. Some investigators have suggested stimulatory effects of G-CSF on immature pluripotent progenitor cells (48). IL-3 (49,50) and GM-CSF (51) are multipotential growth factors supporting the **in vitro** survival, proliferation and differentiation of multipotential hematopoietic progenitor cells (CFU-GEMM) and committed precursors of multiple maturation lines, e.g. BFU-e, CFU-Eo and CFU-MK (30,52-54). IL-3 is a basophil differentiation inducer as well (55). IL-3, GM-CSF, G-CSF, M-CSF and IL-5 also support survival of mature cells. HGFs may recruit neutrophils (GM-CSF and G-CSF), monocytes (IL-3, GM-CSF and M-CSF) or eosinophils (IL-3 and IL-5) from the circulation and activate phagocytosis and killing of bacteria, parasites or antibody-coated tumor cells (53,54,56-59).

Other cytokines that have been cloned have an important role in the regulation of lymphopoiesis, i.e. IL-1 (α and β) (60), IL-2 (61), IL-4 (62), IL-6 (63), IL-7 (64) and TNF (α and β) (65,66). However, apparently, some of these are involved in myelopoiesis as well:

- IL-1 acts on hematopoietic and nonhematopoietic target cells (67). An important function of IL-1 in murine and human hematopoiesis is the induction of cytokine production. IL-1 stimulates the release of GM-CSF, G-CSF or IL-6 in a variety of cell types, e.g. lymphocytes, fibroblasts and endothelial cells (54,57, 68-71). IL-1, previously also designated hemopoietin-1 (H-1) (72,73), is a cofactor for IL-3 in the stimulation of primitive murine stem cells to generate blast cell colonies **in vitro** (74). These colonies contain multipotential and committed precursors.

TABLE 2. Human hematopoietic growth factors.

Factor	Protein size (KD)	Ref.	Hematopoietic precursor target cell	Ref.	Producer cells	Ref.
IL-3	14-18	49,50	CFU-GEMM	30,53	- lectin activated T lymphocytes	50
			CFU-Eo	30,53		
			BFU-E	30,53		
			CFU-MK	53,85		
			CFU-blast	48,74-76		
GM-CSF	14-35	51	CFU-GEMM	52,54	- lectin or IL-1 activated T lymphocytes	54,70
			CFU-Eo	52,54	- TNF or IL-1 activated endothelial cells	54,68
			BFU-E	52,54	- TNF or IL-1 activated fibroblasts	54,69
			CFU-MK	53,85		
G-CSF	18-22	42,43	CFU-G	42,43,57	- IL-4, GM-CSF, IL-3 or LPS activated monocytes	57
			CFU-blast	48	- TNF or IL-1 activated endothelial cells	57,68
					- TNF or IL-1 activated fibroblasts	57,69
M-CSF	18-26 (2x) 35-45 (2x)	44	CFU-M	44,58	- IL-3, GM-CSF, TNF, γinterferon, IL-4 or LPS activated monocytes	58
Epo	36	45,46	CFU-E	45,46	- peritubular cells in the kidney	93
			BFU-E	45,46		
IL-5	20 (2x)	47	CFU-Eo	47,59	- lectin activated T lymphocytes	59

M-CSF and IL-5 have been isolated as dimers.
Two distinct forms of M-CSF with the same activity have been demonstrated.

IL = Interleukin; CSF = colony stimulating factor; Epo = erythropoietin; TNF = tumor necrosis factor; LPS = lipopolysaccharide.

TABLE 3. Human lymphoid growth and differentiation factors.

Factor	Protein size(KD)	Ref.	Hematopoietic precursor target cell	Ref.	Lymphoid target cells	Ref.	Producer cells	Ref.
IL-1	17	60			B and T lymphocytes	67	- LPS activated monocytes	67
							- IL-1 activated endothelial cells	67
IL-2	15	61			B and T lymphocytes	77	- lectin or IL-1 activated T lymphocytes	77
IL-4	15	62	CFU-G	81	B and T lymphocytes	62	- lectin activated T lymphocytes	81
IL-6	26	63	CFU-blast	74-76	B lymphocytes	83	- lectin activated T lymphocytes	83
			CFU-M	84			- LPS activated monocytes	83
			CFU-MK	85			- IL-1 or TNF activated endothelial cells	83
							- IL-1 or TNF activated fibroblasts	83
IL-7	17	64			T lymphocytes	87	- bone marrow stroma cells	64
TNF	17	65,66			B lymphocytes	90	- LPS activated monocytes	65
							- lectin activated T lymphocytes	66

- Two forms of IL-1 have been recognized,i.e. IL-1 α and IL-1 β.
- Two types of TNF have been isolated, i.e. TNF α and TNF β (lymphotoxin).

Thus, following replating, the cells from these blast colonies may produce new colonies that contain mature cells of the different lineages. Presently, it is uncertain whether IL-1 exerts this costimulatory effect with IL-3 on human primitive stem cells **in vitro** as well. On the other hand, human as well as murine blast cell colonies can be generated from immature marrow precursors following costimulation by IL-6 and IL-3 (74-76). In fact the synergistic effects of IL-1 with IL-3 in the stimulation of primitive murine stem cells may be indirect (74,76). IL-1 may stimulate the production of IL-6 which could then synergize with IL-3.

- IL-2 is a stimulator of lymphopoiesis (77). Receptors of IL-2, i.e. the low affinity chains or Tac antigens (p55), have been demonstrated on monocytes (78), AML cells (79) and on CML precursors (80). Whether these receptors have a role in the growth of myelomonocytic cells has not been settled.
- IL-4, a growth factor of lymphopoietic cells (62), exerts certain effects on progenitor cells from other lineages as well. Although it has no colony stimulating effect on its own, it may act synergistically with G-CSF in stimulating CFU-G (81). These effects may either be direct or mediated via accessory cells. Furthermore, IL-4 is an inhibitor of myelopoiesis (82).
- IL-6 is a stimulator of B cells (83). IL-6 is also a costimulator with IL-3 for primitive hematopoietic precursors generating blast cell colonies (74-76) and it costimulates with M-CSF macrophage colony formation (84). Furthermore, it is a stimulator of megakaryocyte formation (85).
- IL-7 has a role in lymphopoiesis. It activates the growth of precursor cells of the B lymphoid lineage and induces the proliferation of T cells (64,86,87). Whether it has a regulatory role in the proliferation of cells of other lineages remains to be elucidated.
- IL-8 activates chemotaxis of neutrophils and it inhibits neutrophil-endothelial cell interactions (88).
- IL-9 stimulates the proliferation of a human megakaryocytic cell line and it may stimulate erythroid precursors (89,90).
- TNFs stimulate the proliferation of B cells (91). TNFs inhibit normal marrow and AML colony growth when stimulated with crude sources of HGFs, i.e. cell line or peripheral blood conditioned media (92). Furthermore, TNFs are regulators of cytokine production. For instance, TNFs stimulate endothelial cells, fibroblasts, T lymphocytes or monocytes to produce

GM-CSF, G-CSF or M-CSF (54,57,58).

One of the goals of the studies presented here is to determine the role of different growth factors in the proliferation and maturation of AML cells in vitro. The following questions have been addressed in this thesis:

- Is AML proliferation regulated by the growth factors IL-3, GM-CSF, G-CSF and M-CSF (Chapters 4 and 5)?
- Is the maturation blockade of AML cells absolute or, do the cells mature towards neutrophils when stimulated by G-CSF, alone or combined with other HGFs (Chapter 6)?
- Is IL-1 a growth regulator for AML, and if so, does it stimulate AML proliferation indirectly, i.e. via the induction of endogenous growth factors (autocrine mechanism) (Chapters 8, 9 and 10)?

1.6. *Inhibitors of hematopoiesis*

Blood cell formation is controlled by stimulators and inhibitors of proliferation. Relatively little is known about growth inhibitors of hematopoiesis in man, although several molecules have been characterized and in some instances the cDNAs cloned. These include IL-4 (62,82), TNF (65,66,92), interferon α and γ (94). Recently, leukemia inhibitory factor (LIF) was cloned (95). This inhibitor arrests the growth of a myeloid cell line (the M-1 cell line), but inhibitory effects on normal and leukemic marrow cells have not been observed (unpublished observations). Other inhibitors of normal bone marrow and AML progenitor cell proliferation are: Prostaglandin E (PGE) and TGF-β (96,97).

A mechanism to explain how AML cells outgrow normal hematopoietic cells is that the leukemia cells may have become insensitive to growth inhibitors. It will therefore be of interest to investigate the inhibitory effects of these compounds on the growth of AML when stimulated with the distinct HGFs. In Chapter 7 the effects of TNF on IL-3, GM-CSF, G-CSF and M-CSF stimulated proliferation of AML cells have been investigated.

1.7. *Receptor binding and signal transduction*

Growth factors bind to specific receptors that are anchored in the plasma membrane of the cells (98). The formation of dimeric or oligomeric receptor complexes, either homologous (e.g. M-CSF-R) (99) or heterologous (IL-2-R, IL-6-R, IL-3-R, IL-4-R, GM-CSF-R or Epo-R) (100-105) appears obligatory for high affinity binding of a growth factor and for receptor signalling. Following receptor activation intracellular messengers are generated, which further convey signals to the nucleus modulating DNA synthesis and gene expression, leading to cell proliferation, differentiation and function (98,106). An important step following ligand-receptor binding is the activation of protein kinases, which phosphorylate cellular substrates (98,106). Phosphorylation alters the functions of proteins and is essential in the transduction of intracellular signals. Substrates for protein kinases include GF-receptors, enzymes (e.g. other kinases), cytoskeletal proteins and nuclear proteins (107). In fact, three major routes of signal transduction have been identified:

- The adenylate cyclase/cyclic adenosine monophosphate (AC/cAMP) pathway (108). Following binding of the ligand to the receptor (e.g. β-adrenergic receptor) AC, which is associated with the receptor and is under the control of G-proteins, converts ATP into cAMP. Cyclic AMP is a "second messenger" that activates protein kinase A (PK-A).
- The inositol phospholipid breakdown pathway (109). Following receptor activation (e.g. epidermal growth factor (EGF) receptor (110) and platelet derived growth factor (PDGF) receptor (111)) phosphatidyl inositol 4,5 biphosphate (PIP_2) is hydrolyzed by phospholipase C. Hydrolysis of PIP_2 results in the generation of inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG). These two second messengers frequently trigger two synergistically acting signal pathways. IP_3 stimulates or enhances the influx of intracellular Ca^{2+} ions, whereas DAG is a second messenger that may activate Ca^{2+}/phospholipid dependent protein kinase-C (PK-C).
- Receptors with intrinsic tyrosine kinase activity. EGF receptors (110), PDGF receptors (111) and M-CSF receptors (99) are tyrosine kinases. Following binding of the ligand, the receptors become activated, autophosphorylated and cellular substrates will be phosphorylated at tyrosine residues.

These pathways probably operate in close association rather than as separate mechanisms and may be switched on in parallel following receptor activation. For instance, EGF- and PDGF receptors have intrinsic tyrosine kinase activity but following receptor activation the inositol phospholipid breakdown pathway will be activated as well (110,111).

Following stimulation of murine IL-3 and GM-CSF dependent cell lines with their ligands, receptors as well as intracellular substrates become phosphorylated at tyrosine sites (112-114). At the same time, PK-C may also be activated following stimulation with these ligands (115). Thus multiple pathways may be involved in IL-3 and GM-CSF receptor activation. Whether human IL-3 and GM-CSF receptors follow the same pathways as their murine homologues upon stimulation remains to be elucidated. Relatively little is known about intracellular signals that follow G-CSF receptor activation. The M-CSF or CSF-1 receptor which has been studied thoroughly is a tyrosine kinase (99,116). Although different pathways may be switched on following activation of a receptor, it appears that tyrosine phosphorylation is the most important signal for proliferation induction (98).

1.8. *Mechanisms of neoplastic growth in experimental leukemias*

Abnormal growth factor control is considered to be important in cancer growth. Evidence for this comes from studies of the last decade showing that oncogenes code for products that are involved in the growth factor-receptor transduction pathways (106,117). Oncogenes were first discovered in tumorigenic retroviruses (118) capable of infecting and transforming eukaryotic cells, e.g. murine, feline or chicken cells. The viral RNA is transcribed into DNA and then integrated into the host chromosomal DNA. Oncogenes represent normal cellular genes (proto-oncogenes) that during the viral life cycle have been transduced into the viral genome (118). In fact, oncogenes code for growth factors (e.g. v-sis; homologous to PDGF) (119), growth factor receptors (e.g. v-erb or v-fms; homologous to the EGF receptor and M-CSF receptor, respectively) (120,121), G-binding proteins (e.g. v-ras) (122), tyrosine kinases (e.g. v-src or v-abl) (123,124) or nuclear proteins that may have a role in intracellular signal transduction (e.g. v-fos or v-myc) (125,126). Certain genes are mutated resulting in altered (e.g. aminoacid substituted or truncated) products with transforming abilities (127). The products coded by the viral oncogenes are usually overexpressed, as the genes are controlled by viral promotors and enhancers which lead to immortalization

of cells and tumor formation (127).

Unregulated expression of cellular equivalents of the viral oncogenes, the proto-oncogenes, may lead to tumor formation as well (118,127). This may occur in cells that are infected by RNA viruses that do not contain oncogenes themselves. The viral DNA may be integrated into the host genome so that proto-oncogenes become controlled by viral regulatory elements. This mechanism is called insertional mutagenesis. Alternatively, abnormal proto-oncogene expression may be the result of the transposition of genes due to chromosomal translocations, e.g. in human burkitt lymphomas with translocation t(8;14) and CML with t(9;22), the c-myc and the c-abl proto-oncogenes have been transposed respectively and are abnormally expressed (128-131).

Studies with experimental leukemias (animals) have provided evidence that altered responses to HGFs or even loss of susceptibility to exogenous growth factors may lead to **in vitro** immortalization of blood cells and subsequently tumor formation. How may hematopoietic cells escape normal control of HGFs and become tumorigenic? Several murine growth factor (IL-3, GM-CSF or G-CSF) dependent cell lines have been generated following infection with retroviruses (132-136). When introduced into syngeneic animals these HGF dependent cell lines are not neoplastic. However, these cells have undergone at least one step in a cascade that may lead to tumor formation, since they have become immortalized **in vitro.** Immortalized cells have partly or completely lost their ability to mature in response to HGFs in favor of their selfrenewal capacity in vitro. Subsequent loss of exogenous growth factor requirement in vitro usually leads to neoplastic growth, i.e. following injection into syngeneic animals. Cells may lose their requirement of exogenous growth factors by different mechanisms (Table 4):

- Autocrine stimulation

  Cell lines may become HGF independent by acquiring the ability to produce their own growth factor. This may be achieved by:
  - insertional mutagenesis; growth factor genes become controlled by viral transcriptional regulators (132,137);
  - introduction of growth factor genes by using retroviral vectors (138-141);
  - introduction of v-onc genes, e.g. v-ras or v-mil, that, by yet unknown mechanisms, may activate growth factor production (142,143).

- Activation of intracellular receptors

  Although the RNA messengers for growth factors are demonstrated in certain transformed cells that grow autonomously, the factor independent growing cells do not produce their own HGFs and are not stimulated by an autocrine mechanism (138). A possible explanation for these observations is that receptors may be activated by the growth factors intracellularly, leading to proliferation independent of exogenous factors. In fact, the mechanism of intracellular receptor activation has recently been demonstrated (144). Introduction of a gene coding for a modified IL-3 protein that cannot be excreted renders the cells, of an IL-3 dependent cell line, completely factor independent. This suggests that receptors are activated before they reach the surface membrane.

- Altered membrane receptors that are permanently activated

  This mechanism has initially been described for the EGF receptor (120). The viral oncogene v-erb codes for a truncated EGF receptor that does not need to be activated by EGF, but is permanently turned on. Cells that express this structurally abnormal EGF receptor proliferate autonomously. Similarly the v-fms oncogene codes for an altered CSF-1 (M-CSF) receptor that is permanently activated independent of its ligand (99,121). When introduced into factor dependent cell lines, cells are generated that proliferate factor independent (145).

- Overexpression of membrane receptors for HGFs

  Certain hematopoietic cell lines that have been transformed by retroviruses containing the v-H-ras oncogene are tumorigenic and grow factor independent **in vitro** (146). Although a causal relation with tumorigenicity has not been shown, these cell lines express extremely high numbers of IL-3, GM-CSF or M-CSF receptors. Similarly, introduction of the normal human M-CSF receptor into certain IL-3 dependent cell lines results in the generation of factor independent cell lines that are tumorigenic and that express extraordinary numbers of M-CSF receptors (147). Possibly dimerization of receptors occurs spontaneously in those instances due to their high numbers, which results in factor independent signalling.

- Introduction and permanent activation of tyrosine kinases

  M-CSF receptors are tyrosine kinases (99). Following stimulation with its ligand, IL-3 receptors and cellular substrates are tyrosine

phosphorylated as well (112-114). Receptor growth control may be bypassed by the introduction of permanently activated tyrosine kinases, such as v-src (148), v-abl (149,150) or v-trk (114).

Cell lines that become factor independent give tumors when injected into syngeneic animals, whereas the factor dependent control lines are nontumorigenic. These results illustrate that tumor formation in these experimental leukemias is at least a two-step process, i.e. 1) immortalization as a result of insertional mutagenesis, or introduction of a certain oncogene and 2) loss of requirement of exogenous growth factors, e.g. by introduction or activation of a (proto)oncogene product, expression of altered receptors, overexpression of receptors or induction of an autocrine mechanism (possibly by activation of intracellular receptors). The idea that tumorigenesis in mice is indeed a multistep process, is confirmed by investigations showing that introduction of IL-3 genes into murine bone marrow progenitors renders them autocrine but does not create acute leukemias (151). Thus the cells are not immortalized and a second event is needed to fully transform these cells.

TABLE 4. Mechanisms that determine loss of requirement of exogenous HGFs, leading to neoplastic growth of HGF dependent nontumorigenic animal cell lines.

Mechanisms	Examples	References
- Autocrine stimulation (possibly by activation of intracellular receptors)	IL3, GM-CSF	128,133-140
- Permanently activated (mutated) HGF receptors	M-CSF receptor (fms oncogene)	141
- Overexpression of HGF receptors	IL3, GM-CSF or M-CSF receptors	142,143
- Permanently activated tyrosine kinases	v-src, v-abl, v-trk oncogenes	144-147

Whether the mechanisms of leukemogenesis observed in animals are also involved in neoplastic growth of primary human AMLs is largely unknown. Detailed insight into the responses of AML cells to growth factors, receptor binding studies, investigations of intracellular signal transduction pathways following receptor activation, e.g. phosphorylation of HGF receptors and of cellular substrates should provide information about the mechanisms that may determine neoplastic growth of AML. The studies presented in this thesis deal with a detailed analysis of the growth factor responses of AML cells in vitro (Chapters 4-9). Since loss of exogenous growth factor requirement is an important feature of experimental leukemias, it is investigated whether in certain cases of human AML the cells proliferate independent of HGFs as well (Chapter 5). In Chapter 10 it is studied whether spontaneous proliferation of AML cells, which is evident in approximately 50% of the cases as determined in a ^{3}H-TdR uptake assay (Chapter 5), can be attributed to autocrine stimulation or to non-autocrine autonomous growth of the cells.

1.9. *Introduction to the experimental work*

The studies presented in this thesis deal with characteristics of primary human AML cells **in vitro.** The first experiments are concerned with the phenotypic characterization of AML subsets capable of proliferation **in vitro.** The expression of α-L-fucose, determined by the fucose binding lectin Ulex europaeus agglutinin (UEA) (Chapter 2), CD34 (My10 or BIC3-5) and CD_w65 (Vim-2) (Chapter 3) on AML progenitor cells (AML-CFU) was studied. The presence of these markers on AML-CFUs was compared with surface marker expression on normal bone marrow progenitor cells (CFU-GEMM, CFU-GM and BFU-e). These studies were carried out with the purpose to define discrepancies between normal CFUs and AML-CFUs, which could be of use in the diagnosis of AML, in particular for the detection of minimal numbers of AML cells in remission bone marrow. To verify the utility of phenotypic differences for the detection of minimal residual disease, experiments were carried out to trace low numbers of AML cells in artificial mixtures with normal marrow cells. In Chapter 4 it was investigated whether IL-3, GM-CSF and G-CSF can replace feeder leukocytes in the PHA leukocyte feeder assay (PHA l.f.) and stimulate AML-CFU proliferation. In a subsequent study (Chapter 5), a serum-free culture method is introduced and the role of the five HGFs, IL-3, GM-CSF, G-CSF, M-CSF and Epo in the growth of AML was investigated. These experiments were conducted with single as well as combined factors in colony cultures (methylcellulose) and in suspension cultures (^{3}H-TdR incorporation). The ability of these factors to induce maturation of AML cells **in vitro** was examined in the study presented in Chapter 6. In Chapter 7 the role of TNF, described as an inhibitor of hematopoietic cell growth (92), was investigated. The effects of TNF on factor dependent proliferation was assessed serum-free for each of the growth factors, IL-3, GM-CSF, G-CSF and M-CSF. In Chapters 8 and 9 the role of IL-1, initially also designated hemopoietin-1 and an inducer of growth factor production (68-74) was investigated. It was studied whether IL-1 could stimulate AML proliferation and if so, whether the stimulatory effects of IL-1 were direct or mediated through the induction of GM-CSF (Chapter 8) or TNF production (Chapter 9) by AML blasts. While it appears from the results of these experiments that autocrine growth in AML may be regulated by IL-1, in certain other cases leukemic blast cells may produce their own HGFs constitutively, i.e. nonregulated (152-154). Therefore, in Chapter 10 a comparative analysis of IL-1 regulated and spontaneous growth of AML cells was performed. It was

investigated which endogenously produced HGFs are involved in IL-1 stimulated and spontaneous proliferation of AML blasts. It was also studied whether non-autocrine autonomous growth may occur in certain cases of AML. Several aspects of growth activation of AML cells are discussed in Chapter 11.

1.10. *References*

1. Dexter TM, Allen TD and Lajtha LG. Conditions controlling the proliferation of haemopoietic stem cells in vitro. J Cell Physiol 91: 335, 1977.
2. Clark S and Kamen R. The human hematopoietic colony-stimulating factors. Science 236:1229, 1987.
3. Foon KA and Todd RF, III. Immunologic classification of leukemia and lymphoma. Blood 68:1, 1986.
4. Dongen JJM van, Adriaansen HJ and Hooijkaas H. Immunophenotyping of leukaemias and non-Hodgkin's lymphomas. Immunological markers and their CD codes. Neth J Med 33:298, 1988.
5. French-American-British (FAB) Cooperative Group: Proposals for the classification of the acute leukemias. Br J Haematol 33:451, 1976.
6. French-American-British (FAB) Cooperative Group: proposed revised criteria for the classification of acute myeloid leukemia. Ann Intern Med 103:620, 1985.
7. Fialkow PJ, Jacobson RJ and Papayannopoulou T. Chronic myelocytic leukemia: Clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. Am J Med 63:125, 1977.
8. Bloomfield CD and de la Chapelle A. Chromosome abnormalities in acute nonlymphocytic leukemia: clinical and biological significance. Semin Oncol 14:372, 1987.
9. Fialkow PJ, Singer JW, Adamson JW, Vaidya K, Dow LW, Ochs J and Moohr JW. Acute nonlymphocytic leukemia: heterogeneity of stem cell origin. Blood 57:1068, 1981.
10. Fearon ER, Burke PJ, Schiffer CA, Zehnbauer BA and Vogelstein B. Differentiation of leukemia cells to polymorphonuclear leukocytes in patients with acute nonlymphocytic leukemia. New Engl J Med 315:15, 1986.
11. Fialkow PJ, Singer JW, Raskind WH, Adamson JW, Jacobson RJ, Bernstein ID, Dow LW, Najfeld V and Veith R. Clonal development, stem cell differentiation, and clinical remissions in acute nonlymphocytic leukemia. New Engl J Med 317:468, 1987.
12. Keinänen M, Griffin JD, Bloomfield CD, Machnicki J and de la Chapelle A. Clonal chromosomal abnormalities showing multiple-cell-lineage involvement in acute myeloid leukemia. New Engl J Med 318:1153, 1988.

13. Trujillo JM, Cork A, Ahearn MJ, Youness EL and McCredie KB. Hematologic and cytologic characterization of 8;21 translocation acute granulocytic leukemia. Blood 53:695, 1979.
14. Rowley JD, Golomb HM and Dougherty C. 15;17 translocation, a consistent chromosomal change in acute promyelocytic leukemia. Lancet i:549, 1977.
15. Arthur DC and Bloomfield CD. Partial deletion of the long arm of chromosome 16 and bone marrow eosinophilia in acute nonlymphocytic leukemia: a new association. Blood 61:994, 1983.
16. Holmes R, Keating MJ, Cork A, Broach Y, Trujillo J, Dalton WT, McCredie K and Freireich EJ. A unique pattern of central nervous system leukemia in acute myelomonocytic leukemia associated with inv(16)(p13q22). Blood 65:1071, 1985.
17. Greaves MF, Chan LC, Furley AJW, Watt SM and Molgaard HV. Lineage promiscuity in hemopoietic differentiation and leukemia. Blood 67:1, 1986.
18. Knapp W, Dorken B, Rieber P, Schmidt RE, Stein H and Borne AEGKr von dem. CD antigens 1989. Blood 74:1448, 1989.
19. Griffin JD, Mayer RJ, Weinstein HJ, Rosenthal DS, Coral FS, Beveridge RP, Schlossman SF. Surface marker analysis of acute myeloblastic leukemia: Identification of differentiation-associated phenotypes. Blood 62:557, 1983.
20. Reijden HJ van der, Rhenen DJ van, Lansdorp PM, Veer MB van 't, Langenhuijsen MMAC, Engelfriet CP and Borne AEGKr von dem. A comparison of surface marker analysis and FAB classification in acute myeloid leukemia. Blood 61:443, 1983.
21. Perussia B, Trinchieri G, Lebman D, Jankiewicz J, Lange B and Rovera G. Monoclonal antibodies that detect differentiation surface antigens on human myelomonocytic cells. Blood 59:382, 1982.
22. Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF and Shaper JH. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J Immunol 133:157, 1984.
23. Tindle RW, Nichols RAB, Chan L, Campana D, Catovsky D and Birnie GD. A novel monoclonal antibody BI-3C5 recognises myeloblasts and non-B non-T lymphoblasts in acute leukemias and CGL blast crises, and reacts with immature cells in normal bone marrow. Leuk Res 9:1, 1985.

24. Lange B, Ferrero D, Pessano S, Palumbo A, Faust J, Meo P and Rovera G. Surface phenotype of clonogenic cells in acute myeloid leukemia defined by monoclonal antibodies. Blood 64:693, 1984.
25. Sabbath KD, Ball ED, Larcom P, Davis RB and Griffin JD. Heterogeneity of clonogenic cells in acute myeloblastic leukemia. J Clin Invest 75:746, 1985.
26. Griffin JD and Löwenberg B. Clonogenic cells in acute myeloblastic leukemia. Blood 68:1185, 1986.
27. Pike BL and Robinson WA. Human bone marrow colony growth in agar gel. J Cell Physiol 76:77, 1970.
28. Robinson WA, Kurnick JE and Pike BL. Colony growth of human leukemic peripheral blood cells in vitro. Blood 38:500, 1971.
29. Fauser AA and Messner HA. Granuloerythropoietic colonies in human bone marrow, peripheral blood and cord blood. Blood 52:1243, 1978.
30. Bot FJ, Dorssers L, Wagemaker G and Löwenberg B. Stimulating spectrum of human recombinant multi-CSF (IL-3) on human marrow precursors: importance of accessory cells. Blood 71:1609, 1988.
31. Minden MD, Till JE and McCulloch EA. Proliferative state of blast cell progenitors in acute myeloblastic leukemia (AML). Blood 52:592, 1978.
32. Francis GE, Berney JJ, Tuma GA, Wing MA and Hoffbrand AV. Divergent sensitivities of leukaemic cells to human placental conditioned medium and leukocyte feeder layers. Leuk Res 4:531, 1980.
33. Hoang T and McCulloch EA. Production of leukemic blast growth factor by a human bladder carcinoma cell line. Blood 66:748, 1985.
34. Moore MAS and Metcalf D. Cytogenetic analysis of human acute and chronic myeloid leukemic cells cloned in agar culture. Int J Cancer 11:143, 1973.
35. Moore MAS, Spitzer G, Williams N, Metcalf D and Buckley J. Agar culture studies in 127 cases of untreated acute leukemia. The prognostic value of reclassification of leukemia according to in vitro growth characteristics. Blood 44:1, 1974.
36. Marie JP, Izaguirre CA, Civin CI, Mirro J and McCulloch EA. Granulopoietic differentiation in AML blasts in culture. Blood 58:670, 1981.
37. Touw IP and Löwenberg B. Variable differentiation of human acute myeloid leukaemia during colony formation in vitro: a membrane marker analysis with monoclonal antibodies. Br J Haematol 59:37, 1985.

38. Dicke KA, Spitzer G and Ahearn MJ. Colony formation in vitro by leukaemic cells in acute myelogenous leukaemia with phytohaem-agglutinin as stimulating factor. Nature 259:129, 1976.
39. Dicke KA, Tindle SE, Davis FM, Jasgannath S, Tucker S, Lilien M, Leeuwen P van, Verma DA and Vellekoop L. Leukemic cell colony formation in soft agar by bone marrow cells and peripheral blood cells from untreated acute leukemia patients. Exp Haematol 11:341, 1983.
40. Löwenberg B, Swart K and Hagemeijer A. PHA-induced colony formation in acute non-lymphocytic and chronic myeloid leukemia. Leuk Res 4:143, 1980.
41. Platzer E. Human hemopoietic growth factors. Eur J Haematol 42:1, 1989.
42. Nagata S, Tsuchiya M, Asano S, Kaziro Y, Yamazaki T, Yamamoto O, Hirata Y, Kubota N, Oheda M, Nomura H and Ono M. Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor. Nature 319:415, 1986.
43. Souza LM, Boone TC, Gabrilove J, Lai PH, Zsebo KM, Murdock DC, Chazin VR, Bruszewski J, Lu H, Chen KK, Barendt J, Platzer E, Moore MAS, Mertelsmann R and Welte K. Recombinant human granulocyte colony stimulating factor: effects on normal and leukemic myeloid cells. Science 232:61, 1986.
44. Kawasaki ES, Ladner MB, Wang AM, Arsdell J van, Warren MK, Coyne MY, Schweickart VL, Lee MT, Wilson KJ, Boosman A, Stanley ER, Ralph P and Mark DF. Molecular cloning of a complementary DNA encoding human macrophage specific colony-stimulating factor (CSF-1). Science 230:291, 1985.
45. Lin FK, Suggs S, Lin CH, Browne J, Smalling R, Egrie JL, Chen K, Fox G, Martin F, Stabinsky Z, Badrawi S, Lai PH and Goldwasser E. Cloning and expression of the human erythropoietin gene. Proc Natl Acad Sci USA 82:7580, 1985.
46. Jacobs K, Shoemaker C, Rudersdorf R, Neill SD, Kaufman RJ, Mufson A, Seehra J, Jones SS, Hewick R, Fritsch EF, Kawakita M, Shimizu T and Miyake T. Isolation and characterization of genomic and cDNA clones of human erythropoietin. Nature 313:806, 1985.
47. Campbell HD, Tucker WQJ, Hort Y, Martinson ME, Mayo G, Clutterbuck EJ, Sanderson CJ and Young IG. Molecular cloning, nucleotide sequence, and expression of the gene encoding human eosinophil differentiation factor (interleukin-5). Proc Natl Acad Sci USA 84:6629, 1987.

48. Ikebuchi K, Clark SC, Ihly JN, Souza LM and Ogawa M. Granulocyte colony stimulating factor enhances interleukin-3-dependent proliferation of multipotential hemopoietic progenitors. Proc Natl Acad Sci USA 85:3445, 1988.

49. Yang YC, Ciarletta AB, Temple PA, Chung MP, Kovacic S, Witek-Giannotti JS, Leary AC, Kriz R, Donahue RE, Wong GG and Clark SC. Human IL-3 (Multi-CSF): Identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. Cell 47:3, 1986.

50. Dorssers L, Burger H, Bot F, Delwel R, Geurts van Kessel AHM, Löwenberg B and Wagemaker G. Characterization of a human multi-CSF cDNA clone identified by a conserved noncoding sequence in mouse interleukin-3. Gene 55:115, 1987.

51. Wong GG, Witek JS, Temple PA, Wilkins KM, Leary AC, Luxenberg DP, Jones SS, Brown EL, Kay RM, Orr EC, Shoemaker C, Golde DW, Kaufman RJ, Hewick RM, Wang EA and Clark SC. Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science 228:810, 1985.

52. Bot FJ, Eijk L van, Schipper P and Löwenberg B. Human granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates immature marrow precursors but no CFU-GM, CFU-G, or CFU-M. Exp Hematol 17:292, 1989.

53. Morris CF, Young IG and Hapel AJ. Molecular and cellular biology of interleukin-3. In: Colony Stimulating Factors. Molecular and Cellular Biology. Eds. Dexter TM, Garland JM and Testa NG. 49:177, 1990.

54. Gough NM and Nicola NA. Granulocyte-Macrophage Colony-Stimulating Factor. In: Colony Stimulating Factors. Molecular and Cellular Biology. Eds: Dexter TM, Garland JM and Testa NG. 49:111, 1990.

55. Valent P, Schmidt G, Besemer J, Mayer P, Zenke G, Liehl E, Hinterberger W, Lechner K, Maurer D and Bettelheim P. Interleukin-3 is a differentiation factor for human basophils. Blood 73:1763, 1989.

56. Lopez AF, Williamson DJ, Gamble JR, Begley CG, Harlan JM, Klebanoff SJ, Waltersdorph A, Wong G, Clark SC and Vadas MA. Recombinant human granulocyte-macrophage colony-stimulating factor stimulates in vitro mature human neutrophil and eosinophil function, surface receptor expression, and survival. J Clin Invest 78:1220, 1986.

57. Nicola NA. Granulocyte Colony-Stimulating Factor. In: Colony Stimulating Factors. Molecular and Cellular Biology. Eds: Dexter TM, Garland JM and Testa NG. 49:77, 1990.

58. Ralph P, Warren MK, Nakoinz I, Lee MT, Brindley L, Sampson-Johannes A, Kawasaki ES, Ladner MB, Strickler JE, Boosman A, Csejtey J and White TJ. Biological properties and molecular biology of human macrophage growth factor, CSF-1. Immunobiology 172:194, 1986.
59. Sanderson CJ. Eosinophil differentiation factor (Interleukin-5). In: Colony Stimulating Factors. Molecular and Cellular Biology. Eds: Dexter TM, Garland JM and Testa NG. 49:231, 1990.
60. March CJ, Mosley B, Larsen A, Cerretti DP, Braedt G, Price V, Gillis S, Henney CS, Kronheim SR, Grabstein K, Conlon PJ, Hopp TP and Cossman D. Cloning, sequence and expression of distinct human interleukin-1 complementary DNAs. Nature 315:641, 1985.
61. Devos R, Plaetnick G, Geroutre H, Simons G, Degrave W, Taveriner J, Remant E and Fiers W. Molecular cloning of human interleukin-2 cDNA and its expression in E. coli. Nucleic Acids Res 11:4307, 1983.
62. Yokota T, Otsuka T, Mosmann T, Banchereau J, DeFrance T, Blanchard D, Vries JE de, Lee F and Arai KI. Isolation and characterization of a human interleukin cDNA clone, homologous to mouse B-cell stimulatory factor 1, that expresses B-cell and T-cell-stimulating activities. Proc Natl Acad Sci USA 83:5894, 1986.
63. Brakenhoff JPJ, Groot ER de, Evers RF, Pannekoek H and Aarden LA. Molecular cloning and expression of hybridoma growth factor in Escherichia coli. J Immunol 139:4116, 1987.
64. Goodwin RG, Lupton S, Schemierer A, Hjerrild KJ, Jerzy R, Clevenger W, Gillis S, Cosman D and Namen AE. Human interleukin 7: Molecular cloning and growth factor activity on human and murine B-lineage cells. Proc Natl Acad Sci USA 86:302, 1989.
65. Pennica D, Nedwin GE, Hayflick JS, Seeburg PH, Derynck R, Palladino MA, Kohr WJ, Aggerwal BB and Goeddel DV. Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature 312:724, 1984.
66. Gray PW, Aggerwal BB, Benton CV, Bringman TS, Henzel WJ, Jarrett JA, Leung DW, Moffat B, Ng P, Svedersky LP, Palladino MA and Nedwin GE. Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity. Nature 312:721, 1984.
67. Oppenheim JJ, Kovacs EJ, Matsushima K and Durum SK. There is more than one interleukin-1. Immunol Today 7:45, 1986.

68. Broudy VC, Kaushansky K, Harlan JM and Adamson JW. Interleukin-1 stimulates human endothelial cells to produce granulocyte-macrophage colony stimulating factor and granulocyte colony stimulating factor. J Immunol 139:464, 1987.
69. Kaushansky K, Lin N and Adamson JW: Interleukin-1 stimulates fibroblasts to synthesize granulocyte-macrophage and granulocyte colony stimulating factors. J Clin Invest 81:92, 1988.
70. Herrmann F, Oster W, Meuer SC, Lindemann A and Mertelsmann RH. Interleukin-1 stimulates T-lymphocytes to produce granulocyte-monocyte colony stimulating factor. J Clin Invest 81:1415, 1988.
71. Damme J van, Cayphas S, Opdenakker G, Billiau A and van Snick J. Interleukin-1 and poly(rI).poly(rC) induce production of a hybridoma growth factor by human fibroblasts. Eur J Immunol 17:1-7.
72. Stanley ER, Bartocci A, Patinkin D, Rosendaal M and Bradley TR. Regulation of very primitive, multipotent, hemopoietic cells by hemopoietin-1. Cell 45:667, 1986.
73. Mochizuki DY, Eisenman JR, Conlon PJ, Larsen AD and Tushinski RJ. Interleukin-1 regulates hematopoietic activity, a role previously ascribed to hemopoietin-1. Proc Natl Acad Sci USA 84:5267, 1987.
74. Ikebuchi K, Ihle JN, Hirai Y, Wong GG, Clark SC and Ogawa M. Synergistic factors for stem cell proliferation: further studies of the target stem cells and the mechanisms of stimulation by interleukin-1, interleukin-6, and granulocyte colony-stimulating factor. Blood 72:2007, 1988.
75. Ikebuchi K, Wong GG, Clark SC, Ihly JN, Hirai Y and Ogawa M. Interleukin-6 enhancement of interleukin-3-dependent proliferation of multipotential hemopoietic progenitors. Proc Natl Acad Sci USA 84:9035, 1987.
76. Laery AG, Ikebuchi K, Hirai Y, Wong GG, Yang YC, Clark SC and Ogawa M. Synergism between interleukin-6 and interleukin-3 in supporting proliferation of human hematopoietic stem cells: comparison with interleukin-1α. Blood 71:1759, 1988.
77. Smith KA. Interleukin-2: inception, impact and implications. Science 240:1169, 1988.
78. Herrmann F, Cannistra SA, Levine H and Griffin JD. Expression of interleukin-2 receptors and binding of interleukin-2 by gamma interferon-induced human leukemic and normal monocytic cells. J Exp Med 162:1111, 1985.

79. Yamamoto S, Hattori T, Matsuoka M, Ishii T, Asou N, Okada M, Tagaya Y, Yodoi J and Takatsuki K. Induction of Tac antigen and proliferation of myeloid leukemic cells by ATL-derived factor: comparison with other agents that promote differentiation of human myeloid or monocytic leukemic cells. Blood 67:1714, 1986.
80. Visani G, Delwel R, Touw IP, Bot FJ and Löwenberg B. Membrane receptors for interleukin-2 on hematopoietic precursors in chronic myeloid leukemia. Blood 69:1182, 1987.
81. Cosman D. Colony-stimulating factors in vivo and in vitro. Immunol Today 9:97, 1988.
82. Vellenga E, Wolf JThM de, Beentjes JAM, Esselink MT, Smit JW and Halie MR. Divergent effects of interleukin-4 (IL-4) on the granulocyte colony-stimulating factor and IL-3-supported myeloid colony formation from normal and leukemic bone marrow cells. Blood 75:633, 1990.
83. Kishimoto T. The biology of Interleukin-6. Blood 74:1, 1989.
84. Bot FJ, Eijk L van, Broeders L, Aarden LA and Löwenberg B. Interleukin-6 synergizes with M-CSF in the formation of macrophage colonies from purified human marrow progenitor cells. Blood 73:435, 1989.
85. Hoffman R. Regulation of megakaryocytopoiesis. Blood 74:1196, 1989.
86. Touw I, Pouwels K, Agthoven T van, Gurp R van, Budel L, Hoogerbrugge H, Delwel R, Goodwin R, Namen A and Löwenberg B. Interleukin-7 is a growth factor of precursor B and T acute lymphoblastic leukemia. Blood 75:2097, 1990.
87. Welch PA, Namen AE, Goodwin RG, Armitage R and Cooper MD. Human IL-7: a novel T cell growth factor. J Immunol 143:3562, 1989.
88. Gimbrone MA, Obin MS, Brock AF, Luis EA, Hass PE, Hébert CA, Yip YK, Leung DW, Lowe DG, Kohr WJ, Darbonne WC, Bechtol KB and Baker JB. Endothelial interleukin-8: a novel inhibitor of leukocyte- endothelial interactions. Science 246:1601, 1989.
89. Yang YC, Ricciardi S, Ciarletta J, Calvetti J, Kelleher K and Clark SC. Expression cloning of a cDNA encoding a novel human hematopoietic growth factor: human homologue of murine T-cell growth factor P40. Blood 74:1880, 1989.
90. Donahue RE, Yang Y, Paul S and Clark SC. Human interleukin-9 is capable of stimulating erythroid colony formation in vitro. Blood 74 (suppl.1):116a, 1989.

91. Kehrl JH, Alvarez-Mon M, Delsing GA and Fauci AS. Lymphotoxin is an important T cell-derived growth factor for human B cells. Science 238:1144, 1987.
92. Murase T, Hotta T, Saito H and Ohno R. Effect of recombinant human tumor necrosis factor on the colony growth of human leukemia progenitor cells and normal hematopoietic progenitor cells. Blood 69:467, 1987.
93. Goldwasser E, Beru N and Smith D. Erythropoietin. In: Colony Stimulating Factors. Molecular and Cellular Biology. Eds: Dexter TM, Garland JM and Testa NG. 49:257, 1990.
94. Rigby WFC, Ball ED, Guyre PM and Fanger MW. The effects of recombinant-DNA-derived interferons on the growth of myeloid progenitor cells. Blood 65:858, 1985.
95. Gough NM, Gearing DP, King JA, Willson TA, Hilton DJ, Nicola NA and Metcalf D. Molecular cloning and expression of the human homologue of the murine gene encoding myeloid leukemia-inhibitory factor. Proc Natl Acad Sci USA 85:2623, 1988.
96. Taetle R and Koessler A. Effects of cyclic nucleotides and prostaglandins on normal and abnormal human myeloid progenitor proliferation. Cancer Res 40:1223, 1980.
97. Ottman OG and Pelus LM. Differential proliferative effects of transforming growth factor-β on human hematopoietic progenitor cells. J Immunol 140:2661, 1988.
98. Ullrich A and Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. Cell 61:203, 1990.
99. Sherr CJ. Colony-stimulating factor-1 receptor. Blood 75:1, 1990.
100. Hatakeyama M, Tsudo M, Minamoto S, Kono T, Doi T, Miyata T, Miyasaka M and Taniguchi T. Interleukin-2 receptor β chain gene: Generation of the three receptor forms by cloned human α and β chain cDNA's. Science 244:551, 1989.
101. Taga T, Hibi M, Hirata Y, Yamasaki K, Yasukawa K, Matsuda T, Hirano T and Kishimoto T. Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. Cell 58:573, 1989.
102. Itoh N, Yonehara S, Schreurs J, Gorman DM, Maruyama K, Ishii A, Yahara I, Arai KI and Miyajima A. Cloning of an interleukin-3 receptor gene: a member of a distinct receptor gene family. Science 247:324, 1990.

103. Mosley B, Beckman MP, March CJ, Idzerda RL, Gimpel SD, VandenBos T, Alpert A, Anderson D, Jackson J, Wignall JM, Smith C, Gallis B, Sims JE, Urdal D, Widmer MB, Cosman D and Park LS. The murine interleukin-4 receptor: molecular cloning and characterization of secreted and membrane bound forms. Cell 59:335, 1989.
104. Gearing DP, King JA, Gough NM and Nicola NA. Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor. EMBO J 8:3667, 1989.
105. D'Andrea AD, Lodish HF and Wong GG. Expression cloning of the receptor of the murine erythropoietin receptor. Cell 57:277, 1989.
106. Gaustin AC, Leof EB, Shipley GD and Moses HL. Growth factors and cancer. Cancer Res 46:1015, 1986.
107. Lord JM, Brunce CM and Brown G. The role of protein phosphoryation in the control of cell growth and differentiaton. Br J Cancer 58:549, 1988.
108. Neer EJ and Clapham DE. Roles of G protein subunits in transmembrane signalling. Nature 333:129, 1988.
109. Berridge MJ and Irvine RF. Inositol phosphates and cell signalling. Nature 341:197, 1989.
110. Stoscheck CM and King LE. Role of epidermal growth factor in carcinogenesis. Cancer Res 46:1030, 1986.
111. Williams LT. Signal transduction by the platelet-derived growth factor receptor. Science 243:1564, 1989.
112. Morla AO, Schreurs J, Miyajima A and Wang JYJ. Hematopoietic growth factors activate the tyrosine phosphorylation of distinct sets of proteins in interleukin-3-dependent murine cell lines. Mol Cell Biol 8:2214, 1988.
113. Isfort RJ, Stevens D, May WS and Ihle JN. Interleukin-3 binds to a 140-kDa phosphotyrosine-containing cell surface protein. Proc Natl Acad Sci USA 85:7982, 1988.
114. Isfort RJ, Huhn RD, Frackelton AR and Ihle JN. Stimulation of factor-dependent myeloid cell lines with interleukin-3 induces tyrosine phosphorylation of several cellular substrates. J Biol Chem 263:19203, 1988.
115. Whetton AD, Monk PN, Consalvey SD, Huang SJ, Dexter TM and Downes CP. Interleukin-3 stimulates proliferation via protein kinase C activation without increasing inositol lipid turnover. Proc Natl Acad Sci USA 85:3284, 1988.

116. Rettenmier RM, Roussel MF and Sherr CJ. The colony stimulating factor 1 (CSF-1) receptor (c-fms proto-oncogene product) and its ligand. J Cell Sci Suppl 9:27, 1988.
117. Druker BJ, Mamon HJ and Roberts TM. Oncogenes, growth factors, and signal transduction. N Engl J Med 321:1383, 1989.
118. Bishop JM. The molecular genetics of cancer. Science 235:305, 1987.
119. Waterfield MD, Scrace GT, Whittle N, Stroobant P, Johnsson A, Wasteson A, Westermark B, Heldin CH, Huang JS and Deuel TF. Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. Nature 304:35, 1983.
120. Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ulrich A, Schlessinger J and Waterfield MD. Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature 307:521, 1984.
121. Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT and Stanley ER. The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell 41:665, 1985.
122. Weinberg RA. RAS oncogenes and the molecular mechanisms of carcinogenesis. Blood 64:1143, 1984.
123. Witte ON, Dasgupta A and Baltimore D. Abelson murine leukemia virus protein is phosphorylated in vitro to form phosphotyrosine. Nature 283:826, 1980.
124. Collett MS and Erikson RL. Protein kinase activity associated with avian sarcoma virus src gene product. Proc Natl Acad Sci USA 75:2021, 1978.
125. Curran T, Mac Connell WP, Straaten F van and Verma IM. Structure of the FBJ murine virus genome: molecular cloning of its associated helper virus and the cellular homolougs of the v-fos gene from mouse and human cells. Mol Cell Biol 3:914, 1983.
126. Alitalo K, Ramsay G, Bishop JM, Pfeifer SO, Colby WW and Levinson AD. Identification of nuclear protein encoded by viral and cellular myc oncogenes. Nature 306:274, 1983.
127. Bishop JM. The molecular genetics of cancer. Leukemia 2:199, 1988.
128. Leder P, Battey J, Lenoir G, Moulding C, Murphy W, Potter H, Stewart T and Taub R. Translocations among antibody genes in human cancer. Science 222:767, 1983.
129. Klein G and Klein E. Evolution of tumours and the impact of molecular oncology. Nature 315:190, 1985.

130. Heisterkamp N, Stephenson JR, Groffen J, Hansen PF, Klein A de, Bartram CR and Grosveld G. Localization of the c-abl oncogene adjacent to a translocation break point in chronic myelocytic leukaemia. Nature 306:239, 1983.
131. Shtivelman E, Lifshitz B, Gale RP and Canaani E. Fused transcript of abl and bcr genes in chronic myelogenous leukaemia. Nature 315:550, 1985.
132. Stocking C, Löliger C, Kawai M, Suciu S, Gough N and Ostertag W. Identification of genes involved in growth autonomy of hematopoietic cells by analysis of factor independent mutants. Cell 53:869, 1988.
133. Ihly JN, Rein A and Mural R. Immunologic and virologic mechanisms in retrovirus induced murine leukemogenesis. In: G Klein (ed): Advances in viral oncology, vol 4. New York, Raven: 95, 1984.
134. Dexter TM, Garland JM, Scott D, Scolnick E and Metcalf D. Growth of factor dependent hemopoietic precursor cell lines. J Exp Med 152:1036, 1980.
135. Greenberger JS, Hapel A, Nabel G, Eckner RJ, Newburger PE, Ihly J, Denburg J, Moloney WC, Sakakeeny M and Humphries K. Effect of murine leukemia virusses on establishment, growth and differentiation of permanent factor dependent committed and pluripotential hematopoietic stem cell lines in vitro. In: SJ Baum, GD Ledner and S Thierkelder (eds) Experimental hematology today. Karger S, New York: 195, 1982.
136. Weinstein Y, Ihly JN, Lavu S and Reddy EP. Truncation of the c-myb gene by a retroviral integration in an interleukin 3-dependent myeloid leukemia cell line. Proc Natl Acad Sci USA 85:5010, 1986.
137. Ymer S, Tucker WQJ, Sanderson CJ, Hapel AJ, Campbell HD and Young IG. Constitutive synthesis of interleukin-3 by leukaemia cell line WEHI-3B is due to retroviral insertion near the gene. Nature 317:255, 1985.
138. Browder TM, Abrams JS, Wong PMC, Nienhuis AW. Mechanisms of autocrine stimulation in hematopoietic cells producing interleukin-3 after retrovirus mediated gene transfer. Mol Cell Biol 9:204, 1989.
139. Wong PMC, Chung SW and Nienhuis AW. Retroviral transfer and expression of the interleukin-3 gene in hemopoietic cells. Genes Develop 1:358, 1987.
140. Lang RA, Metcalf D, Gough NM, Dunn AR and Gonda TJ. Expression of a hemopoietic growth factor cDNA in a factor dependent cell line results in autonomous growth and tumorigenicity. Cell 43:531, 1985.

141. Laker C, Stocking C, Bergholz U, Hess N, DeLamarter JF and Ostertag W. Autocrine stimulation after transfer of the granulocyte/macrophage colony-stimulating factor gene and the autonomous growth are distinct but interdependent steps in the oncogenic pathway. Proc Natl Acad Sci USA: 84:8458, 1987.

142. Nair APK, Diamantis ID, Conscience JF, Kindler V, Hofer P and Moroni C. A v-H-ras-dependent hemopoietic tumor model involving progression from a clonal stage of transformation competence to autocrine interleukin-3 production. Mol Cell Biol 9:1183, 1989.

143. Graf T, Weizsaecker F von, Grieser S, Coll J, Stehelin D, Patschinsky T, Bister K, Bechade C, Calothy G and Leutz A. v-mil induces autocrine growth and enhanced tumorigenicity in v-myc-transformed avian macrophages. Cell 45:357, 1986.

144. Dunbar CE, Browder TM, Abrams JS and Nienhuis AW. COOH-terminal modified interleukin-3 is retained intracellularly and stimulates autocrine growth. Science 245:1493, 1989.

145. Wheeler EF, Rettenmier CW, Look AT and Sherr CJ. The v-fms oncogene induces factor independence and tumorigenicity in CSF-1 dependent murine macrophage cell line. Nature 324:377, 1986.

146. Klingler K, Johnson GR, Walker F, Nicola NA, Decker T and Ostertag W. Macrophage cell lines transformed by the malignant histiocytosis sarcoma virus: increase of CSF receptors suggests a model for transformation. J Cell Physiol 132:22, 1987.

147. Kato JY, Roussel MF, Ashmun RA and Sherr CJ. Transduction of human colony-stimulating factor-1 (CSF-1) receptor into interleukin-3 dependent mouse myeloid cells induces both CSF-1-dependent and factor-independent growth. Mol Cell Biol 9:4069, 1989.

148. Watson JD, Eszes M, Overell R, Conlon P, Widmer M and Gillis S. Effect of infection with murine recombinant retroviruses containing the v-src oncogene on interleukin 2- and interleukin 3-dependent growth states. J Immunol 139:123, 1987.

149. Cook WD, Metcalf D, Nicola NA, Burgess AW and Walker F. Malignant transformation of a growth factor-dependent myeloid cell line by abelson virus without evidence of an autocrine mechanism. Cell 41:677, 1985.

150. Oliff A, Agranovsky O, McKinney MD, Murty VVVS and Bauchwitz R. Friend murine leukemia virus-immortalized myeloid cells are converted into tumorigenic cell lines by abelson leukemia virus. Proc Natl Acad Sci USA 82:3306, 1985.

151. Wong PMC, Chung SW, Dunbar CE, Bodine DM, Ruscetti S and Nienhuis AW. Retrovirus-mediated transfer and expression of the interleukin-3 gene in mouse hematopoietic cells results in a myeloproliferative disorder. Mol Cell Biol 9:798, 1989.

152. Young DC, Wagner K and Griffin JD. Constitutive expression of the GM-CSF gene in acute myeloblastic leukemia. J Clin Invest 79:100, 1987.

153. Young DC, Demetri GD, Ernst TJ, Cannistra SA and Griffin JD. In vitro expression of colony-stimulating factor genes by human acute myeloblastic leukemia cells. Exp Hematol 16:378, 1988.

154. Oster W, Cicco NA, Klein H, Hirano T, Kishimoto T, Lindemann A, Mertelsmann RH and Herrmann F. Participation of the cytokines IL-6, TNF-alpha and IL-1-beta secreted by acute myelogenous leukemia blasts in autocrine and paracrine leukemia growth control. J Clin Invest 84:451, 1989.

CHAPTER 2

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Fucose Binding Lectin for Characterizing Acute Myeloid Leukemia Progenitor Cells

Fucose Binding Lectin for Characterizing Acute Myeloid Leukemia Progenitor Cells

By Ruud Delwel, Ivo Touw, Freek Bot, and Bob Löwenberg

The reactivity of acute myeloid leukemia cells (AML) was determined in 29 patients using the fucose binding lectin *Ulex europaeus* agglutinin (UEA) as surface marker. We show a marked heterogeneity in the UEA-binding abilities of the cells in these patients as determined by fluorescence analysis of the blasts labeled with the UEA coupled to the fluorescent molecule FITC. The results suggest a correlation between the capability of AML blast cells to bind UEA and cytologic maturation, because in 1 of 10 M1, 3 of 8 M2, 6 of 8 M4, and 1 of 3 M5 cytology types UEA binding to the leukemic cells was apparent. In 13 cases, the cells gave rise to colonies in vitro. The amount of UEA binding to AML colony-forming cells (AML-CFU) was determined by cell sorting and subsequent colony culture of UEA-negative, intermediately positive, and highly fluorescent cells. AML-CFU from none of the four patients with M1 cytology were UEA positive, whereas they showed intense reactivity with the lectin in 1 of 4 cases with M2 cytology and in all 4 cases of M4. In these five cases with strongly UEA positive AML-CFU, the fluorescence distribution of the colony formers differed from that of the total leukemia population, indicating that AML-CFU represent a subpopulation of AML cells with specific UEA-binding properties. Normal bone marrow myeloid and multipotential colony-forming cells (CFU-GM, CFU-GEMM) showed low or no binding of UEA. UEA-FITC appears a useful reagent for membrane analysis of AML-CFU. In certain cases, UEA-FITC labeling may be applied to discriminate AML-CFU from normal hematopoietic progenitors.

SEVERAL STUDIES have dealt with the detailed phenotyping of acute myeloid leukemia colony-forming cells (AML-CFU) in relation to normal bone marrow colony formers (CFU-GM, CFU-GEMM).[1-3] The results of those studies indicate that AML-CFU can be recognized as the leukemic counterparts of normal hematopoietic progenitor cells. These investigations were performed with monoclonal antibodies, which have become the most common tools to identify surface phenotypes.[4-14]

Lectins may also be of value to detect surface antigens. For example, the fucose-binding lectin from *Lotus tetragonolobus* (FBL-L) has been shown to react with human myeloid cells.[15-18] The amount of cellular binding of the lectin increases progressively from myeloblasts toward mature granulocytes; monocytes show intermediate reactivity. CFU-GM mainly belong to the low FBL-L–binding bone marrow fraction. No information exists on the usefulness of the lectin for analysis of AML-CFU.

In the present study, we labeled AML blasts with the fucose binding lectin (FBL) *Ulex europaeus* agglutinin coupled to fluorescein-isothiocyanate (UEA-FITC) to determine whether UEA-binding to AML-CFU is different from that of normal CFU-GM and CFU-GEMM. We also compared UEA binding patterns of AML-CFU with that of the total AML blast population and with morphological classification of AML according to the criteria of the French-American-British (FAB) group.[19]

It is shown that: (a) in certain cases, UEA binding to AML-CFU is markedly discrepant from that of normal CFU-GM and CFU-GEMM; (b) binding patterns of AML-CFU can be distinguished from those of total blast populations; and (c) UEA binding capacity of AML-CFU is positively correlated to cytologic (FAB) maturation.

MATERIALS AND METHODS

Cell separation. Cells from 29 patients with newly diagnosed AML and from three subjects without hematological disease were studied. AML cytology was classified according to the FAB criteria.[19] All individuals had given consent to donate bone marrow. AML blasts from bone marrow or peripheral blood were separated to >97% purity following discontinuous bovine serum albumin (BSA) density fractionation and E rosette sedimentation.[20-22] The cells were then cryopreserved in aliquots of 30×10^6 cells in 7.5% dimethyl sulfoxide (DMSO) and 20% fetal calf serum (FCS) using a controlled freezing apparatus and storage in liquid nitrogen as described.[23] Normal bone marrow cells were recovered from the interface after Ficoll-Isopaque centrifugation.[24]

Colony cultures for AML-CFU, CFU-GM, and CFU-GEMM. Colonies from AML cells were cultured in the PHA-leukocyte feeder (PHA-LF) system.[20,21,25] Colonies in this system from purified blasts are always of leukemic nature as indicated by morphology, immunophenotypes,[5] and cytogenetic markers.[20,21,25] Myeloid colonies (CFU-GM) from normal marrow were grown in a semisolid culture containing human placenta conditioned medium (HPCM; 20% vol/vol) as a source of colony-stimulating factor (CSF).[26,27]

Mixed colonies (CFU-GEMM) were grown as described by Fauser and Messner with slight modifications.[28] Normal bone marrow cells were cultured in a 1-mL mixture of Iscove's modified Dulbecco's medium (IMDM, GIBCO, Gent, Belgium), 1.1% methylcellulose, 30% autologous heparinized plasma, 7.5% of a medium conditioned by leukocytes in the presence of 1% PHA (PHA-LCM), 1 U sheep erythropoietin per milliliter (step III, Connaught, Willowdale, Ontario, Canada), BSA, transferrin, lecithin, sodium selenite, and 2-mercapto-ethanol. Dishes were incubated at 37 °C and 100% humidity in an environment of 5% CO_2 in air. Colonies were scored at day 15. Mixed colonies were defined as containing red hemoglobinized cells plus at least 5% nonhemoglobinized translucent cells of various sizes. Disputable mixed colonies were always verified cytologically after they were lifted off the plate with a fine-drawn Pasteur pipette.

Labeling of cells with UEA-FITC and FACS. AML blast cells or bone marrow nucleated cells were suspended in phosphate-buffered saline (PBS) containing 5% FCS at a cell concentration of

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2×10^7 cells/mL. The suspension was incubated for 30 minutes at 4 °C with 20 μL of UEA-FITC (Polysciences Inc, Warrington, Pa). The cells were then washed three times with PBS and resuspended in PBS with 5% FCS at a cell concentration of 2×10^6 cells per milliliter. Cell sorting was performed on a FACS 440 (Becton Dickinson, Sunnyvale, Calif). Excitation was performed with the laser at 488 nm (0.4 W). For the fluorescence measurements, a 530/30 band pass filter was used. The instrument was calibrated with fluorescent standard beads (1.0 μm and 2.83 μm in diameter) (Polysciences Inc).

Dead cells among the leukemic population were excluded from analysis by elevating the threshold level of the forward light scatter. Fractions of different fluorescence intensity were sorted following a sterile procedure and plated separately in colony culture for determining the number of colony-forming cells as a function of the amount of UEA binding. Usually, between 1.5 to 3.0×10^5 cells per fraction were collected. Sorting rate was between 1,500 and 2,000 cells/second. Data analysis was performed using a Hewlett Packard 86B system.

May-Grünwald-Giemsa staining. After FACS, normal bone marrow cells or peripheral blood cells were suspended in 50% FCS in PBS (4×10^5 cells in 0.1 mL) and centrifuged onto clean glass slides (500 rpm for 5 minutes). The slides were air dried and, after fixation in methanol (1½ minutes), they were stained with May-Grünwald's solution for 3 minutes and then counterstained with 4% (vol/vol) Giemsa in PBS. Slides were then washed and air dried. Differential counts were performed at ×1,000 magnification under oil, counting 250 cells.

RESULTS

UEA-binding to AML-blasts and AML progenitors. The reactivity of purified AML cells to the fluorescent FBL (UEA-FITC) was studied. In 11 of 29 cases, the AML blasts reacted with the lectin (Table 1). The data are indicative of a correlation between the binding of UEA and morphologic maturation of AML cells according to the FAB classification. The blasts of only 1 of 10 patients with AML-M1 cytology were UEA reactive, whereas in 3 of 8 cases of AML-M2, 6 of 8 AML-M4, and 1 of 3 AML M5, the cells showed significant UEA binding.

Of the complete series of patients, in 13 cases the cells were capable of giving rise to colonies in vitro. To determine the quantitative level of surface binding of UEA-FITC to AML-CFU, cells were sorted into separate fractions of varying fluorescence intensity and seeded into PHA-LF. AML-CFU from subjects with a relatively undifferentiated AML morphology, ie, all four M1 cases, showed no or minimal capacity for UEA-binding (Table 2), whereas AML precursor cells from one of four patients with M2 and four of four patients with M4 cytology exhibited high UEA binding. The colony formers of the single patient with acute monoblast leukemia (M5) did not bind detectable UEA to their surface. In Fig 1, four typical examples are given of the fluorescence distribution profiles of AML blast cells and AML-CFU. The cells from three patients also formed colonies in the other culture system, ie, with HPCM as stimulator. The distributions of AML-CFU as assessed in these cultures were found to be identical to those of AML-CFU grown in the PHA-LF method (data not shown).

UEA binding to normal bone marrow cells, CFU-GM, and CFU-GEMM. For comparison, the UEA-binding properties of normal marrow cells, CFU-GM and CFU-GEMM were determined. Figure 2 gives an example of the UEA-FITC fluorescence distribution of Ficoll-separated normal marrow cells and CFU-GM. CFU-GEMM, not plotted, showed essentially identical UEA binding properties as CFU-GM. It is evident from this and the data from the separate experiments shown in Table 2 that low amounts of UEA-FITC bind to the cell surface of normal CFU-GM and CFU-GEMM.

Table 1. Binding of UEA-FITC in Relation to FAB Classification of AML

Classification*	UEA Negative	UEA Positive†
M1 (n = 10)	9	1
M2 (n = 8)	5	3
M4 (n = 8)	2	6
M5 (n = 3)	2	1
Total N = 29	18	11

UEA, *Ulex europaeus* agglutinin; AML, acute myelogenous leukemia.

*Morphologic subtypes based on the classification of the French-American-British (FAB) Cooperative Group.[19]

†Of the 11 UEA-positive cases, 100% of the cells were UEA reactive in 7 patients (1 M1, 2 M2, 4 M4), and 15% to 20% of the cells reacted with the lectin in the other 4 cases (1 M2, 2 M4, 1 M5).

Table 2. Distribution of Nucleated Cells, AML-CFU, CFU-GM, and CFU-GEMM Relative to the Amount of UEA Binding

		UEA Negative (−)†		UEA Weakly Positive (+)‡		UEA Strongly Positive (++)§	
Patient	FAB*	Nucleated Cells (%)	CFU (%)	Nucleated Cells (%)	CFU (%)	Nucleated Cells (%)	CFU (%)
1	M1	100	100	0	0	0	0
2	M1	95	100	4	0	1	0
3	M1	97	100	2	0	1	0
4	M1	97	100	2	0	1	0
5	M2	91	100	8	0	1	0
6	M2‖	26	54	53	46	21	0
7	M2	94	100	6	0	0	0
8	M2	39	18	27	26	34	56
9	M4	22	10	45	18	33	72
10	M4	24	8	21	29	55	63
11	M4	13	0	33	20	54	80
12	M4	49	5	33	33	18	63
13	M5	98	100	2	0	0	0
Normal BM	CFU-GM¶	64	51	16	46	14	3
Normal BM	CFU-GM#	54	26	36	66	15	8
Normal BM	CFU-GM	49	44	30	52	21	4
Normal BM	CFU-GEMM	49	13	30	80	21	7
Normal BM	CFU-GEMM	62	29	17	57	21	14

Abbreviations as in Table 1. BM, bone marrow.

Percentage of total recovered (%).

*Classification according to the FAB nomenclature.[19]

†Negative fluorescence (log scale); channel 0–90.

‡Weakly positive fluorescence; channel 91–130.

§Strongly positive fluorescence; channel 131–255.

‖Channel numbers of sorted fractions: (0–90), (91–126), (127–255).

¶Channel numbers of sorted fractions: (0–90), (91–142), (143–255).

#Channel numbers of sorted fractions: (0–90), (91–147), (148–255).

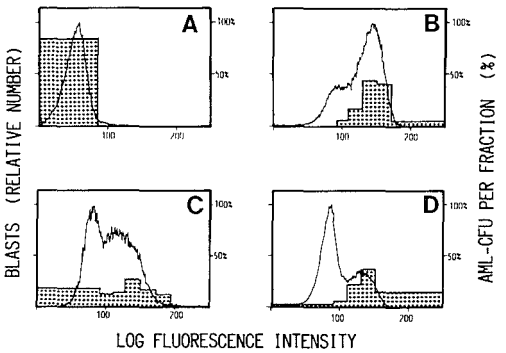

Fig 1. Distribution of acute myelogenous leukemia (AML) blast cells (——) and AML-CFU (▨) as a function of the fluorescence intensity following *Ulex europaeus* agglutinin (UEA)–FITC labeling (four representative profiles). Fractions: 0 through 90; 91 through 110; 111 through 130; 131 through 150; 151 through 170; and 171 through 255. (A) Patient 4 (FAB-M1): AML blasts and AML-CFU are UEA negative. (B) Patient 11 (FAB-M4): heterogeneous quantities of UEA are bound to AML blasts. AML-CFU are recovered from the blast cell fraction, intensively reactive with UEA. (C) Patient 8 (FAB-M2): broad peak of UEA binding indicating cellular heterogeneity. Most AML-CFU are UEA reactive. (D) Patient 12 (FAB-M4): most AML blasts are weakly UEA positive, but most AML-CFU are selectively recovered from the strongly UEA-positive subpopulation.

In addition, the relative density of UEA binding of different marrow cells was determined following cell sorting and subsequent differential counting of the smear (Table 3). Nucleated red cells were highly reactive with the lectin, whereas >50% of the monocytes showed intermediate to strong UEA binding. Only part of the promyelocytes/myelocytes were weakly UEA positive. All other cell types were found in the negative fraction. Peripheral blood monocytes and most of the eosinophils showed high UEA reactivity whereas neutrophilic granulocytes were intermediately positive for UEA. Basophilic granulocytes were equally distributed; lymphocytes were UEA negative.

Table 3. UEA-FITC Binding of Normal Bone Marrow Cells

Fraction* (Cell Type)	Percentage of Recovery of Different Cell Types as a Function of UEA-Binding Intensity							
	1	2	3	4	5	6	7	Total
Myeloblasts	70	27	0	0	0	3	0	100 (6)†
Promyelocytes, myelocytes	59	37	1	1	1	1	0	100 (13)
Metamyelocytes, polymorphs	93	5	1	0	1	0	0	100 (10)
Monocytes	40	10	16	15	10	6	3	100 (8)
Nucleated red cells	0	0	1	0	1	10	88	100 (<1)
Lymphocytes	97	3	0	0	0	0	0	100 (64)

UEA, *Ulex europaeus* agglutinin.

*Ficoll-separated bone marrow nucleated cells were sorted into fractions on the basis of *Ulex europaeus* (UEA)-FITC fluorescence intensity. Channel numbers of sorted fractions: fraction 1 (0–90, negative), 2 (91–110), 3 (111–130), 4 (131–150), 5 (151–170), 6 (171–190), and 7 (191–255, strongly positive).

†Total of each cell type was set at 100%. Recoveries of different cell types from Ficoll-separated marrow are indicated in parentheses.

DISCUSSION

In this study, we examined the reactivity of the UEA to AML blast cells and AML-CFU. The findings were related to the maturation stage of AML (FAB) and to UEA reactivity of normal bone marrow nucleated cells, CFU-GM and CFU-GEMM. Lectin binding to the surface of AML blast cells among the patients (N = 29) showed considerable variability. The cell samples from 18 patients were UEA negative; the blasts from 11 patients were UEA positive. Fluorescence histograms indicate that UEA reactivity is heterogeneously distributed among blasts, indicating subsets with relatively low and high UEA surface binding.

Although the number of cases was limited, a correlation is suggested as regards the capacity of the cells to bind UEA and their morphology. The M1 cases with one exception were negative, whereas cells from three of eight M2 cases and six of eight M4 cases reacted with the lectin. The cells from one of three cases of an acute monoblastic leukemia (M5), showed UEA reactivity. The data are compatible with the results from investigations with FBL-L.[28] In the latter studies, it was demonstrated that 5 of 6 M4 cases and the 1 M2 case reacted with this FBL-L, whereas their 1 M5 case was FBL-L negative. No AML cells classified as M1 were examined in that particular study. FBL-L and UEA are both reactive with α-L fucose. UEA can be washed from the cells with this sugar (data not shown). The reactivity of the two lectins, however, differs somewhat. FBL-L has a higher binding capacity for granulocytes than for monocytes.[16–18] Our results show the opposite for UEA, ie, intense binding to monocytes and an intermediate binding to neutrophilic granulocytes. Studies in larger numbers of patients appear of interest in order to evaluate the usefulness of UEA as a reagent in the classification of AML.

The fluorescence histograms shown in Table 1 reveal three discrete types of reactivity among the leukemias: a group of cases of fully UEA-negative AML (N = 18), a second group of AML containing ~20% UEA-positive cells (N = 4) and a

Table 4. UEA-FITC Binding to Peripheral Blood Cells

Fraction* (Cell Type)	Percentage of Recovery of Different Cell Types as a Function of UEA Binding Intensity					
	1	2	3	4	5	Total
Neutrophils	13	44	25	7	11	100 (87)†
Basophils	2	2	13	27	56	100 (6)
Eosinophils	15	14	19	20	32	100 (1)
Monocytes	1	4	21	31	43	100 (1)
Lymphocytes	86	7	1	2	4	100 (5)

UEA, *Ulex europaeus* agglutinin.

*Methylcellulose sedimented peripheral blood nucleated cells were sorted into fractions on the basis of UEA-FITC fluorescence intensity. Channel numbers of sorted fractions: fraction 1 (0–90, negative), 2 (91–110), 3 (111–130), 4 (131–150), and 5 (151–255, strongly positive).

†Total of each cell type was set at 100%. Recoveries of different cell types from methylcellulose sedimented blood are indicated in parentheses.

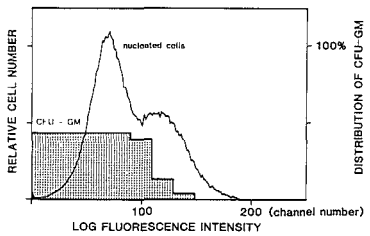

Fig 2. Distribution of normal bone marrow nucleated cells and CFU-GM as a function of fluorescence intensity following *Ulex europaeus* agglutinin (UEA)-FITC labeling. Fractions: 0 through 90; 91 through 110; 111 through 130; 131 through 150; and 151 through 255.

third series of seven cases, in which 100% of the cells reacted with the lectin. Because we had no sufficient cell material to study colony growth among cases of each of these three groups, we were not able to correlate these classes to colony-forming abilities or AML-CFU phenotypes. However, it appeared that of the fully UEA-positive AML cases with colony growth, the majority of the AML-CFU were recovered from the UEA weakly positive fraction in one case and, in the other 5 cases, from the UEA strongly positive fraction.

Sorting experiments using UEA-FITC fluorescence show that in several cases AML-CFU can be recognized as a subgroup of cells with specific lectin-binding properties. This is consistent with the results of immunophenotyping, which have indicated that AML-CFU are frequently different from the total AML population in their antigenic cell surface profile.[9,12,13]

The relation between UEA binding and FAB categories holds up when binding is expressed for the AML-CFU subpopulation. In 4 M1 cases, UEA binding to AML-CFU was low (Table 2), whereas in 1 of 4 M2 and 4 of 4 M4 patients, a high level of UEA binding sites was demonstrated on the cell membrane. Variations in the expression of membrane antigens of AML-CFU have been noticed by different groups.[12-14] Correlations between immunophenotypes of AML-CFU and AML cytology have been established but have provided controversial results. On the basis of the antigenic cell surface structure, Lange and co-workers classified AML-CFU as counterparts of the in vitro multipotential stem cell (CFU-GEMM), "early" CFU-GM or "late" CFU-GM. These investigators did not find a correlation with FAB classification of AML cells.[1] However, using another panel of monoclonal antibodies, Sabbath and colleagues concluded that the antigenic makeup of AML-CFU tended to correlate with the maturation stage of the cells.[2] We demonstrate that CFU-GM and CFU-GEMM were generally UEA negative or weakly positive. We suggest that AML-CFU with little UEA reactivity are generally of a more immature phenotype, whereas AML-CFU with high binding capacities are more differentiated into monocytic directions.

Morstyn and colleagues have shown that early as well as late CFU-GM have low affinity for FBL-L.[18] In our studies, AML-CFU in 5 patients (1 AML-M2, 4 AML-M4) showed high reactivity with the lectin UEA and, in 8 cases, AML-CFU were recovered from the UEA low-reactive or negative fractions. It is possible that the discrepant UEA-binding capacities of AML-CFU and normal colony formers can be exploited in certain cases for recognizing AML clonogenic cells in the bone marrow during complete remission.

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REFERENCES

1. Lange B, Ferrero D, Pessano S, Palumbo A, Faust J, Meo P, Rovera G: Surface phenotype of clonogenic cells in acute myeloid leukemia defined by monoclonal antibodies. Blood 64:693, 1984

2. Sabbath KD, Ball ED, Larcom P, Davis RB, Griffin JD: Heterogeneity of clonogenic cells in acute myeloblastic leukemia. J Clin Invest 75:746, 1985

3. Löwenberg B, Bauman JGS: Further results in understanding the subpopulation structure in AML: Clonogenic cells and their progeny identified by differentiation markers. Blood 66:1225, 1985

4. Pagliardi GL, Pessano S, Palumbo A, Levis A, Bottero L, Ferrero D, Lange B, Rovera G: Differentiation, maldifferentiation or arrested differentiation in human acute myelogenous leukemias, in Mirand EA (ed): Symposia of 13th International Cancer Congress. New York, Liss, 1983, p 65 (part C)

5. Touw IP, Löwenberg B: Variable differentiation of human acute myeloid leukemia during colony formation in vitro. A membrane marker analysis with monoclonal antibodies. Br J Haematol 59:37, 1985

6. Linker-Isreali M, Billing RJ, Foon KA, Terasaki PI: Monoclonal antibodies reactive with acute myelogenous leukemia cells. J Immunol 127:2473, 1981

7. Griffin JD, Ritz J, Nadler LM, Schlossman SF: Expression of myeloid differentiation antigens on normal and malignant myeloid cells. J Clin Invest 68:932, 1981

8. Bernstein ID, Andrews RG, Cohen SF, McMaster BE: Normal and malignant human myelocytic and monocytic cells identified by monoclonal antibodies. J Immunol 128:876, 1982

9. Reyden HJ van der, Rhenen DJ van, Lansdorp PM, Veer MB van't, Langenhuijsen MMAC, Engelfriet CP, Borne AEGK von dem: A comparison of surface marker analysis and FAB classification in acute myeloid leukemia. Blood 61:443, 1983

10. Ball ED, Fanger MW: The expression of myeloid specific antigens on myeloid leukemia cells: Correlations with leukemia subclasses and implications for myeloid differentiation. Blood 61:456, 1983

11. Griffin JD, Mayer RJ, Weinstein HJ, Rosenthal DS, Coral FS, Beveridge RP, Schlossman SF: Surface marker analysis of acute myeloblastic leukemia: Identification of differentiation-associated phenotypes. Blood 62:557, 1983

12. Griffin JD, Larcom P, Schlossman SF: Use of surface markers to identify a subset of acute myelomonocytic leukemia cells with progenitor cell properties. Blood 62:1300, 1983

13. Wouters R., Löwenberg B: On the maturation order of AML cells: A distinction on the basis of self-renewal properties and immunologic phenotypes. Blood 63:684, 1984

14. Griffin JD, Linch D, Sabbath K, Schlossman SF: A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. Leuk Res 4:521, 1984

15. Tung KSK, Epps DE van: Identification of a human polymorphonuclear leukocyte specific marker using fluoresceinated fucose binding lectin form Lotus tetragonolobus seeds. J Clin Lab Immunol 2:171, 1979

16. Nicola NA, Morstyn G, Metcalf D: Lectin receptors on human blood and bone marrow cells and their use in cell separation. Blood Cells 6:563, 1980

17. Epps DE van, Brazil R, Tung K, Warner N: Identification by flow cytometry of human monocytes with fucose binding lectin (FBL) from *Lotus tetragonolobus* seeds. J Reticuloendothel Soc 34:113, 1983

18. Morstyn G, Nicola NA, Metcalf D: Purification of haemopoietic progenitor cells from human marrow using a fucose binding lectin and cell sorting. Blood 56:798, 1980

19. French-American-British (FAB) Cooperative Group: Proposals for the classification of the acute leukemia. Br J Haematol 33:451, 1976

20. Löwenberg B, Swart K, Hagemeijer A: PHA-induced colony formation in acute non-lymphocytic and chronic myeloid leukemia. Leuk Res 4:143, 1980

21. Swart K, Hagemeijer A, Löwenberg B: Acute myeloid leukemia colony growth in vitro: Differences of colony forming cells in PHA-supplemented and standard leukocyte feeder cultures. Blood 59:816, 1982

22. Swart K, Löwenberg B: Feeder cell requirements for leukemic cell colony formation in cultures supplemented with phytohaemagglutinin. Cancer Res 44:657, 1984

23. Schaefer UW, Dicke KA, Bekkum DW van: Recovery of hemopoiesis in lethally irradiated monkeys by frozen allogeneic bone marrow grafts. Rev Eur Etude Clin Biol 17:443, 1972

24. Böyum A: Separation of leukocytes from blood and bone marrow. Immunol Clin Lab Invest 27:77, 1968

25. Löwenberg B, Hagemeijer A, Swart K: Karyotypically distinct subpopulations in acute leukemia with specific growth requirements. Blood 59:641, 1982

26. Burgess AW, Wilson EMA, Metcalf D: Stimulation by human placental conditioned medium of hemopoietic colony formation by human marrow cells. Blood 49:573, 1977

27. Touw IP and Löwenberg B: No stimulative effect of adipocytes on haematopoiesis in long-term human marrow cultures. Blood 61:770, 1983

28. Fauser AA, Messner HA: Granuloerythropoietic colonies in human bone marrow, peripheral blood, and cord blood. Blood 52:1243, 1978

29. Elias L, van Epps DE: Analysis of myelomonocytic leukemic differentiation by a cell surface maker panel including a fucose-binding lectin from *Lotus tetragonolobus*. Blood 63:1285, 1984

CHAPTER 3

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Phenotyping of Acute Myelocytic Leukemia (AML) Progenitors: An Approach for Tracing Minimal Numbers of AML Cells Among Normal Bone Marrow

Phenotyping of Acute Myelocytic Leukemia (AML) Progenitors: An Approach for Tracing Minimal Numbers of AML Cells Among Normal Bone Marrow

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Previous studies have shown that the phenotypes of progenitors of human AML (AML-CFU) are variable, reflecting arrests at different stages of maturation. We were interested to seek discrepancies between the surface properties of AML precursors and normal bone marrow colony formers in order to detect minimal numbers of AML cells among normal bone marrow cells in remission bone marrow. Therefore, we selected two surface markers, the MoAb CD34, reactive with blast cells, and Vim-2, a surface marker reactive with mature myeloid cells, and determined the antigen density of these markers (relative fluorescence intensity using fluorescence-activated cell sorting) for normal marrow and AML progenitors. While these markers defined an identical phenotype ($CD34^{++}/Vim\text{-}2^{-/+}$) for a broad spectrum of normal progenitors, i.e., CFU-GEMM, BFU-e, day 15 CFU-GM, and day 7 CFU-GM, referred to as the "normal" progenitor phenotype, AML progenitors frequently exhibited different phenotypes. In 12 of 20 cases the phenotypes of the majority of AML progenitors were discrepant from the normal surface profile, i.e., according to one marker in 8 cases ($CD34^{-/+}/Vim\text{-}2^{-/+}$ or $CD34^{++}/Vim\text{-}2^{++}$) and two markers in 4 cases ($CD34^{-/+}/Vim\text{-}2^{++}$). Since these data indicate that AML and normal progenitors were frequently distinguishable, we then determined the potential utility of these phenotypic dissimilarities for detection of minimal disease. Artificial mixtures of normal bone marrow and minimal numbers (0.1–1%) of AML cells were prepared. Based upon the phenotypic discrepancies, AML metaphases were successfully demonstrated in these mixtures following cell sorting and culture. Thus, it appears that minimal numbers of AML mitoses can be identified with an approximate 10^{-2} to 10^{-3} sensitivity by taking advantage of differential coexpression of surface antigens.

INTRODUCTION

In AML a minor population of the blasts is capable of colony formation in vitro. It is likely that these colony-forming cells (AML-CFU) represent cells which contribute to the growth of AML in vivo (1–4). Antigenic differences in the cell surface distinguish AML-CFU from the nonproliferating AML cell compartment (3–9). Moreover, immunophenotyping has revealed that the phenotypes of AML-CFU vary among individual patients. This heterogeneity reflects the diversity of normal hematopoietic progenitor cells as a function of maturity (7, 8). Thus, AML-CFU correlate with either the "late" CFU-GM (day 7), the "early" CFU-GM (day 14), or the multipotential precursors CFU-GEMM and appear to represent the neoplastic equivalents of different normal bone marrow progenitor cells.

In the present study we have searched for surface phenotypes of AML progenitors which are distinct from those of normal hematopoietic precursors, with the purpose of using these phenotypic discrepancies in the detection of minimal residual disease. AML and normal bone marrow progenitors were phenotyped by flow cytometry, cell sorting, and culture, using two MoAbs, CD34, which particularly reacts with immature marrow cells (10–12), and Vim-2, which is reactive with mature cells of the myeloid lineage (13, 14). When phenotypic discrepancies between normal and leukemic progenitors were evident, we investigated whether these differences could be used in the detection of minimal residual disease and examined artificial mixtures of AML and normal bone marrow following cell sorting.

MATERIALS AND METHODS

Cell Isolation. AML cells were isolated from bone marrow and peripheral blood by BSA density gradient centrifugation, followed by an E-rosette Ficoll sedimentation to remove T cells (15, 16). The percentage of E-positive cells was always reduced to less than 1% (200 cells counted). The cells were then cryopreserved and stored in liquid nitrogen (9). Normal bone marrow cells were recovered from the interface after Ficoll-Isopaque gradient centrifugation (17). All individuals had given consent to donate bone marrow and blood.

Culture Systems. AML cells were cultured in the PHA-leukocyte feeder assay (15, 18, 19). Normal bone marrow colony-forming cells, i.e., CFU-GEMM (erythroid, myelomonocytic, and megakaryocytic), "early" CFU-GM (myelomonocytic), and BFU-e (erythroid) were grown simultaneously in culture system described by Fauser and Messner (20), with slight modifications (9). Normal bone marrow "late" CFU-GM were determined in triplicate in semisolid culture containing human placental conditioned medium (30%, v/v) as the source of colony-stimulating factors (21, 22) and scored at day 7 of culture.

Tritiated thymidine incorporation was determined essentially as described (23). A total of 3×10^4 cells or the representative fractions sorted were cultured in 100 µl α-medium containing 10% FCS and 10% PHA-leukocyte conditioned medium, in 96-well round-bottom microtiter trays (Greiner, Nurtringen, FRG) for 90 and 180 hr. Sixteen hours before harvesting, 0.1 µCi [^{3}H]TdR (2 Ci/mmol; Amersham, U.K.) was added. All experiments were performed in triplicate, and data are expressed as means ± SD. Pre-irradiated (30 Gy) AML cells were run as controls to assess the background [^{3}H]TdR incorporation.

Mixtures of AML and normal bone marrow cells were cultured in a suspension of α-medium (Gibco; Paisly, U.K.) containing 10% GCT conditioned medium (Gibco) as a source of colony-stimulating factors. Metaphases were harvested after 3–6 days of culture.

MoAbs. The MoAb CD34 (IgG1, My-10) reacts with a surface antigen present on blast cells in normal marrow including the in vitro colony-forming cells CFU-GEMM, CFU-GM, and BFU-e and was provided by Dr. C. I. Civin (Johns Hopkins University School of Medicine, Baltimore, MD) (10, 11). Another CD34, MoAB BI3C5

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Abbreviations: GEMM, granulocyte erythrocyte macrophage megakaryocyte; GAM, goat anti-mouse.

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(12) (IgG1, Sera Lab, Crawley Down, U.K.) was used in the double labeling experiments. Vim-2 (IgM), kindly donated by Dr. W. Knapp (Institute of Immunology, University of Vienna, Vienna, Austria), reacts with monocytes and granulocytic cells more mature than "early" CFU-GM (13, 14).

Labeling of the Cells with Fluorescent Markers and FACS. Cells were labeled with the MoAbs and with the second step reagent GAM-FITC (Nordic, Tilburg, The Netherlands) as described (3, 4). Following incubation, the cells were washed three times in PBS plus 1% BSA. FACS was performed on a FACS 440 (Becton Dickinson, Mountain View, CA). Cells were suspended in PBS plus 5% FCS at a cell concentration of 2×10^6 cells/ml. After the instrument had been calibrated with 1.0- and 2.83-μm-diameter fluorescent standard beads (Polysciences Inc.), cells were analyzed and subsequently sorted into fractions of different fluorescence intensity.

Dual labeling was performed following incubation with CD34 and Vim-2 biotin. In a second incubation the cells were labeled with avidin-phycoerythrin (20 μl/10^6 cells) (Becton Dickinson) and finally with GAM-FITC (IgG1 specific; Nordic; 1/40). As controls, cells were incubated with CD34 alone, Vim-2 biotin alone, or without antibodies. Always in these control groups the cells were incubated with avidin-phycoerythrin and GAM-FITC (IgG1).

Biotinylation of Vim-2 was performed in 0.2 M borate buffer, pH 8.5. A 1-ml disposable syringe was filled with Sephadex G25 (Pharmacia, Uppsala, Sweden) in borate buffer. Glass-wool was used to plug the bottom hole of the syringe. The syringe was placed in a 50-ml Falcon tube (Becton Dickinson) and then centrifugated for 1 min at $700 \times g$, to settle the gel. A 100-μl sample, containing 1 mg/ml protein, was applied on top of the gel and the syringe, placed in a second 50-ml tube, and centrifuged again. The sample was collected and the MoAb was biotinylated following the addition of 10 μl (1 mg/ml) *N*-hydroxysuccinimidobiotin (Sigma, St. Louis, MO) in dimethyl sulfoxide and gently stirring for 3 hr at room temperature. Excess *N*-hydroxysuccinimidobiotin was removed by centrifugation as described above, but with Sephadex G25 in PBS.

Cytogenetic Analysis. After 3 to 5 days in culture the metaphases were harvested according to standard techniques on glass slides (24). The spread metaphases were stained with 1 mg Atebrin (BDH Chemicals, Poole, U.K.) in 100 ml ethanol, and the male/female ratio of metaphases was determined.

RESULTS

Surface Markers on AML Progenitors and Normal Hematopoietic Colony-forming Cells, CFU-GEMM, CFU-GM ("Early" and "Late"), and BFU-e. Samples of 20 patients with AML were tested for in vitro growth in the PHA-leukocyte feeder system (Table 1). Colony numbers ranged from 27 to 1074 (mean 210) per 10^5 cells. The presence of CD34 and Vim-2 antigens on the surface of AML cells was then determined by flow cytometric analysis. Frequently, the fluorescence distributions of the negative and the positive populations overlapped (Figs. 1, B and C, and 2, B and C), so that an absolute separation between negative and positive cells was not possible. Therefore, we determined the relative marker density expressed on the surface of AML precursors and normal bone marrow progenitors. The strongly positive (++) cells were separated from negative/weakly reactive (−/+) cells by sorting according to fluorescence intensity. The recoveries of progenitor cells in CD34 strongly positive (++) and Vim-2 strongly positive (++) fractions for all 20 cases of AML and 2 normal bone marrow samples are collected in Table 1. Normal CFU-GEMM, CFU-GM, and BFU-e expressed CD34 antigens at an intense density (Fig. 1D). Similarly, in 15 cases the majority of AML-CFU were strongly positive for CD34 (Fig. 1C). In

Table 1. Percentage Distribution of AML Precursors and Normal Bone Marrow Colony-forming Cells with Intense Surface Marker Expression

AML Patient	FAB[a]	Colonies per 10^5 Cells	Percentage Recovery in Strongly Positive (++) Fractions	
			CD34	Vim-2
1	M2	1074	100	0
2	M4	67	100	31
3	M4	269	63	18
4	M4	115	80	38
5	M4	27	100	61
6	M2	127	99	82
7	M1	122	96	64
8	M1	179	67	80
9	M1	64	100	98
10	M2	40	100	54
11	M1	780	63	52
12	M4	212	0	56
13	M5	306	0	100
14	M2	123	0	56
15	M1	50	0	55
16	M4	29	30	3
17	M1	33	68	0
18	M2	439	100	0
19	M2	54	100	2
20	M1	91	76	4
Normal marrow CFU-GEMM	Donor 1	7	87	0
	Donor 2	9	100	0
"early" CFU-GM	Donor 1	74	87	0
	Donor 2	129	92	0
"late" CFU-GM	Donor 1	29	70	22
	Donor 2	42	88	12
BFU-e	Donor 1	42	88	0
	Donor 2	408	92	0

The percentage of AML precursors with strong marker reactivity (++) based on fluorescence intensity were determined (upper section). Cells were sorted into strongly positive (++) versus weakly positive (+) plus negative (−) fractions and then cultured. The recoveries of AML-CFU in the strongly positive fractions, expressed as the percentages of the total sorted (−/+ and ++; 100%) AML-CFU, are presented. The results of experiments with normal marrow are presented in the lower part of the table. The distributions of colony-forming cells among antigen-positive cells of donors 1 and 2, as given in the lower section, are representative of five individual experiments with normal bone marrow which gave similar results. Boxes indicate marker expressions of AML precursors different from normal marrow progenitors. The results are based on two experiments in 10 cases and one experiment in the other 10 cases. The fluorescence channels of the strongly positive fractions were 105–255 (CD34) and 110–255 (Vim-2).

[a] Cytological classification of AML according to the FAB nomenclature (25, 26).

4 cases of AML (patients 12, 13, 14, and 15), AML-CFU were not recovered from the $CD34^{++}$ fraction (Table 1; Fig. 1, A and B). In a fifth case (patient 16), most (i.e., 70%) AML-CFU were $CD34^{-/+}$ (Table 1). In summary, using CD34 as a single marker, AML-CFU were distinguishable from normal bone marrow progenitors in 5 of 20 cases by low or negative CD34 expression (Table 1). It should be noted that these differences are not absolute, since small proportions of normal marrow colony formers were present in the negative or weakly positive fractions of normal marrow as well.

Normal progenitors were predominantly $Vim\text{-}2^{-/+}$ (Table 1, Fig. 2D). Sorting and culture of AML cells revealed, similarly, that in 9 cases of AML, blast progenitors were predominantly recovered from the $Vim\text{-}2^{-/+}$ fraction (Fig. 2, A and B). In the other cases the major proportion of AML precursors was found in the strongly Vim-2 positive (++) fraction (Fig. 2C). Thus, in 11 of 20 cases AML progenitors were distinguishable from normal colony-forming cells by intense Vim-2 fluorescence (Table 1).

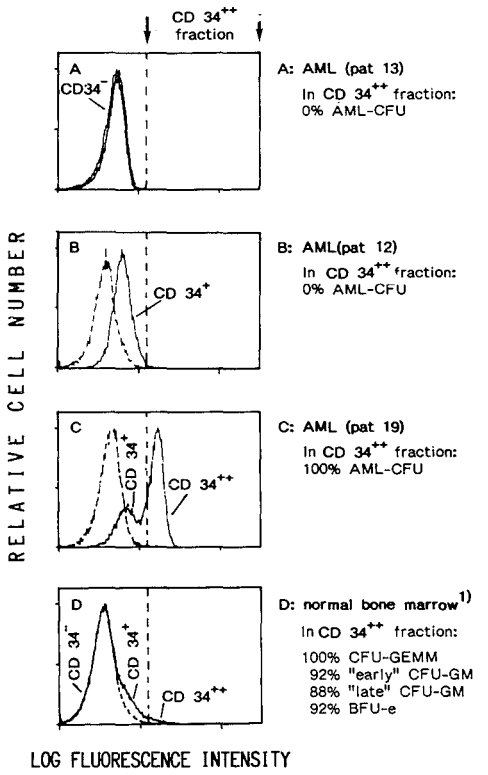

Figure 1. Fluorescence histograms of AML (3 cases) and normal marrow (1 case) labeled with anti-CD34. AML (A to C) and normal bone marrow cells (D) were labeled with the MoAb CD34 and GAM-FITC (——). As controls, cells incubated with GAM-FITC without the MoAb were used (– – –). AML cells in A were CD34 negative. The fluorescence profiles indicated in B and C reveal that in these cases AML cells were CD34 positive, but these cells could not be sharply distinguished from CD34-negative cells. CD34 strongly positive cells (fraction 105–255), i.e., beyond the vertical dashed lines, were sorted and then cultured. The recoveries (%) of the progenitors in the latter fraction with highest antigenic surface reactivity are indicated.

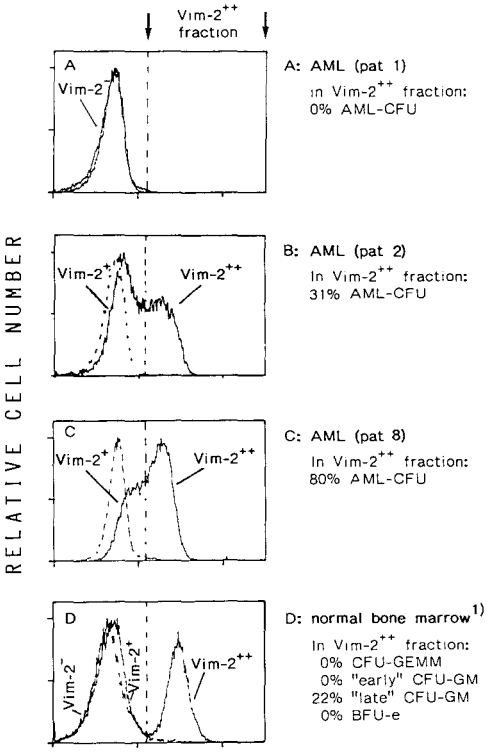

Figure 2. Fluorescence histograms of AML (3 cases; A to C) and normal marrow (1 case; D) labeled with Vim-2. For explanation see Figure 1. AML cells in A were Vim-2 negative. The fluorescence profiles of B and C reveal that in these cases AML cells were Vim-2 positive, but these could not be sharply distinguished from Vim-2 negative AML cells. The sorts of strongly positive cells are indicated by the dashed lines at channel 110.

AML-CFU were determined in the PHA-leukocyte feeder assay because of the low proliferative capacity of AML-CFU in semisolid cultures. However, the quantitative analysis of colony formation in the PHA-leukocyte feeder assay may be hampered to a certain extent by agglutination (27, 28). Therefore, in 7 cases of AML (i.e., patients 2, 4, 8, 10, 13, 14, and 15) we also analyzed the proliferative capacities of cells from the sorted fractions by radioactive thymidine uptake at day 4 and day 7 of culture (Table 2). In all cases a significant [^{3}H]TdR incorporation in AML cells was evident and the distributions of DNA-synthesizing cells, following cell sorting, were similar to the distributions of AML-CFU.

Detection of Leukemic Metaphases in Artificial Mixtures of AML and Normal Bone Marrow Cells. We then investigated whether these phenotypic discrepancies between AML and normal hematopoietic progenitors could be used to detect minimal numbers of AML cells among normal bone marrow. AML cells of a male or a female were mixed with normal bone marrow cells of the opposite sex in ratios of 1:100, 1:200, or 1:1000. Cells were labeled with CD34 (FITC) and Vim-2 biotin (phycoerythrin), and the desired fractions were sorted (Table 3). The sorted cells were cultured for 3 to 6 days with 10% GCT medium and the ratios of AML to normal metaphases (male/female or vice versa) were determined (n = 5 cases). Leukemic metaphases could readily be detected following sorting and culture of the CD34$^{-/+}$/Vim-2^{++} fraction of mixtures with AML cells from cases 13, 14, and 15, whereas among the unsorted cells no or minimal numbers of leukemic metaphases were demonstrated. In cases 8 and 10, leukemic metaphases were detected following sorting of the CD34^{++}/Vim-2^{++} subset of cells as well. Although cell sorting increased the ratio of AML/normal marrow mitoses in case 10, cytogenetic analysis of the cultured nonsorted cells of the 1:100 mixture also revealed leukemic metaphases.

Table 2. Percentage Distribution of [^{3}H]TdR-incorporating AML Cells with Intense Surface Marker Expression

Patient No.	Harvested at Day	[^{3}H]TdR Uptake of Unsorted Cells[a]	Control[b]	Percentage Recovery in Strongly Positive (++) Fractions[c]	
				CD34	Vim-2
2	4	341.0 ± 17.7	3.4 ± 0.3	93	15
	7	136.0 ± 7.7	2.3 ± 0.1	66	39
4	4	230.0 ± 4.5	4.5 ± 0.1	81	39
	7	88.4 ± 4.2	3.7 ± 0.3	91	16
8	4	92.7 ± 1.9	1.7 ± 0.1	ND	91
	7	68.3 ± 19.8	1.1 ± 0.2	ND	97
10	4	193.6 ± 9.7	3.0 ± 0.4	88	74
	7	119.7 ± 7.0	0.8 ± 0.2	70	63
13	4	96.2 ± 5.8	1.8 ± 0.4	2	88
	7	46.8 ± 4.7	0.4 ± 0.1	1	42
14	4	155.9 ± 12.0	8.8 ± 1.3	0	ND
	7	156.9 ± 4.1	2.7 ± 0.8	1	ND
15	4	44.4 ± 8.8	2.4 ± 0.4	2	64
	7	135.1 ± 16.8	3.2 ± 0.4	2	68

AML cells were sorted (see legend to Table 1) and cultured for 4 or 7 days in the presence of PHA-leukocyte conditioned medium (10%). ND = not done.

[a]The numbers indicate dpm × 10^{-2} (± SD) of 3 × 10^4 cells/well.

[b]Pre-irradiated cells (3 × 10^4) were cultured as controls in the presence of PHA-leukocyte conditioned medium.

[c]The recoveries of [^{3}H]TdR-incorporating AML cells in the strongly positive fractions are presented as the percentage of the total sorted (−/+ and ++) fractions (100%).

DISCUSSION

In the present study we have phenotyped precursor cells in 20 cases of human AML with the two MoAbs, CD34 and Vim-2 by FACS and subsequent culture. The proportions of precursor cells with intense or negative/weak antigen expression on their surface were estimated in order to identify phenotypic discrepancies between the main portions of AML and normal hematopoietic progenitors. The analysis of the expression of membrane surface antigens revealed that in 12 cases, phenotypes of AML precursor cells differed from those of the majority of normal hematopoietic clonogenic cells.

Phenotypic heterogeneity of AML precursors using combinations of monoclonal antibodies has been reported before (4, 7, 8). These studies had indicated that the variability of AML-CFU correlates with the phenotypes characteristic of CFU-GEMM, "early" CFU-GM, or "late" CFU-GM (7, 8). Experimental data in support of the existence of AML-CFU phenotypes which deviate from normal, however, have not been obtained. That we were able to disclose such dissociations between the immunophenotypes of normal marrow and AML progenitors is due not only to the fact that we introduced fluorescence intensity as a more refined phenotypic parameter but also is due to the choice of two surface markers which defined a constant phenotype for a broad range of normal hematopoietic progenitors. The majority of normal marrow progenitors of varying lineage commitment and distinct maturity, i.e., CFU-GEMM, BFU-e, and "early" and "late" CFU-GM, all shared the CD34 strongly positive (++), Vim-2 negative/weakly positive (−/+) phenotype. This common phenotype of normal marrow precursors served as a standard for demonstrating differences with AML.

The data of these investigations provide practical clues for separating AML precursors from the great majority of normal precursors for diagnostic purposes. These clues are: CD34 negativity or weak positivity (5 of 20 cases) and Vim-2 strong positivity of AML progenitor cells (11 of 20 cases). These markers provide possibilities for separating AML precursors from normal hematopoietic progenitors by a single marker in 8 cases and by 2 discrepant markers in 4 of 20 cases (Table 1). In the latter cases the two markers can be simultaneously applied to separate AML precursors from normal progenitors in remission bone marrow.

Table 3. Recovery of AML Metaphases in Artificial Mixtures of AML Cells and Normal Bone Marrow Cells Following Cell Sorting and Cell Culture

Patient No.	Sex	AML/NBM Ratio[a]	Sorted Fraction[b]			Unsorted AML-NBM Cell Mixture[c]	
			Phenotype Sorted	Number of Metaphases		Number of Metaphases	
				Total[d]	AML[e]	Total	AML
8	Male	1:1000	CD34^{++}/Vim-2^{+-}	22	1 (5%)	202	0 (0%)
10	Male	1:100	CD34^{++}/Vim-2^{++}	31	9 (29%)	26	2 (8%)
		1:1000		17	0 (0%)	14	0 (0%)
13	Male	1:200	CD34^{-+}/Vim-2^{++}	27	7 (26%)	18	0 (0%)
		1:1000		23	3 (13%)	21	0 (0%)
14	Male	1:200[f]	CD34^{-+}/Vim-2^{++}	65	4 (6%)	50	0 (0%)
		1:1000		90	0 (0%)	ND[a]	ND
15	Female	1:200	CD34^{-+}/Vim-2^{++}	106	19 (18%)	45	1 (2%)
		1:1000		45	5 (11%)	45	0 (0%)

[a]NBM = normal bone marrow; ND = not done.

[b]Ten to 20 × 10^6 cells were run through the FACS.

[c]One to 2 × 10^6 cells of the unsorted mixture were cultured.

[d]Total number of metaphases scored.

[e]Number of leukemic metaphases.

[f]In this case we also verified the leukemic nature of the metaphases. All the male cells were abnormal (i.e., 50, xy, +6,t(9:11), +13, +21, +22). The fluorescence channels of the strongly positive fractions were 105–255 (CD34) and 110–255 (Vim-2).

In addition to colony cultures, similar experiments were conducted to assess proliferation by measuring DNA synthesis in 7 patients, and consistent results were obtained (Table 2). Thus, the proliferating AML cells exhibited identical immunophenotypes in both PHA-leukocyte feeder and [^{3}H]TdR uptake assays.

To investigate whether the phenotypic discrepancies that appeared between normal and leukemic precursors could be employed for detection of a minor AML blast population, we conducted sorting experiments with artificial mixtures of AML cells and normal bone marrow. It appeared that in mixtures of AML and normal marrow (1:100), sorting of AML-proliferating cells which were discrepant from normal by only one marker (i.e., $CD34^{+}{}^{+}/Vim\text{-}2^{+}{}^{+}$) resulted in an approximately 4-fold enrichment of leukemic metaphases as compared to unsorted mixtures in case 10 (Table 3). In case 8 one leukemic metaphase was found in a 1:1000 mixture after cell sorting, whereas no leukemic mitoses were detected in the unsorted mixture. Sorting of AML-proliferating cells from the $CD34^{-/+}/Vim\text{-}2^{+}{}^{+}$ phenotype was applied to mixtures of normal marrow and AML cells from three cases (i.e., patients 13, 14, and 15) and resulted in a high recovery of leukemic metaphases as compared to unsorted mixtures (Table 3). Thus, according to results of the latter experiments, the separation based upon two discrepant markers appeared more efficient for detecting AML metaphases. The recoveries of AML metaphases from the 1:1000 sorted fractions in cases 13 and 15 were nonproportionally high as compared to the 1:200 mixtures following cell sorting (Table 3). These differences, however, are determined by the scoring of a small number of leukemic metaphases. Obviously, to allow for precise quantitation of minimal residual tumor loads, greater cell numbers should be screened cytogenetically. We believe that the approach outlined can be utilized for improved detection of minimal numbers of AML-proliferating cells. Previously, we had suggested that the lectin Ulex Europaeus agglutinin might also be a useful marker for the separation of normal and leukemic progenitor cells (9). Investigations of a series of bone marrow samples of AML patients in remission are currently in progress to examine whether leukemic metaphases can be demonstrated in clinical samples. On the basis of the data presented, it is likely that residual AML cells can indeed be traced when they are present at 0.1 to 1% concentrations.

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REFERENCES

1. Buick RN, Minden MD, McCulloch EA. Self renewal in cultures of proliferative blast progenitor cells in acute myeloblastic leukemia. Blood **1979**;54:95–104.
2. Chang LJA, Till JE, McCulloch EA. The cellular basis of self renewal in culture by human acute myeloblastic leukemia blast cell progenitors. J Cell Physiol **1980**;102:217–222.
3. Wouters R, Löwenberg B. On the maturation order of AML cells: a distinction on the basis of self-renewal properties and immunologic phenotypes. Blood **1984**;63:684–689.
4. Löwenberg B, Bauman JGJ. Further results in understanding the subpopulation structure in AML: clonogenic cells and their progeny identified by differentiation markers. Blood **1985**;66:1225–1232.
5. Touw IP, Löwenberg B. Variable differentiation of human acute myeloid leukemia during colony formation in vitro. A membrane marker analysis with monoclonal antibodies. Br J Haematol **1985**;59:37–44.
6. Griffin JD, Larcom P, Schlossman SF. Use of surface markers to identify a subset of acute myelomonocytic leukemia cells with progenitor cell properties. Blood **1983**;62:1300–1303.
7. Lange B, Ferrero D, Pessano S, Palumbo A, Faust J, Meo P, Rovera G. Surface phenotype of clonogenic cells in acute myeloid leukemia defined by monoclonal antibodies. Blood **1984**;64:693–700.
8. Sabbath KD, Ball ED, Larcom P, Davis RB, Griffin JD. Heterogeneity of clonogenic cells in acute myeloblastic leukemia. J Clin Invest **1985**;75:746–753.
9. Delwel R, Touw I, Bot F, Löwenberg B. A fucose binding lectin (UEA) for characterizing acute myeloid leukemia progenitor cells. Blood **1986**;68:41–45.
10. Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, Shaper JH. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J Immunol **1984**;133:157–165.
11. Strauss LC, Rowley SK, Loken MR, Stuart RK, Civin CI. Primitive normal lymphohematopoietic progenitor cells express the My-10 antigen: further characterization. Blood **1984**;64:117a.
12. Katz FE, Tindle R, Sutherland OR, Greaves MF. Identification of a membrane glycoprotein associated with haematopoietic progenitor cells. Leuk Res **1985**;9:191.
13. Majdic O, Bettelheim P, Stockinger H, Aberer W, Liszka K, Lutz D, Knapp W. M2, a novel myelomonocytic cell surface antigen and its distribution on leukemic cells. Int J Cancer **1984**;33:617–623.
14. Peschel C, Konwalinks G, Geissler D, Majdic O, Stockinger H, Braunsteiner H, Knapp W. Studies on differentiation of committed hemopoietic progenitor cells with monoclonal antibodies directed against myeloid differentiation antigens. Exp Hematol **1985**;13:1211–1216.
15. Swart K, Hagemeijer A, Löwenberg B. Acute myeloid leukemia colony growth in vitro: differences of colony forming cells in PHA supplemented and standard leukocyte feeder cultures. Blood **1982**;59:816–821.
16. Swart K, Löwenberg B. Feeder cell requirements for leukemic cell colony formation in cultures supplemented with phytohaemaglutinin. Cancer Res **1984**;44:657–660.
17. Böyum A. Separation of leukocytes from blood and bone marrow. Scand J Clin Lab Invest **1968**;21:7–91.
18. Löwenberg B, Swart K, Hagemeijer A. PHA-induced colony formation in acute non-lymphocytic and chronic myeloid leukemia. Leuk Res **1980**;4:143–149.
19. Löwenberg B, Hagemeijer A, Swart K. Karyotypically distinct subpopulations in acute leukemia with specific growth requirements. Blood **1982**;59:641–645.
20. Fauser AA, Messner HA. Granuloerythropoietic colonies in human bone marrow, peripheral blood, and cord blood. Blood **1978**;52:1243–1248.
21. Burgess AW, Wilson EMA, Metcalf D. Stimulation by human placental conditioned medium of hemopoietic colony formation by human marrow cells. Blood **1977**;49:573–583.
22. Touw I, Löwenberg B. No stimulative effect of adipocytes on haematopoiesis in long-term human marrow cultures. Blood **1983**;61:770–774.
23. Touw I, Delwel R, Zanen G, Löwenberg B. Acute lymphoblastic leukemia and non-Hodgkin's lymphoma of T-lineage: colony forming cells retain growth factor (interleukin-2) dependence. Blood **1986**;68:1088.
24. Hagemeyer A, Smit EME, Bootsma D. Improved identification

of chromosomes of leukemic cells in methotrexate treated cells. Cytogenet Cell Genet **1979**;23:208.
25. French-American-British (FAB) Cooperative Group: Proposals for the classification of the acute leukemia. Br J Haematol **1976**;33:451–458.
26. French-American-British (FAB) Cooperative Group: Proposed revised criteria for the classification of acute myeloid leukemia. Ann Intern Med **1985**;103:620–625.
27. Dicke KA, Spitzer G, Ahearn MJ. Colony formation in vitro by leukaemic cells in acute myelogenous leukaemia with phytohaemagglutinin as stimulating factor. Nature **1976**;259:129.
28. Dicke KA, Tindle SE, Davis FM, Jasgannath S, Tucker S, Lilien M, van Leeuwen P, Verma DA, Vellekoop L. Leukemic cell colony formation in soft agar by bone marrow cells and peripheral blood cells from untreated acute leukemia patients. Exp Haematol **1983**;11:341.

CHAPTER 4

Blood, Vol 70: pp 333-336, 1987.

Human Recombinant Multilineage Colony Stimulating Factor (Interleukin-3): Stimulator of Acute Myelocytic Leukemia Progenitor Cells in Vitro

Human Recombinant Multilineage Colony Stimulating Factor (Interleukin-3): Stimulator of Acute Myelocytic Leukemia Progenitor Cells In Vitro

By Ruud Delwel, Lambert Dorssers, Ivo Touw, Gerard Wagemaker, and Bob Löwenberg

Acute myeloid leukemia colony forming cells (AML-CFU) require the addition of colony stimulating factors (CSFs) for in vitro proliferation. Recently, we isolated a human recombinant multilineage CSF (hMulti-CSF). We investigated the ability of hMulti-CSF to stimulate AML clonogenic cells in seven patients in direct comparison with the effects of human granulocyte CSF (hG-CSF), human granulocyte-macrophage CSF (hGM-CSF), and feeder leukocytes. We show that hMulti-CSF is an efficient stimulator of AML colony formation in four of seven cases. In these patients, hGM-CSF was also capable of stimulating AML colonies in vitro. In two of seven cases hMulti-CSF appeared to be a weak stimulus of AML-CFU proliferation. In these latter two cases, however, hG-CSF and in one case hGM-CSF effectively stimulated AML-CFU growth. In one patient none of the hCSFs, either alone or in combination, induced AML colony formation, whereas AML colonies consistently appeared in the phytohemagglutinin (PHA) leukocyte feeder assay. This finding suggests that PHA stimulated leukocytes produce components other than the tested hCSFs that may have a role in the proliferation of AML cells in vitro. Multi-CSF, like hGM-CSF, revealed a limited capacity to induce progressive maturation during AML colony growth, ie, not beyond the promyelocytic stage. On the other hand, in one case, hG-CSF stimulated the growth of AML colonies containing (meta)myelocytes and granulocytes. We conclude that hMulti-CSF is a regulator of AML-CFU proliferation in a significant number of cases. The patterns of responsiveness of AML precursors to the three hCSFs in different patients show a striking variability, which may indicate that AML-CFU are the neoplastic representatives of normal bone marrow progenitors at different stages of maturation and with distinct CSF requirements.

THE COLONY STIMULATING factors (CSFs) comprise a group of hormones that stimulate the proliferation and differentiation of hematopoietic precursor cells in vitro.[1] Recently, we isolated a human multilineage CSF (hMulti-CSF) cDNA clone from a cDNA library of phorbol ester and lectin stimulated human leukocytes.[2] This hMulti-CSF cDNA was identified by virtue of sequence homology occurring within the 3' terminal untranslated region of mouse interleukin-3 (mIL-3). Protein homology between hMulti-CSF and mIL-3 appeared to be low (28%).[2] Expression of this hMulti-CSF cDNA clone in mammalian cells resulted in the production of a CSF that stimulates the in vitro proliferation of normal hematopoietic clonogenic cells, ie, granulocytic, erythroid, and multipotent progenitors.[2] This hMulti-CSF is very similar to a gibbon Multi-CSF described by Yang et al[3] with respect to DNA and protein sequence as well as biologic activity. In the present study we analyzed the role of this factor in the proliferation of acute myelocytic leukemia clonogenic cells (AML-CFU) in vitro. Since two other human CSFs, ie, granulocyte-macrophage CSF (hGM-CSF) and granulocyte CSF (hG-CSF), had become available earlier by recombinant DNA technology and had been demonstrated to stimulate AML colony growth in vitro,[4-9] we examined the effect of hMulti-CSF on AML progenitors in culture in direct comparison with those hCSFs.

MATERIALS AND METHODS

AML blast cells. The blasts from seven adult patients with AML were studied. The AML cases were classified according to the criteria of the French-American-British (FAB) committee.[10,11] AML blasts were purified from bone marrow (patients no. 1, 4, and 6) or blood (patients no. 2, 3, 5, and 7) using a bovine serum albumin (BSA) density gradient centrifugation. Contaminating T lymphocytes were removed from those cell samples by E-rosette sedimentation as described.[12,13] All patients had given informed consent to donate bone marrow and blood.

Recombinant CSF preparations. Recombinant hGM-CSF and hG-CSF preparations were obtained from Genetics Institute (Cambridge, MA). The structural and biological characteristics of these hCSFs have been reported in detail.[4-9,14,15] Recombinant hMulti-CSF was produced in monkey (Cos1) cells by transfection of a eukaryote expression plasmid (pLB4) containing the hMulti-CSF cDNA.[2] Cos/pLB4 conditioned medium was harvested 48 to 72 hours after transfection. Conditioned medium from Cos cells transfected with a vector not containing the hMulti-CSF gene did not stimulate AML-CFU proliferation.

AML colony formation. AML colony numbers were assessed in the standard PHA leukocyte feeder (PHA l.f.) assay.[12,13] Colonies (groups of 50 cells or more) were counted on days 7 through 9 of culture. To study the dependence of AML-CFU on the recombinant hCSFs, the preparations were added separately as well as in combination to the upper layer of the cultures while no feeder cells had been incorporated into the underlayers. After culture, the AML colony cells were mass harvested and analyzed morphologically on May-Grüwald Giemsa stained cytocentrifuge slides.

RESULTS

The optimal concentrations of the hCSFs to induce AML colony formation were determined in dose-response experiments (Fig 1). Based on these data further experiments were performed with 10% (vol/vol) hMulti-CSF, 0.1% (vol/vol)

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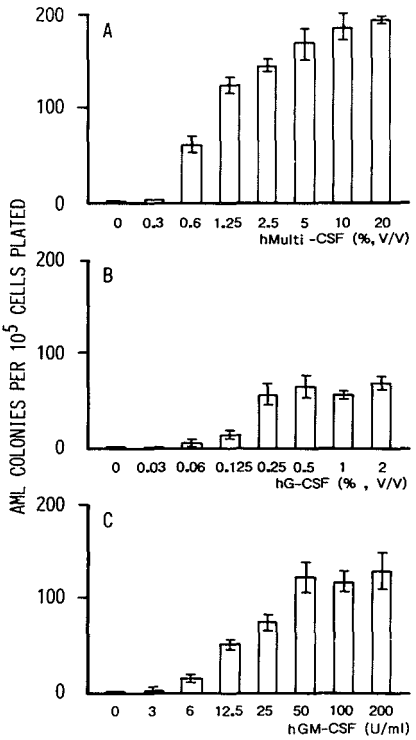

Fig 1. AML colony formation (patient no. 6) with titrated concentrations of the hCSFs. (A) hMulti-CSF, (B) hG-CSF, and (C) hGM-CSF.

hG-CSF, and 100 U/mL hGM-CSF. The effects of hMulti-CSF on AML colony formation as compared with hGM-CSF, hG-CSF, and feeder leukocytes are shown in Table 1. Without the addition of any of the stimuli, no colonies were formed. AML colony formation was stimulated efficiently by hMulti-CSF in cases no. 2, 4, 5, and 6. In patients no. 2 and 6, hMulti-CSF alone induced numbers of AML colonies that exceeded the values of the standard PHA l.f. assay. In the other two cases (no. 4 and 5) suboptimal colony formation was observed, as compared with feeder leukocyte stimulation. In patients no. 1 and 3, a consistent but minimal colony response to hMulti-CSF was apparent. In one case (no. 7) hMulti-CSF failed to induce AML colonies.

The stimulatory activity of hMulti-CSF on AML-CFU proliferation paralleled that of hGM-CSF, with the exception of case no. 1, in which hGM-CSF stimulated larger numbers of colonies than did hMulti-CSF. On the other hand, hMulti-CSF and hG-CSF stimulation patterns were opposite. In the cases showing a significant colony response to hMulti-CSF (cases no. 2, 4, 5, and 6) no or considerably fewer numbers of colonies were induced by hG-CSF, whereas in the hG-CSF high responders (cases no. 1 and 3) the effect of hMulti-CSF was minimal.

The data from Table 1 indicate that in most cases more than one recombinant hCSF stimulated AML colony formation. To investigate the possibility that the different hCSFs acted on AML-CFU in an additive fashion the preparations were also tested in combination. Only in cases no. 2 and 6 was an indication of additive stimulation by hMulti-CSF and hGM-CSF obtained. In the other cases the stimulatory effect of the single most efficient stimulator was not enhanced as a consequence of using a mixture of the three hCSFs in culture.

In cases no. 1, 3, 4, and 5 it appeared that AML colonies were induced more efficiently in the PHA l.f. assay than in the cultures with the complete mixture of recombinant hCSFs. Moreover, AML-CFU from patient no. 7 did not respond to the three hCSFs, whereas significant numbers of colonies appeared in the PHA l.f. assay. These data suggest that feeder leukocytes may produce at least one other component regulating AML-CFU proliferation.

The AML colony cells of patients no. 1, 3, and 6 were harvested and analyzed morphologically to assess whether the CSFs differ in their capacity to induce maturation (Table 2, Fig 2). In these cases the colony cells from the cultures stimulated by either hMulti-CSF or hGM-CSF showed limited differentiation, ie, not beyond the promyelocytic stage. In contrast, hG-CSF-stimulated colonies from patient no. 6 contained cells that had matured towards (meta)myelocytes and granulocytes (Fig 2).

DISCUSSION

We investigated the stimulative effects of hMulti-CSF, in comparison with the effects of hG-CSF and hGM-CSF and feeder leukocytes on the proliferation and differentiation of

Table 1. Induction of AML Colony Formation

	Case No. 1 (M2)*	Case No. 2 (M1)	Case No. 3 (M1)	Case No. 4 (M4)	Case No. 5 (M2)	Case No. 6 (M1)	Case No. 7 (M1)
No addition	0	0	0	0	0	0	0
hMulti-CSF	5 ± 2†	169 ± 13	9 ± 3	22 ± 2	24 ± 9	197 ± 23	0
hG-CSF	70 ± 8	0	68 ± 17	0	0	24 ± 6	0
hGM-CSF	104 ± 13	100 ± 3	2 ± 0	12 ± 2	22 ± 0	123 ± 6	0
hMulti-CSF + hG-CSF + hGM-CSF	125 ± 11	207 ± 7	70 ± 13	26 ± 1	34 ± 4	323 ± 12	0
PHA l.f.	256 ± 23	94 ± 9	103 ± 12	50 ± 12	61 ± 13	107 ± 6	24 ± 8

The effects of hMulti-CSF, hG-CSF, and hGM-CSF on AML colony formation were studied in a modified PHA l.f. system from which feeder leukocytes were omitted. Standard PHA l.f. cultures for AML colony formation were run in parallel.

*Classification according to the FAB Cooperative Group.[10,11]

†Colony numbers per 10^5 cells (mean ± SD).

Table 2. Differential Counts of MGG-Stained AML Colony Cells

	Case No. 1*			Case No. 3*			Case No. 6*		
	Multi-CSF	GM-CSF	G-CSF	Multi-CSF	GM-CSF	G-CSF	Multi-CSF	GM-CSF	G-CSF
Myeloblasts	2†	1	4	25	19	18	62	58	20
Promyelocytes	92	96	84	68	68	73	26	26	17
(Meta)myelocytes	0	3	2	0	1	4	2	2	43
Granulocytes	0	0	0	0	0	0	0	0	8
Monocytes/macrophages	6	0	10	7	12	5	7	14	12
Other	0	0	0	0	0	0	3	0	0

*The purified AML samples preculture contained 51% (case no. 1), 96% (case no. 3), and 98% (case no. 6) myeloblasts. The AML sample from case no. 1 preculture contained 46% promyelocytes.

†Percentages of 250 cells counted.

AML-CFU in colony culture. Human Multi-CSF, hG-CSF, and hGM-CSF were all capable of stimulating AML colony formation in vitro, although the responses of AML-CFU of different individuals to these hCSFs were variable.

The results indicate that AML colony formation in only one of these patients was stimulated predominantly by one of the three hCSF tested (case no. 3). Usually two (cases no. 1, 2, 4, and 5) or all three hCSFs (case no. 6) were able to induce AML-CFU proliferation. In most of these patients the presence of the three hCSFs in the cultures simultaneously did not result in an increased colony response as compared with the single most active factor, suggesting that the different hCSFs acted on overlapping AML-CFU target populations. However, exceptions were patients no. 2 and 6 in whom additive effects of hMulti-CSF and hGM-CSF on AML colony formation were observed. Thus, in these cases different AML-CFU subpopulations were apparently stimulated by different growth stimuli. The data in Table 1 do not provide evidence that the combination of the three factors can enhance colony numbers synergistically. To explore further the possibility of a synergistic action of the hCSFs, we set up cultures to which suboptimal concentrations of the different growth stimuli were added simultaneously. These studies also did not indicate synergistic stimulation of AML colony formation by these hCSFs (data not shown).

An interesting observation was that in patient no. 7 AML colony growth could not be induced by the recombinant hCSFs, whereas colonies appeared consistently in the standard PHA l.f. assay. The PHA l.f. colony culture has been an efficient assay for clonogenic AML cells,[12,13] although the active stimulatory components of the system are probably multiple and not exactly defined. The fact that colony growth in patient no. 7 appeared only in the PHA l.f. assay could imply that components other than these hCSFs have a critical role in stimulating the proliferation of AML precursor cells. This suggestion is also supported by the fact that in

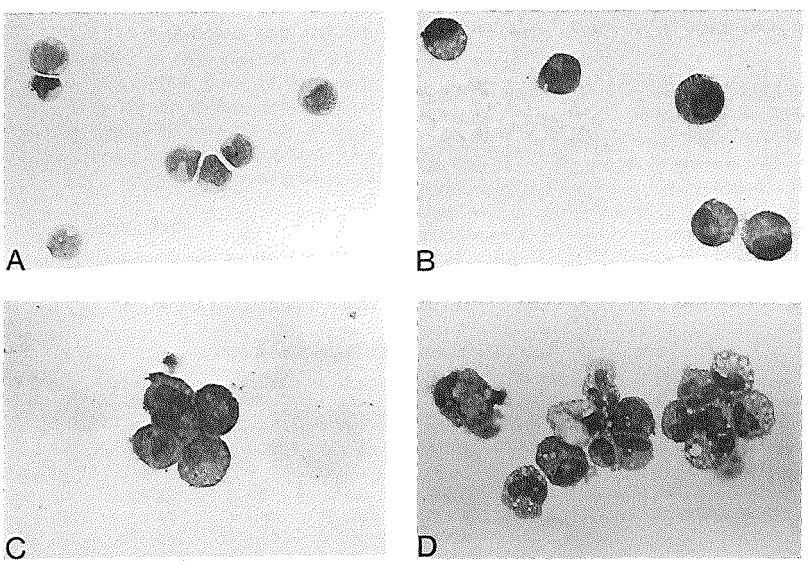

Fig 2. May-Grünwald Giemsa morphological analysis of AML cells (patient no. 6). (A) Preculture, (B) cultured with hMulti-CSF, (C) cultured with hGM-CSF, and (D) cultured with hG-CSF.

4 cases (no. 1, 3, 4, and 5) the PHA l.f. system supported the outgrowth of larger numbers of colonies than was achieved with the mixture of recombinant hCSFs.

Morphologic analysis of the AML colony cells of three patients revealed that progressive maturation occurred occasionally in the hG-CSF stimulated but not in the hMulti-CSF and hGM-CSF stimulated cultures (Table 2). Since the three factors have been reported to induce granulocytic or monocytic differentiation in normal bone marrow cultures, this could indicate that in patient no. 6, the AML blasts respond abnormally to hMulti-CSF and hGM-CSF but normally to hG-CSF, as regards the induction of maturation.[1,2] However, at this stage, the possibility cannot be excluded that differentiation towards granulocytic end cells, observed in the normal bone marrow cultures stimulated by hMulti-CSF or hGM-CSF, is not caused by direct effects of these two factors but by endogenously produced G-CSF. Thus, it remains unclear whether the variations of CSF responses of AML progenitors either represent leukemic characteristics of the cells or reflect the features of normal bone marrow progenitor subsets at different maturation stages with distinct CSF requirements. To discriminate between these two possibilities it will be essential to study in detail the effects of the recombinant hCSFs on highly purified normal bone marrow progenitors.

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REFERENCES

1. Metcalf D: The molecular biology and functions of the granulocyte-macrophage colony-stimulating factors. Blood 67:257, 1986

2. Dorssers L, Burger H, Bot F, Delwel R, Geurts van Kessel A, Löwenberg B, Wagemaker G: Characterization of a human multi-CSF cDNA clone identified by a conserved noncoding sequence in mouse interleukin 3. Gene, 1987 (in press)

3. Yang YC, Ciarletta AB, Temple PA, Chung MP, Kovacic S, Witek-Gianotti JS, Leary AC, Kriz R, Donahue RE, Wong GG, Clark SC: Human IL 3 (Multi-CSF): Identification by expression cloning of a novel hematopoietic growth factor related to murine IL 3. Cell 47:3, 1986

4. Wong GG, Witek JS, Temple PA, Wilkens KM, Leary AC, Luxenberg DP, Jones SS, Brown EL, Kay RM, Orr EC, Shoemaker C, Golde DW, Kaufman RJ, Hewick RM, Wang EA, Clark SC: Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science 228:810, 1985

5. Nagata S, Tsuchiya M, Asano S, Kaziro Y, Yamazaki T, Yamamoto O, Hirata Y, Kubota N, Oheda M, Nomura H, Ono M: Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor. Nature 319:415, 1986

6. Souza LM, Boone TC, Gabrilove J, Lai PH, Zsebo KM, Murdock DC, Chazin VR, Bruszewski J, Lu H, Chen KK, Barendt J, Platzer E, Moore MAS, Mertelsmann R, Welte K: Recombinant human granulocyte colony-stimulating factor: Effects on normal and leukemic myeloid cells. Science 232:61, 1986

7. Griffin JD, Young D, Herrmann F, Wiper D, Wagner K, Sabbath KD: Effects of recombinant human GM-CSF on proliferation of clonogenic cells in acute myeloblastic leukemia. Blood 67:1448, 1986

8. Hoang T, Nara N, Wong G, Clark S, Minden MD, McCulloch EA: Effects of recombinant GM-CSF on the blast cells of acute myeloblastic leukemia. Blood 68:313, 1986

9. Vellenga E, Delwel R, Touw I, Löwenberg B: Patterns of AML colony growth in response to recombinant granulocyte-macrophage colony stimulating factor (rGM-CSF). Exp Hematol, 1987 (in press)

10. French-American-British (FAB) Cooperative Group: Proposals for the classification of the acute leukemia. Br J Haematol 32:451, 1976

11. French-American-British (FAB) Cooperative Group: Proposed revised criteria for the classification of acute myeloid leukemia. Ann Intern Med 103:626, 1985

12. Swart K, Hagemeyer A, Löwenberg B: Acute myeloid leukemia colony growth in vitro: differences of colony forming cells in PHA supplemented and standard leukocyte feeder cultures. Blood 59:816, 1982

13. Löwenberg B, Hagemeyer A, Swart K: Karyotypically distinct subpopulations in acute leukemia with specific growth requirements. Blood 59:641, 1982

14. Sieff CA, Emerson SG, Donahue RE, Nathan DG, Wang EA, Wong GG, Clark SC: Human recombinant granulocyte-macrophage colony stimulating factor: A multilineage haematopoietin. Science 230:1171, 1985

15. Tomonaga M, Golde DW, Gasson JL: Biosynthetic (recombinant) human granulocyte-macrophage colony-stimulating factor: Effect on normal bone marrow and leukemic cell lines. Blood 67:31, 1986

CHAPTER 5

Blood, Vol 72: pp 1944-1949, 1988.

Growth Regulation of Human Acute Myeloid Leukemia: Effects of Five Recombinant Hematopoietic Factors in a Serum-Free Culture System

Growth Regulation of Human Acute Myeloid Leukemia: Effects of Five Recombinant Hematopoietic Factors in a Serum-Free Culture System

By Ruud Delwel, Mohammad Salem, Carin Pellens, Lambert Dorssers, Gerard Wagemaker, Steven Clark, and Bob Löwenberg

The response of human acute myeloid leukemia (AML) cells to the distinct hematopoietic growth factors (HGFs), ie, recombinant interleukin-3 (IL-3), granulocyte-macrophage-CSF (GM-CSF), granulocyte-CSF (G-CSF), macrophage-CSF (M-CSF), and erythropoietin (Epo) was investigated under well-defined serum-free conditions. Proliferative responses to these factors, when added separately as well as in combinations, were analyzed in 25 cases of human AML using ^{3}H-thymidine incorporation and colony assays. The ^{3}H-thymidine uptake data revealed that IL-3, GM-CSF, G-CSF, and M-CSF were stimulators of AML proliferation in 19, 15, 13, and 4 cases, respectively. Epo only stimulated DNA synthesis in the cells of the single erythroleukemia case. GM-CSF stimulation was seen only in IL-3 reactive cases and GM-CSF, when combined with IL-3, could not further elevate the DNA synthesis evoked by IL-3 alone. On the other hand, in six cases, G-CSF enhanced the IL-3– or GM-CSF–stimulated thymidine uptake. These results suggest that subpopulations of AML cells that are activated by distinct CSFs (eg, IL-3/GM-CSF–responsive cells and G-CSF–responsive cells) coexist. The ^{3}H-thymidine incorporation assay was more sensitive for measuring CSF responses than methylcellulose colony cultures, since activation of DNA synthesis was more frequently seen than induction of colony formation. DNA synthesis experiments revealed eight different CSF response patterns among these 25 cases. CSF phenotyping may be a useful addition to the morphologic classification of AML, since these patterns directly reflect the ability of the proliferating subsets of AML cells to respond to the CSFs.

HEMATOPOIETIC growth factors (HGFs) are essential for the proliferation and maturation of bone marrow and blood cells.[1,2] The availability of recombinant pure HGFs allows a detailed study of growth regulation of leukemic hematopoiesis in vitro.[3,4] It has been shown in initial studies that the in vitro proliferation of acute myeloid leukemia colony forming cells (AML-CFU) can be stimulated by recombinant granulocyte-macrophage colony stimulating factor (GM-CSF),[5-7] granulocyte-CSF (G-CSF),[8,9] and interleukin-3 (IL-3).[10-12] Macrophage-CSF (M-CSF) has not been shown to be a frequent stimulator of AML growth in vitro. Erythropoietin (Epo) induces the multiplication of progenitor cells in erythroleukemias.[13] These studies have been carried out in serum-containing systems. In the present study, we investigated 25 cases of human AML and the effects of each of these growth factors, ie, IL-3, GM-CSF, G-CSF, M-CSF, and Epo on AML cell proliferation in vitro when added individually and in various combinations. Both tritiated thymidine incorporation (^{3}H-TdR) assays (to assess DNA synthesis) and colony cultures (to assess clonogenic cell proliferation) were used. To avoid the interference of any undefined component, a standardized culture system absent of serum was used.[14]

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MATERIALS AND METHODS

Patients and preparation of AML cells. AML was diagnosed according to the French-American-British Committee (FAB) (Table 1).[15,16] The leukemic cells from 25 patients were separated following bovine serum albumin (BSA) density gradient[17,18] or Ficoll-Isopaque centrifugation (case nos. 10, 13, 16, 18, 20, 21, and 24).[19] T lymphocytes were then removed by rosetting the cells with 2-aminoethylisothiouronium bromide (AET; Sigma Chemicals Co, St Louis)-treated sheep erythrocytes as described.[17,18] These AML samples usually contained >90% blasts. In six cases (nos. 13, 18, 19, 21, 22, and 26), between 80% and 90% blasts were present. In four cases (nos. 12, 16, 23, and 24), the samples contained 67%, 67%, 50%, and 67% blasts, respectively. Following this procedure the cells were cryopreserved in 7.5% dimethyl sulfoxide (DMSO) and 20% fetal calf serum (FCS; Flow Laboratories, Isrike, UK).[20,21] AML cells from bone marrow or blood were obtained after receiving permission of the patients.

Hematopoietic growth factors. Recombinant human IL-3, GM-CSF, G-CSF, and M-CSF were prepared at the Genetics Institute (Cambridge, MA). Another IL-3 preparation was obtained following expression of the human IL-3 cDNA[22] in bacteria (Dorssers et al, manuscript in preparation). Human recombinant Epo was obtained from Amgen (Thousand Oaks, CA) and used at a concentration of 1 U/mL.

Serum-Free Culture Medium. A serum-free medium that was recently proposed for AML cell growth in vitro[14] was used throughout the experiments, ie, in suspension cultures as well as in colony assays.

Tritiated thymidine incorporation. DNA synthesis was measured essentially as described.[23] Two $\times 10^4$ cells were cultured in 100 μL serum-free medium, with or without the addition of CSFs, in 96-well round-bottom microtiter trays (Greiner, Nürtingen, FRG) for 90 hours. Four hours before harvesting, 0.1 μCi tritiated thymidine (^{3}H-TdR 2 Ci/mmol; Amersham International, Amersham, UK) was added. Cells were harvested on nitrocellulose paper using a Titertek harvester 550 (Flow). Radioactivity was determined with a Beckman LS 3800 scintillation counter (Beckman Instruments, Inc, Fullerton, Ca). All experiments were performed in triplicate, and data are expressed as means ± SD. Preirradiated (30 Gy) AML cells were run as controls to assess the background ^{3}H-TdR incorporation. Data obtained from cultures with CSFs were compared with those without CSF. Data obtained from the cultures not supplemented

Table 1. The Effects of Individual CSFs on ^{3}H-TdR Uptake by AML Cells

FAB	Patient No.	Source*	No. CSF	IL-3	GM-CSF	G-CSF	M-CSF	Irradiated (30 Gy) Control
M1	1	BM	1.2	1.6	1.1	3.5	5.8	0.5
	2	PB	0.7	4.4	2.0	2.0	0.9	0.4
	3	BM	4.5	15.1	10.2	6.6	4.1	1.1
	4	PB	4.0	11.0	9.0	14.4	4.5	0.7
	5	BM	44.9	36.7	23.5†	54.6	27.8†	0.6
M2	6	PB	0.7	0.8	0.5	1.8	0.4	0.5
	7	BM	0.1	16.5	0.5	6.5	0.2	0.1
	8	BM	1.1	171.3	156.5	36.4	1.8	0.3
	9	PB	90.8	164.8	163.5	175.6	111.0	0.7
	10	BM	31.0	90.0	91.0	221.0	53.0	1.2
	11	BM	3.3	213.3	219.5	87.7	6.1	1.8
M3	12	PB	8.0	23.5	11.2	9.8	7.2	0.5
	13	PB	14.8	214.0	149.1	243.7	17.1	1.1
M4	14	BM	1.0	178.0	24.1	7.5	5.9	0.8
	15	PB	37.5	108.8	91.4	87.3	45.3	8.8
	16	BM	14.5	41.5	26.3	17.1	12.0	0.4
	17	BM	9.9	11.4	12.0	24.2	13.1	1.2
	18	BM	19.1	57.5	38.9	93.0	24.4	1.1
	19	PB	46.3	60.0	65.0	121.3	52.7	0.7
	20	PB	11.1	20.2	23.2	9.0	8.0	0.5
	21	BM	11.9	92.0	32.2	20.1	11.9	1.4
	22	PB	39.2	88.9	78.4	99.4	40.5	0.4
M5	23	BM	3.3	17.4	6.8	2.3	2.8	0.6
	24	BM	11.4	26.9	13.0	13.6	11.8	0.7
M6	25	BM	0.7	4.2	1.1	2.1	0.4	1.0
No. of cases positive			14/25	19/25	15/25	13/25	4/25	

^{3}H-TdR incorporation by AML cells from 25 cases was determined in cultures with the individual CSFs (IL-3, GM-CSF, G-CSF, M-CSF) as a measure of cell proliferation. Values indicate dpm $\times 10^{-2}$. The results were compared with the values in AML cell cultures without any exogenous stimulus (first column). The latter were set against cultures with preirradiated (30 Gy) AML cells (last column). Positive responses of ^{3}H-TdR uptake are indicated by brackets. The AML cases are classified according to the FAB cytology.

*Leukemic cells were isolated from peripheral blood (PB) or bone marrow (BM).

†AML cells from patient no. 5 showed significant inhibition of DNA synthesis when cultured with GM-CSF and M-CSF.

with CSFs were set against the background control values. A Student t test ($P < 0.05$) was used to determine significant differences.

Colony cultures. The effects of the recombinant CSFs on AML colony formation were also determined. In these experiments, 0.8×10^5 cells were plated per dish containing 0.8 mL of the serum-free medium in 0.9% methylcellulose and cultured in a humidified 5% CO_2 atmosphere at 37°C. Colonies of ten cells or more were scored at days 12 through 14 of culture. In a separate series of experiments, colonies were also scored at days 5 through 7. Colony numbers represent means of triplicate cultures. The effects of the CSFs on colony size were analyzed as well. Four catagories of colonies were distinguished, ie, colonies containing ten to 20 cells, 20 to 50 cells, 50 to 200 cells, and 200 cells or more.

RESULTS

Activation of DNA synthesis in AML cells in vitro: The effects of individual CSFs. The stimulative effects of optimal concentrations of human IL-3 (1:1,000), GM-CSF (200 U/mL), G-CSF (1:1,000), and M-CSF (1:1,000) on the DNA synthesis of AML cells (n = 25 cases) in vitro (^{3}H-TdR incorporation) were assessed in comparison with the cultures without exogenous stimuli (Table 1). In 14 of 25 cases, significant DNA synthesis was observed in the unstimulated cultures. IL-3, GM-CSF, or G-CSF could each activate AML cells above these baseline values in 19 of 25, 15 of 25, and 13 of 25 cases, respectively. M-CSF stimulated DNA synthesis in AML cells only in a minority of cases (4 of 25).

Not only was IL-3 more frequently capable of inducing DNA synthesis, but it also usually induced the highest level of DNA synthesis (ie, in n = 10 cases) when compared with GM-CSF, G-CSF, or M-CSF. In eight other cases, G-CSF was the most active stimulator of ^{3}H-TdR uptake in AML.

In case no. 5, thymidine incorporation was significantly suppressed when the AML cells were cultured with GM-CSF or M-CSF. Suppression of DNA synthesis in this patient coincided with progressive maturation of the cells toward macrophages.[24]

Activation of DNA synthesis in AML cells in vitro: The effects of combined CSFs. In all cases, we also examined the effects of combinations of growth factors on ^{3}H-TdR uptake in AML (Table 2). In none of the cases were the stimulatory effects of IL-3 and GM-CSF additive. In contrast, G-CSF enhanced the IL-3 (4 of 25) or the GM-CSF response (4 of 25) in certain cases of AML. M-CSF further elevated the rate of DNA synthesis resulting from the GM-CSF stimulation in 2 of 25 cases. No further enhancement of DNA synthesis was apparent when three or four factors were added.

Colony formation of AML cells in vitro in response to the CSFs. Spontaneous colony formation, ie, when no exogenous CSFs were supplied, was found in three cases. In five cases, we did not assess colony formation in the presence of

Table 2. The Effects of Combined CSFs on ^{3}H-TdR Uptake by AML Cells

FAB	Patient No.	IL-3, GM-CSF	IL-3, G-CSF	IL-3, M-CSF	GM-CSF, G-CSF	GM-CSF, M-CSF	IL-3, GM-CSF, G-CSF	IL-3, GM-CSF, M-CSF	IL-3, GM-CSF, G-CSF, M-CSF
M1	1	1.3	3.6	4.7	3.2	5.5	4.2	6.3	10.3
	2	4.7	6.8	4.7	4.0	2.3	7.4	4.9	6.7
	3	13.7	19.7	14.7	15.4	11.2	20.3	15.5	17.5
	4	11.2	19.3	11.0	18.4	9.8	19.6	12.2	20.1
	5	21.3	37.2	21.2	19.9	16.0	22.2	12.8	18.5
M2	6	0.7	2.1	0.7	1.9	0.9	3.0	1.1	2.2
	7	16.6	22.8	15.4	10.8	0.6	22.4	19.1	19.7
	8	230.3	227.8	198.8	198.9	201.4	245.4	251.6	237.2
	9	155.0	175.7	145.3	172.2	151.0	176.7	160.3	184.9
	10	98.2	251.5	108.1	229.1	85.2	253.4	98.0	258.7
	11	224.3	226.4	238.3	290.9	239.3	250.2	225.4	214.9
M3	12	21.9	27.9	23.0	14.8	11.5	28.7	24.4	33.8
	13	218.6	275.7	189.7	276.9	223.0	276.6	236.9	290.2
M4	14	257.5	262.2	198.0	53.6	65.6	286.1	218.7	245.3
	15	102.5	114.8	101.1	140.4	99.9	127.9	113.7	137.5
	16	45.7	42.4	40.4	26.7	23.9	48.1	47.3	47.3
	17	10.7	19.2	13.6	17.0	14.6	16.1	10.1	20.1
	18	50.4	105.8	61.1	95.2	41.6	102.5	61.3	106.4
	19	54.8	127.5	62.2	118.3	63.9	124.9	61.5	119.9
	20	20.4	16.5	17.4	17.2	14.9	17.5	13.6	16.6
	21	92.4	80.7	86.7	38.6	37.7	92.1	96.4	90.5
	22	118.3	136.3	109.5	156.5	77.4	183.0	115.2	152.3
M5	23	14.9	14.4	14.3	5.4	6.2	15.5	13.4	15.5
	24	18.2	25.1	21.6	13.0	12.3	25.2	23.0	27.2
M6	25	3.6	6.2	5.1	3.8	4.3	6.8	5.1	nd

Values from cultures containing two CSFs were compared with incubations with the single CSFs (Table 1). The data of cultures with three factors were compared with those containing two CSFs, and finally values obtained from experiments using four CSFs were related to those obtained with three stimulators. Positive responses (ie, significant enhancement of ^{3}H-TdR uptake) are indicated by brackets. For further explanations, see Table 1.

Abbreviation: nd, not determined.

Table 3. Induction of Colony Formation in AML by Individual and Combinations of CSFs

FAB	Patient No.	No. CSF	IL-3, CSF	GM-CSF	G-CSF	M-CSF	IL-3, GM-CSF	IL-3, GM-CSF, G-CSF	IL-3, GM-CSF, G-CSF, M-CSF	IL-3, GM-CSF, G-CSF, M-CSF (+FCS)
M1	1	0	nd	nd	nd	nd	nd	nd	0	1
	2	0	nd	nd	nd	nd	nd	nd	0	0
	3	0	nd	nd	nd	nd	nd	nd	0	0
	4	0	0	0	0	0	0	0	0	0
	5	4	16	1	5	8	0	0	0	0
M2	6	0	0	0	0	0	0	0	0	0
	7	0	0	16	0	0	55	65	48	2
	8	0	75	1,216	1	0	1,407	1,036	1,265	701
	9	45	119	117	61	20	62	80*	91	12
	10	0	0	0	35	0	0	13	20	13
	11	0	20	133	19	0	188	147	173	117
M3	12	0	655	581	0	121	780*	780	>1,000	>1,000
	13	2	221	551	246	23	1,017	2,285	1,426	2,500
M4	14	0	1	0	0	0	44	60	22	3
	15	0	2,725	2,638	301	0	4,120	4,275	3,150	5,000
	16	46	58	76	129	47	95	127*	129	162
	17	0	7	3	0	1	11	19	18	33
	18	0	2	14	10	0	20	23	36	134
	19	0	0	0	2	0	0	15	17	14
	20	0	nd	nd	nd	nd	nd	nd	11	11
	21	0	4	0	0	0	11	6	9	20
	22	0	0	0	0	0	0	0	0	0
M5	23	0	0	0	0	0	0	0	0	0
	24	11	nd	nd	nd	nd	nd	nd	0	3
M6	25	0	16	0	0	0	28	40	45	1

AML colony formation in culture in the presence of IL-3, GM-CSF, G-CSF, and M-CSF, and combinations of these stimuli were assessed in 25 cases of AML. Values are mean numbers of colonies per 10^5 cells from triplicate experiments. The data from CSF-containing cultures were compared with cultures without CSF additions, and significant increases in colony numbers are indicated by brackets. Also, elevations of colony numbers (as compared with the effects of single CSFs) resulting from incubations with combined factors are indicated by brackets. Only in case nos. 9, 12, 13, 15, and 16 were colonies of 50 cells or more formed. In the other cases, the colonies contained <50 cells (clusters).

*Denotes increase of the size of the colonies as a result of a second or a third CSF.

Abbreviation: nd, not determined.

the single factors, but in these cases the addition of the combination of four CSFs produced few, if any, colonies. IL-3 was an efficient inducer of AML colonies in nine cases. In most of those cases (n = 7), GM-CSF was also active. In two cases, IL-3 was the only stimulator of AML-CFU. In two patients, the reverse pattern was seen: GM-CSF stimulated AML-CFU, whereas IL-3 did not. G-CSF acted as a colony-stimulating factor for AML-CFU in seven cases and was the only active stimulus in one case.

When IL-3 and GM-CSF were added together, colony numbers rose significantly in five cases. When G-CSF was also added, an increase in colony numbers was seen in three cases. An enhancing effect was apparent in one case when M-CSF was also added. From the experiments, it is clear that in 17 of 25 cases, colony formation could be induced when the appropriate CSFs or combinations of CSFs were added. In ten cases, we tested whether colonies appeared after five to seven days of culture in the presence of the four CSFs. In case nos. 15, 16, 18, and 22, we found 31, 26, 5, and 1 colonies containing <50 cells, respectively. In the other cases (ie, nos. 1, 3, 4, 10, 21, and 24), no colonies were present at days 5 through 7 of culture.

The effects of combined CSFs were not only evaluated on colony numbers, but also on colony size. The colony size increased in case no. 12 when IL-3 and GM-CSF were added simultaneously, and in case nos. 9 and 16 when G-CSF was added to the cultures containing IL-3 and GM-CSF. Finally, the results shown in Table 3 confirm that the serum-free assay can replace the serum-supplemented cultures without loss of colony forming efficiency.

Proliferation of AML cells in vitro: The effect of Epo. In 11 patients (nos. 1, 3, 6, 9, 10, 14, 17, 22, 23, and 25), we tested the effect of Epo on ^{3}H-TdR uptake of AML cells. Only in the case of erythroleukemia did Epo stimulate DNA synthesis of AML cells (Fig 1A). Experiments in which IL-3 and Epo were added in combination showed a significant increase of ^{3}H-TdR incorporation as compared with the cultures containing the single factors. Colony formation could be induced by IL-3 or Epo (Fig 1B). When Epo was added to cultures containing IL-3 and GM-CSF, an enhancement of colony numbers was observed.

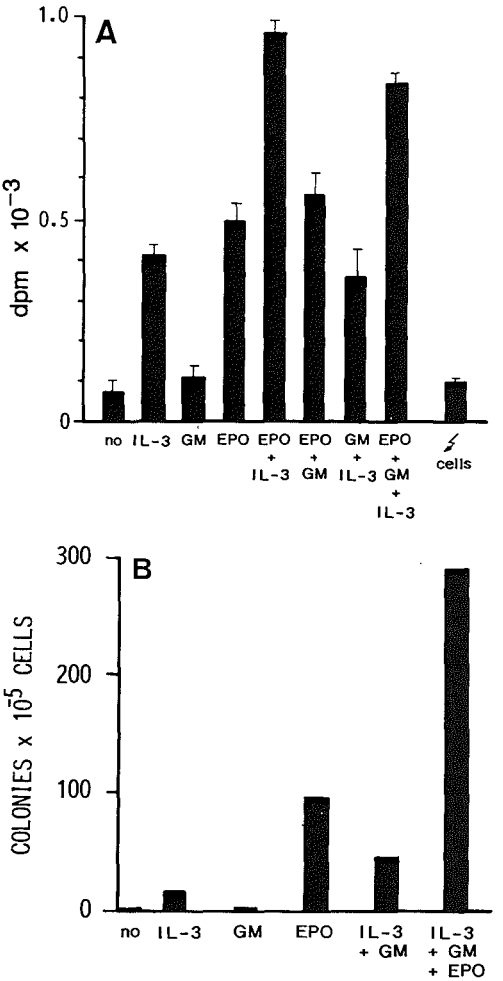

Fig 1. Effect of recombinant Epo, IL-3, and GM-CSF on (A) DNA synthesis ^{3}H-TdR incorporation and (B) colony formation of erythroleukemia cells (M6; case no. 25). The mean values of triplicate experiments ± SD are shown.

DISCUSSION

We investigated the stimulatory effects of five currently known hematopoietic growth factors, ie, IL-3, GM-CSF, G-CSF, M-CSF, and Epo, when supplied individually or simultaneously to AML cell cultures in 25 cases of human AML. The ^{3}H-TdR incorporation assay indicated that IL-3, GM-CSF, and G-CSF activated DNA synthesis in AML cells in 19, 15, and 13 cases, respectively. M-CSF occasionally stimulated ^{3}H-TdR uptake in AML cells (4 of 25 cases). Thus, the malignant cells have retained their ability to proliferate in response to one or more of these CSFs in a high proportion of cases. Previous observations that were made in serum-containing cultures had also shown that IL-3, GM-CSF, and G-CSF stimulated AML-CFU proliferation.[5-12]

Interestingly, whenever GM-CSF stimulated AML cell cycling in suspension cultures, IL-3 was a stimulator as well (Table 1). The linkage of IL-3 and GM-CSF responsiveness is suggestive of a cell population being responsive to both IL-3 and GM-CSF. This suggestion of a common IL-3/GM-CSF responsive subset of AML blasts would also be consistent with the observation that in the cases in which the cells responded to these two CSFs, no additive effects were demonstrable when GM-CSF was supplemented to the cultures with IL-3 (Table 2). In regard to the G-CSF response patterns in AML, the data are in support of a subpopulation of cells that responded to G-CSF alone. In two cases, G-CSF was the only active stimulus, and in six other cases, the significant G-CSF response was demonstrated to be additive to those of either GM-CSF or IL-3 (Table 2). In two cases, a

Table 4. CSF Response Patterns in 25 Cases of AML

No. of Cases	IL-3	GM-CSF	G-CSF	M-CSF	FAB
3	+	+	+	+	M2, M4, M4
8	+	+	+	−	M1 (n = 1), M2 (n = 3), M3 (n = 1), M4 (n = 3)
4	+	+	−	−	M1, M4, M4, M4
1	+	−	+	−	M2
3	+	−	−	−	M3, M5, M5
1	−	−	+	−	M4
1	−	−	−	+	M1
4	−	−	−	−	M1, M1, M2, M6

(+), Indicates a significant increase in ^{3}H-TdR uptake as compared with the cultures without exogenous CSFs.
(−), No effect.

clear enhancement of thymidine uptake induced by GM-CSF or IL-3 appeared when M-CSF was additionally supplemented. Surprisingly, in these two cases, M-CSF alone was incapable to induce any measurable DNA synthesis in AML, indicating a synergistic effect between M-CSF and IL-3 or GM-CSF. On the basis of these data we assume that different subsets of cells exist in human AML with distinct abilities to respond to growth factors. Cell separation studies should provide further support for this hypothesis.

A comparison of the results of ^{3}H-TdR incorporation and colony cultures (Tables 1 and 3) showed that growth factor responses were less frequently evident in the methylcellulose colony assay. In five cases, significant amounts of ^{3}H-TdR were incorporated, whereas no colonies were formed. These differences may be related to the fact that thymidine uptake was measured at day 4 of culture, whereas in a colony assay, only a minority of cells that give rise to progeny following several cell divisions during 12 to 14 days of incubation is assayed. However, in six of six cases that formed no or limited colonies at days 12 through 14 but showed significant ^{3}H-TdR incorporation at day 4, colony formation at days 5 through 7 was absent as well. Therefore, the growth in liquid *v* semisolid medium may also select for the outgrowth of different cell types as a consequence of culture conditions. Existing evidence supports the idea that AML cell growth is indeed better supported in liquid systems than in semisolid cultures.[17,25-28] Since in the suspension cultures the cells are settled at the bottom of round-bottomed microtiter trays and thus show a close cell-to-cell contact, we assume that not only CSFs, but also cell-cell interactions may be essential for the growth of these AML cells. This idea is supported by investigations that have shown stimulative effects on AML proliferation by the addition of membrane fragments of AML cells or irradiated AML cells to the cultures.[28,29]

An interesting observation was the increase of colony size in certain cases when more than one CSF was supplemented to the cultures. Similar effects on AML colonies have been reported before for GM-CSF and G-CSF.[8,9] These data could be explained by the induction of maturation by one factor, resulting in the expression of receptors for the second growth stimulus on the cell surface.

Several investigations[30-34] have shown that AML cells can produce CSFs that regulate proliferation of these cells in an autocrine fashion. In 14 of 25 cases of human AML, "CSF independent" DNA synthesis was observed that was significantly above the background data of cultures with preirradiated cells (Table 1). In ten of ten cases (nos. 5, 9, 10, 11, 16, 19, 20, 21, 23, and 25) tested, depletion of monocytic cells by adherence did not affect the spontaneous ^{3}H-TdR incorporation (data not shown). We assume that AML blasts in those cases incorporate ^{3}H-TdR in response to endogenously produced CSFs.

In the erythroleukemia, the cells responded to recombinant Epo as well as to IL-3. The data were suggestive for the existence of separate populations of AML cells with distinct growth factor requirements (Fig 1), ie, Epo- as well as IL-3–responding AML cells.

In summary, the four CSFs act as regulators of growth of human AML cells. The dependence of the cells on different factors, however, varied a great deal among the 25 cases that were examined. These diverse CSF response patterns in AML may represent abnormalities of growth in AML. Otherwise, they may reflect the response of normal hematopoietic progenitors at different maturation stages to these stimuli. However, we could not find any correlation between CSF response patterns and morphologic classification of AML (FAB) (Table 4). We expect that an exact assessment of growth factor response would provide an important tool for phenotyping AML based on the proliferative reactivity of the cells to the CSFs.

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REFERENCES

1. Metcalf D: The Haematopoietic Colony Stimulating Factors. New York, Elsevier, 1984

2. Metcalf D: The molecular biology and functions of the granulocyte-macrophage colony stimulating factors. Blood 67:257, 1986

3. Sieff CA: Haematopoietic growth factors. J Clin Invest 79:1549, 1987

4. Clark SC, Kamen R: The human haematopoietic colony stimulating factors. Science 236:1229, 1987

5. Griffin JD, Young D, Herrmann F, Wiper D, Wagner K, Sabbath KD: Effects of recombinant human GM-CSF on the proliferation of clonogenic cells in acute myelobastic leukemia. Blood 67:1448, 1986

6. Hoang T, Nara N, Wong G, Clark S, Minden MD, McCulloch EA: Effects of recombinant GM-CSF on the blast cells of acute myeloblastic leukemia. Blood 68:313, 1986

7. Vellenga E, Delwel R, Touw, I, Löwenberg B: Patterns of acute myeloid leukemia colony growth in response to recombinant granulocyte macrophage colony stimulating factor (r GM-CSF). Exp Hematol 15:652, 1987

8. Kelleher C, Miyauchi J, Wong G, Clark S, Minden MD, McCulloch EA: Synergism between recombinant growth factors, GM-CSF and G-CSF acting on blast cells of acute myeloblastic leukemia. Blood 69:1498, 1987

9. Vellenga E, Young DC, Wagner K, Wiper D, Ostapovicz D, Griffin JD: The effects of GM-CSF and G-CSF in promoting growth of clonogenic cells in acute myeloblastic leukemia. Blood 69:1771, 1987

10. Delwel R, Dorssers L, Touw I, Wagemaker G, Löwenberg B: Human recombinant multilineage colony stimulating factor (Interleukin 3): Stimulator of acute myelocytic leukemia progenitor cells in vitro. Blood 70:333, 1987

11. Vellenga E, Ostapovicz D, O'Rourke B, Griffin JD: Effects of recombinant IL-3, GM-CSF and G-CSF on proliferation of leukemic clonogenic cells in short-term and long-term cultures. Leukemia 1:584, 1987

12. Miyauchi J, Kelleher CA, Yang Y, Wong GG, Clark SC, Minden MD, Minkin S, McCulloch EA: The effects of three recombinant growth factors, IL-3, GM-CSF and G-CSF on the blast cells of acute myeloblastic leukemia maintained in short-term suspension culture. Blood 70:657, 1987

13. Suda T, Sato Y, Furakawa Y, Suda I, Mituoki E, Saito M, Miura Y: Myeloid and erythroid lineage expression of haematopoietic progenitors derived from an abnormal clone in erythroleukaemia. Br J Hematol 64:647, 1986

14. Salem M, Delwel R, Touw I, Mahmoud L, and Löwenberg B: Human AML colony growth in serum-free culture. Leuk Res 12:157, 1988

15. French-American-British (FAB) Cooperative Group: Proposals for the classification of the acute leukemia. Br J Hematol 32:451, 1976

16. French-American-British (FAB) Cooperative Group: Proposed revised criteria for the classification of acute myeloid leukemia. Ann Intern Med 103:626, 1985

17. Swart K, Hagemeyer A, Löwenberg B: Acute myeloid leukemia colony growth in vitro: Differences of colony forming cells in PHA supplemented and standard leukocyte feeder cultures. Blood 59:816, 1982

18. Löwenberg B, Hagemeyer A, Swart K: Karyotypically distinct subpopulations in acute leukemia with specific growth requirements. Blood 59:641, 1982

19. Böyum A: Separation of leukocytes from blood and bone marrow. Scand J Clin Lab Invest 21:77, 1968

20. Delwel R, Touw I, Bot F, Löwenberg B: A fucose binding lectin (UEA) for characterizing acute myeloid leukemia progenitor cells. Blood 68:41, 1986

21. Schaefer UW, Dicke KA, van Bekkum DW: Recovery of haemopoiesis in lethally irradiated monkeys by frozen allogeneic bone marrow grafts. Rev Eur Etud Clin Biol 17:443, 1972

22. Dorssers L, Burger H, Bot F, Delwel R, Geurts van Kessel A, Löwenberg B, Wagemaker G: Characterization of a human multicolony stimulating factor cDNA clone identified by a conserved noncoding sequence in mouse interleukin 3. Gene 55:115, 1987

23. Touw I, Delwel R, van Zanen G, Löwenberg B: Acute lymphoblastic leukemia and non-Hodgkin's lymphoma of T lineage: Colony forming cells retain growth factor (interleukin-2) dependence. Blood 68:1088, 1986

24. Salem M, Delwel R, Mahmoud LA, Elbasousy EM, Clark S, Löwenberg B: Maturation of human acute myeloid leukemia in vitro: The response to five haematopoietic factors. (submitted)

25. Taetle R, Caviles A, Koziol J: Response of human myeloid leukemia cells to various sources of colony stimulating activity and phytohaemagglutinin-conditioned medium. Cancer Res 43:2350, 1983

26. Griffin JD, Löwenberg B: Clonogenic cells in acute myeloblastic leukemia. Blood 68:1185, 1986

27. Dicke KA, Spitzer G, Ahearn MJ: Colony formation in vitro by leukemic cells in acute myelogeneous leukemia with pheatohaemoglutinin as stimulating factor. Nature 259:129, 1976

28. Nara N, McCulloch EA: The proliferation in suspension of the progenitors of the blast cells in acute myeloblastic leukemia. Blood 65:1484, 1985

29. Nara N, McCulloch EA: A comparison of the growth supporting capacities of fresh and cultured leukemic blast cells. Leuk Res 10:273, 1986

30. Young DC, Griffin JD: Autocrine secretion of GM-CSF in acute myeloblastic leukemia. Blood 68:1178, 1986

31. Young DC, Wagner K, Griffin JD: Constitutive expression of the GM-CSF gene in acute myeloblastic leukemia. J Clin Invest 79:100, 1987

32. Mannoni P, Mueller T, Torres H, Dubreuil P, de Waal R, Birg F: Expression of IL-3, GM-CSF and G-CSF genes in myeloid leukemia cells. Blood 70:263a, 1987

33. Oster W, Lindemann A, Horn S, Mertellsmann R, Herrmann F: Constitutive expression of genes for hematopoietic growth factors in acute myeloblastic leukemia. Blood 70:266a, 1987

34. Griffin JD, Rambaldi A, Wakamiya N, Vellenga E, Horiguchi J, Warren MK, Kufe DW: Expression of the M-CSF gene by human acute myeloblastic leukemia cells. Blood 70:258a, 1987

CHAPTER 6

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Maturation of Human Acute Myeloid Leukaemia in Vitro: The Response to Five Recombinant Haematopoietic Factors in a Serum-Free System

Maturation of human acute myeloid leukaemia *in vitro*: the response to five recombinant haematopoietic factors in a serum-free system

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Summary. The abilities of human recombinant IL-3, GM-CSF, G-CSF, M-CSF and Epo to induce maturation in human AML cells *in vitro* were investigated using cell specimens from 25 AML patients. The experiments were carried out under exactly defined serum-free culture conditions. In the absence of CSFs, monocytic and/or granulocytic maturation was detected in 14/25 cases. IL-3, GM-CSF, G-CSF and M-CSF elevated the proportions of monocyte/macrophages in 3/25, 2/25, 1/25 and 6/25 cases respectively, and increased the percentages of mature granulocytes in 2/25, 1/25, 1/25 and 0/25 cases, and if so only to a limited extent (values below 50%). The ^{3}H-thymidine (^{3}H-TdR) uptake studies revealed that IL-3, GM-CSF, G-CSF and M-CSF were efficient stimulators of DNA synthesis of AML cells in 19, 15, 13 and four of those cases, respectively. Thus, although the cells in most cases responded to CSFs by activation of DNA synthesis, they were unable to give rise to terminally differentiated stages. Provision of CSFs in combination was more frequently effective in enhancing maturation and also increased the magnitude of maturation response. Monocytic versus granulocytic maturation of AML cells after culture did not correlate with the FAB cytology nor with the type of CSF presented; but generally granulocytic maturation was an infrequent phenomenon. Epo stimulated erythroid differentiation and DNA synthesis only in the case of erythroleukaemia, but it had no effect on the cells of 10 other AML cases. Extrapolation of these *in vitro* findings would suggest that CSFs would have a limited therapeutic utility to induce AML cell maturation *in vivo* and that hazards of stimulating blast cell proliferation with these factors may be anticipated.

Colony stimulating factors (CSFs) and erythropoietin (Epo) are known to regulate the expansion and cell lineage commitment of the haematopoietic progenitor cells. Multi-CSF (IL-3) and granulocyte-macrophage CSF (GM-CSF) exert stimulative effects on the more primitive progenitors in the marrow, and granulocyte CSF (G-CSF), macrophage CSF (M-CSF) and Epo act particularly on maturation and functional activities of the more differentiated cells (Metcalf, 1984; Metcalf & Nicola, 1985; Metcalf, 1986; Clark & Kamen, 1987; Sieff, 1987a). Some apparent overlap in function of these factors has been seen. GM-CSF and G-CSF, for instance, may both activate the functional status of the myelomonocytic end cells (Grabstein *et al*, 1986; Weisbart *et al*, 1986).

The human CSFs (Kawasaki *et al*, 1985; Wong *et al*, 1985; Nagata *et al*, 1986; Yang *et al*, 1986) and Epo (Jacobs *et al*, 1985; Lin *et al*, 1985) have all been obtained as separate entities by molecular cloning. This has inspired an increased interest to analyse the role of these molecules in growth abnormalities of human leukaemia.

It has been demonstrated that one or more of these five recombinant growth factors can stimulate the proliferation of human AML cells *in vitro* (Griffin *et al*, 1986; Hoang *et al*, 1986; Suda *et al*, 1986; Delwel *et al*, 1987; Kelleher *et al*, 1987; Miyauchi *et al*, 1987a, b; Vellenga *et al*, 1987a, b; Delwel *et al*, 1988). Thus the cells in most cases of AML have retained the susceptibility to respond to these stimuli. Here we present the studies that were concerned with the abilities of these factors, when presented individually or in combinations to induce AML cell maturation, and to assess whether—under certain stimulatory situations—the leukaemic blasts could bypass the maturation blockade (Lotem & Sachs, 1974). The cultures were run serum-free (Salem *et al*, 1988) to exclude the interference of any undefined substances.

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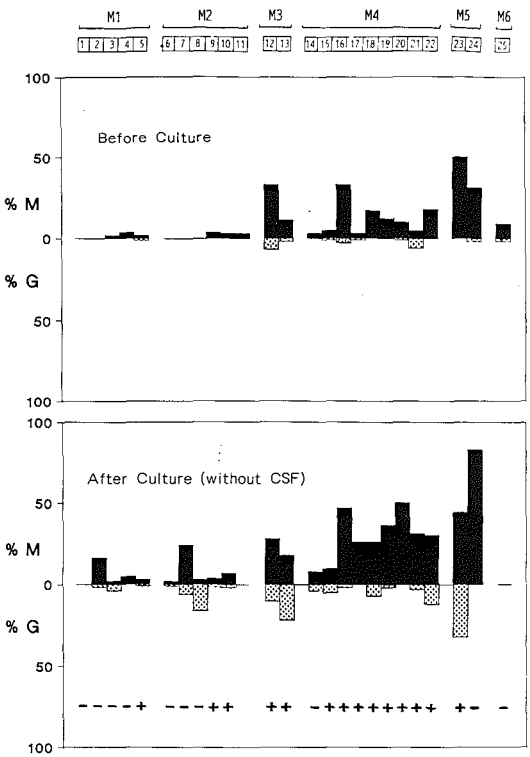


Fig 1. Maturation of AML cells: comparison between preculture (upper panel) and postculture (lower panel) cells in the absence of CSFs. AML blasts were incubated without CSF addition. The percentages of monocyte-macrophage (M) (black bars) and mature granulocytes (G) (dotted bars) were scored in MGG-stained smears before and after culture. The 25 cases of AML are grouped according to their FAB cytology (M1–M6) and the results are indicated per individual patient. (+): cases with significant ($P<0{\cdot}05$) DNA synthesis in the absence of CSFs (as compared to irradiated cells); (−): cases with no spontaneous DNA synthesis.

MATERIALS AND METHODS

Patients and preparation of AML cells. Cells were obtained after permission from 25 untreated cases of AML, classified according to the criteria of the French–American–British (FAB) Cooperative Group (1976, 1985) (Table I and Figs 1–4). AML cells were isolated from peripheral blood (in cases 2, 4, 6, 9, 12, 13, 15, 19, 20 and 22) or bone marrow after bovine serum albumin (BSA) density gradient centrifugation or Ficoll–Hypaque centrifugation (Böyum, 1968) (cases 10, 13, 16, 18, 20, 21 and 24) followed by E-rosette depletion (Löwenberg *et al.* 1982). The cells were then cryopreserved in 7·5% dimethyl sulfoxide (DMSO) and 20% inactivated fetal calf serum (FCS). The differential counts revealed that in 14/25 cases the percentages of blasts in the thawed material were above 90%. In most of M3, M4 and M5 cases, however, mature cells (usually monocytes) represented more than 10% (Fig 1).

Culture medium (serum-free). A serum-free medium that was recently proposed for AML cell growth *in vitro* (Salem *et al.* 1988), was used throughout the experiments. Dulbecco's modified Eagle's minimum essential medium is supplemented with 15 mg/ml dialysed BSA (Cohn fraction V, Sigma), 10^{-7} M sodium selenite (Merck, Darmstadt, F.R.G.), $7{\cdot}7\times10^{-6}$ M iron saturated human transferrin (Behring Institute, Marburg, F.R.G.), 10^{-4} M 2-mercapto-ethanol (Merck), 7·8 μg/ml cholesterol (Sigma), 1 μg/ml insulin (Sigma) and 2·8 μg/ml linoleic acid (Merck).

Haematopoietic growth factors. Human recombinant IL-3, GM-CSF, G-CSF and M-CSF were all obtained from Genetics Institute (Cambridge, Mass.). The molecular cloning and biological characteristics of these CSFs have been published (Kawasaki *et al.* 1985; Wong *et al.* 1985; Nagata *et al.* 1986; Yang *et al.* 1986). Dose–response experiments have established the concentration of each CSF stimulating optimal AML DNA synthesis (Delwel *et al.* 1988). Based upon these data we conducted the experiments using 0·1% (v/v) IL-3, 200 U/ml GM-CSF, 0·1% (v/v) G-CSF, and 0·1% (v/v) M-CSF. These CSFs were tested for their effects upon AML cell maturation as well as DNA synthesis. Control experiments included cultures with no CSFs and cultures with 10% FCS (Flow Laboratories) in addition to the four CSFs. In a series of 11 AML cases, the effects of the addition of 1 U/ml human recombinant Epo (Amgen, Thousand Oaks, Calif.), alone or in combination with IL-3 or GM-CSF, were also tested.

Evaluation of AML cell maturation in vitro. AML cells (2×10^5/ml) were cultured in suspension in polystyrene tubes (Greiner, Nurtingen, F.R.G.), in the presence or absence

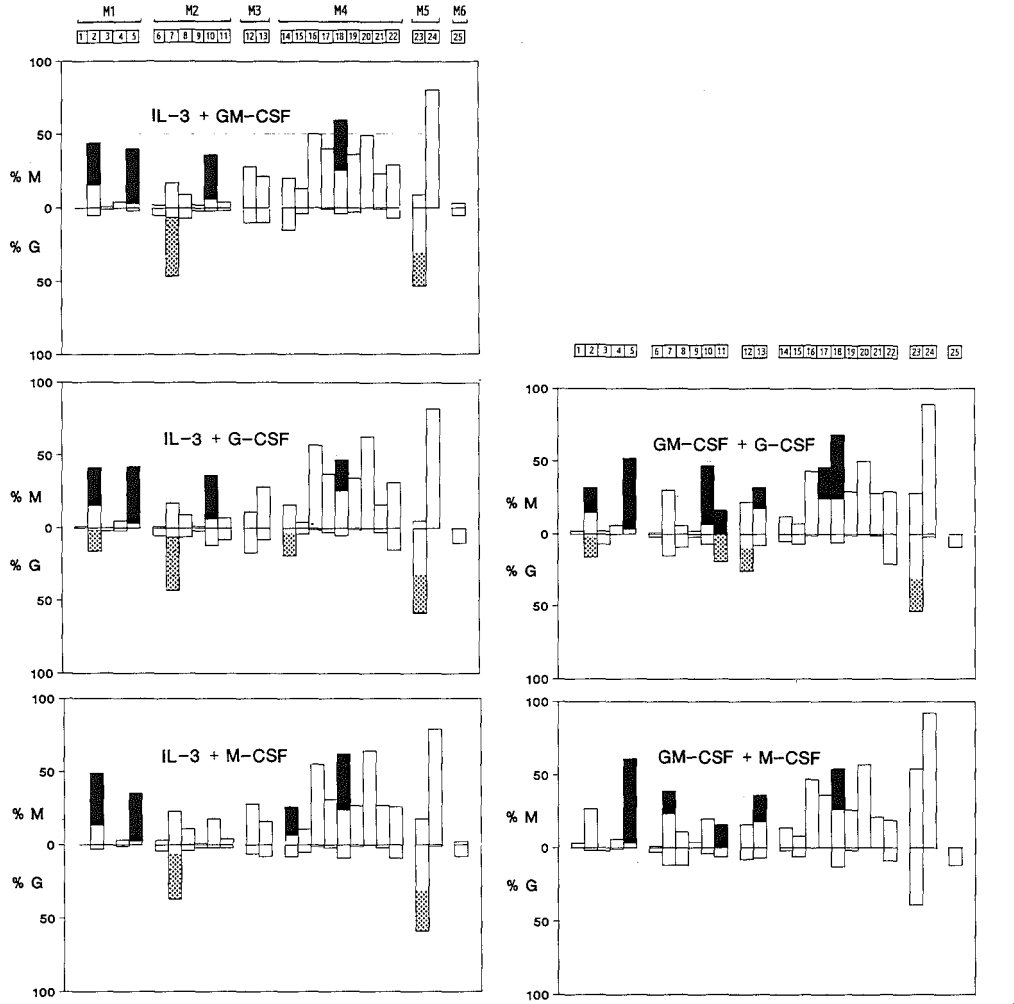


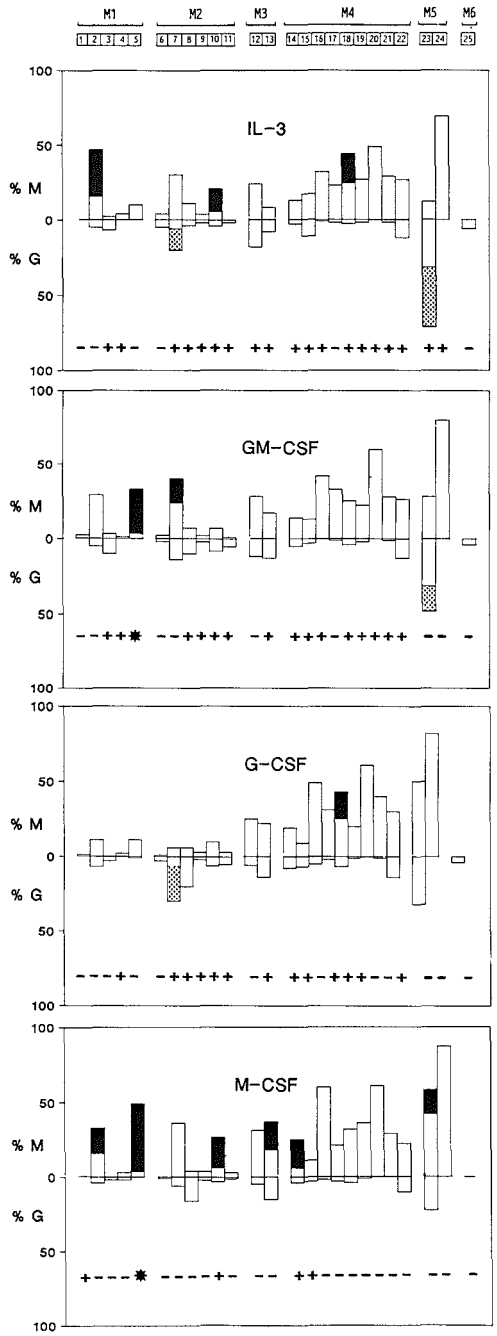
Fig 3. Maturation of AML cells *in vitro*: the response to dual CSF exposure. CSFs were added to the AML cell cultures as two factor combinations. For further explanations see Fig 2.

described (Griffin *et al*, 1986; Salem *et al*, 1988). 2×10^4 cells were cultured in 100 μl serum-free medium, with or without the addition of CSFs, in 96 wells round-bottom microtitre trays (Greiner) for 90 h. 5 h before harvesting 0·1 μCi tritiated thymidine (^{3}H-TdR 2 Ci/mmol; Amersham, U.K.) was added. Cells were harvested on nitrocellulose paper using a Titertek harvester 550 (Flow Laboratories). Radioactivity was determined with a Beckman LS 3800 scintillation counter (Fullerton, Calif.). Data are expressed as means $\pm$ standard deviation (SD) of triplicate cultures. Statistical significance ($P<0·05$) was determined by Student's *t*-test. The results of CSF-supplemented cultures were compared to those of cultures with no CSFs. In order to assess whether in certain cases AML cells exhibited spontaneous proliferation (i.e. in the absence of added CSFs) the data obtained from unstimulated cultures were compared with control values of irradiated (30 Gy) AML cells.

RESULTS

Maturation of AML cells in vitro: no addition of growth factors

In the absence of exogenous stimuli, some granulocyte and/or monocyte-macrophage maturation occurred *in vitro* in 14/25 cases of AML (Fig 1). This 'spontaneous' maturation was more prevalent in cases with M3, M4 and M5 morphology where spontaneous cellular DNA synthesis was as well



of CSFs. Incubations were carried out for 6–8 d in a fully humidified atmosphere of 5% CO_2 at 37°C. In all cases cytocentrifuge slides (Shandon, Cheshire, U.K.) were prepared from pre- and post-culture cells, stained with May-Grünwald-Giemsa stain (MGG), and examined by light microscopy using 100 × lens and immersion oil. At least 200 cells per slide were counted and the percentages of immature cells (blasts, promyelocytes and promonocytes), mature granulocytes (myelocytes, metamyelocytes, band and segmented forms) and monocyte/macrophages were separately scored. The following criteria were considered for morphologic analysis of the cells: cell size, cytoplasmic basophilia, cytoplasmic granules, nuclear/cytoplasmic ratio, nuclear segmentation, chromatin pattern, and the presence or absence of nucleoli. In most cases, alpha-naphthol acetate esterase and chloroacetate esterase stained preparates (Yam *et al*, 1971) were examined in parallel.

The expression of certain surface antigens indicating progressive maturation of cultured AML cells was also assessed in 11/25 cases by indirect immunofluorescence as described (Touw & Löwenberg, 1985). Three murine monoclonal antibodies (MCA) (in appropriate titres) were employed: CD15 (B4·3; reacting with a granulocytic surface antigen; IgM; 1:500) (Reyden *et al*, 1983), CD14 (B44-1; reacting with a surface marker of mature monocytes; IgM; 1:20) (Perrussia *et al*, 1982) and IV B5 (reacting with glycophorin A; IgG; 1:500) (Touw & Löwenberg, 1985). CD15 and IV B5 were gifts from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam; and CD14 was a gift from the Wistar Institute, Philadelphia.

A close concordance was noted between the results of MGG staining, cytochemistry and surface marker analysis. Since the morphology data of MGG films were available in each case, we shall mainly refer to those.

Maturation of AML cells was evaluated in cultures devoid of CSFs as well as in the presence of individual or various combinations of CSFs. Maturation as a result of the addition of CSFs was considered significant (as compared to unstimulated cultures) if an increase of at least 15% in mature granulocytic or monocytic cells was noted.

Tritiated thymidine incorporation. Maturation data were systematically evaluated with reference to the abilities of the AML cells to respond to the growth factors by activation of DNA synthesis. DNA synthesis was estimated essentially as

Fig 2. Maturation of AML cells *in vitro*: the response to individual CSFs. AML blasts were incubated with IL-3, GM-CSF, G-CSF and M-CSF separately. The length of the bars represents the percentages of monocyte-macrophages (M) and mature granulocytes (G) in CSF supplemented cultures. Significant increases in the numbers of mature cells as compared to cultures without CSFs (Fig 1, lower panel) are indicated by black segments (for monocyte-macrophages) or dotted segments (for mature granulocytes). The 25 AML cases are listed individually according to FAB cytology as in Fig 1. (+): cases with significant ($P<0·05$) stimulation of DNA synthesis by individual CSFs (as compared to cultures with no CSF); (−): cases with no proliferative response to CSFs; (*): significant ($P<0·05$) inhibition of DNA synthesis in case 5 after exposure to GM-CSF or M-CSF (as compared to cultures without CSFs).

evident. The direction of maturation did not strictly correlate with the FAB classes. Monocytes and macrophages appeared in culture in cases with M1 (no. 2), M2 (no. 7), M3 (nos. 12 and 13), M4 (nos. 16, 17, 18, 19, 20, 21 and 22) and M5 (nos. 23 and 24) cytology. Granulocytic cells were noted in unstimulated cultures in cases with M2 (no. 8), M3 (no. 13) and M5 (no. 23) AML types.

Maturation of AML cells in vitro: the effects of individual CSFs

It is apparent from Fig 2 that CSFs, when supplied individually, had only a limited and infrequent stimulating effect upon maturation of the cells in AML. IL-3 was able to stimulate monocyte-macrophage maturation in three cases (nos. 2, 10 and 18) and granulocytic maturation in two other cases (nos. 7 and 23). GM-CSF rarely induced monocytic (cases 5 and 7), or granulocytic maturation (case 23). G-CSF, when added as the single growth factor, produced monocytes in case 18 and granulocytic cells in case 7 only. Finally M-CSF stimulated AML cell maturation in six cases (nos. 2, 5, 10, 13, 14 and 23) always along the monocyte/macrophage pathway.

On the contrary, these factors were potent in triggering DNA synthesis of AML cells in most instances. IL-3, GM-CSF and G-CSF were especially effective and stimulated proliferation in 19, 15 and 13 cases respectively. M-CSF was active in 4/25 cases only. Moreover, in the majority of cases (16/25) the ^{3}H-TdR uptake could be induced by two or more of the four CSFs. The data indeed confirm the efficacy of these stimuli in potentiating the *in vitro* proliferation of AML cells. In one instance (case 5), however, GM-CSF and M-CSF inhibited the autonomous growth of cells and this was accompanied by increased production of monocyte/macrophages.

Maturation of AML cells in vitro: the effects of CSF combinations

When two or more CSFs were added simultaneously to the cultures, AML cell maturation was further enhanced in magnitude and in frequency (Figs 3 and 4). For instance, the combination of GM-CSF + G-CSF induced an increase in the percentages of monocyte/macrophages and/or mature granulocytes in nine cases (nos. 2, 5, 10, 11, 12, 13, 17, 18 and 23) (Fig 3). The combination of IL-3 + GM-CSF + G-CSF permitted macrophage maturation in eight cases (nos. 2, 5, 8, 10, 11, 17, 18 and 20), granulocyte maturation in one case (no. 23), and maturation into both directions in another case (no. 7) (Fig 4).

It is of note that granulocytic maturation was generally a rare finding even after the exposure of AML blasts to the four CSFs in combination. Serum supplemented cultures that were performed in direct comparison to the serum-free ones gave similar results (data not shown). Interestingly, the cells of a case of monoblastic leukaemia (no. 23, M5) were found to give rise in culture to cells of granulocytic lineage that were reactive with CD15 MCA. The production of granulocytes in this case was most actively stimulated by IL-3 and—to a less extent—by GM-CSF.

Leukaemic origin of the mature elements that appeared in culture was supported by the demonstration of Auer rods.

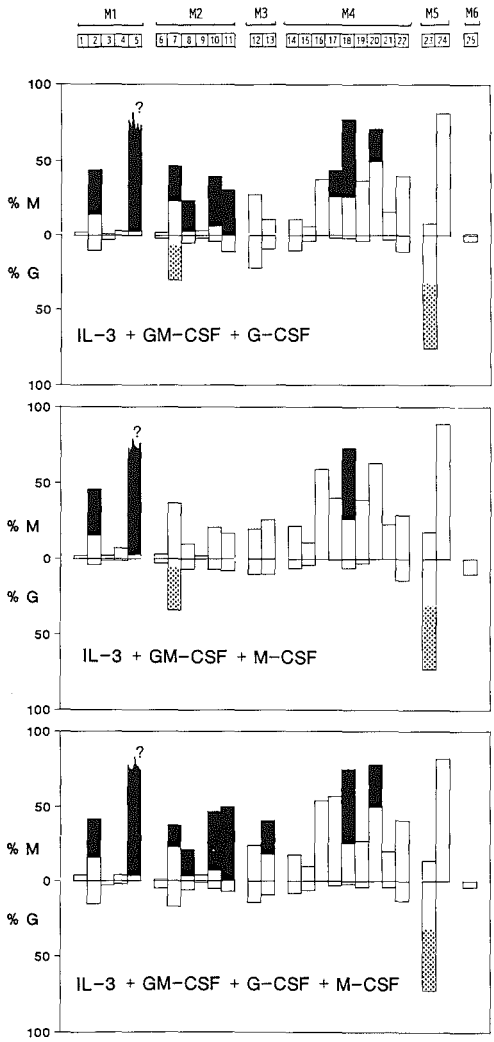


Fig 4. Maturation of AML cells *in vitro*: the response to three or four CSFs. CSFs were added to AML cell cultures as three or four factor combinations: For further explanation see Fig 2. (?): less than 200 cells (macrophages) were recovered so that an exact differential estimate was not done.

Granulocytic cells in two cases (no. 7 with IL-3 or G-CSF, and no. 11 with CSF combinations) and monocyte-macrophages in four cases (no. 11 with CSF combinations, and nos. 16, 20 and 21 in the presence or absence of CSFs) revealed Auer rods postculture: whereas preculture Auer rods had only been seen in blasts.

The effect of Epo. No significant changes in cellular matu-

Table I. Induction of erythroid and myelomonocytic differentiation in erythroleukaemia (case 25) in Epo or IL-3 stimulated cultures

	Myelomonocytic cells (%)			Erythroid cells (%)	
	Immature cells*	Mature granulocytes	Monocyte-macrophages	Proerythroblasts	Normoblasts
Before culture	64	4	6	19	7
After culture					
1. Epo	1	1	1	91	6
2. IL-3	82	6	0	9	3
3. Epo + IL-3	30	1	0	65	4

Differential counts of MGG-stained preparates were performed in pre- and postculture cells. The cultures were stimulated by Epo or IL-3 or both together.

* Immature myelomonocytic cells refer to myeloblasts + promyelocytes + promonocytes.

ration or DNA synthesis were detected after the addition of Epo to the cultures of 10 of 11 AML cases that were tested. However, in patient 25, the only case with erythroleukaemia, Epo alone enhanced the DNA synthesis (stimulation index of 5×) as well as erythroid cell differentiation (Table I). IL-3, which could not significantly activate the ^{3}H-TdR incorporation of these cells (Fig 2), in contrast stimulated the production of myelomonocytic elements. The combination of Epo plus IL-3 further elevated the rate of DNA synthesis by the cells (stimulation index of 9·2×) and yielded intermediate values of erythroid and myeloid cells.

DISCUSSION

A fundamental question in the study of AML is whether these cells are capable, when appropriately stimulated, to mature beyond the blastic stage to the terminally differentiated forms and thus whether the maturation blockade that characterizes leukaemia can be overcome. We have addressed this question in experiments in which AML cells from a cohort of 25 patients were cultured under different conditions of growth factor stimulation and evaluated the abilities of cells to mature to monocyte/macrophages or to granulocytes in response to recombinant IL-3, GM-CSF, G-CSF, M-CSF and Epo. In order to relate the maturation phenomenon most directly to the growth factor effects, these experiments were conducted in a strictly serum-free assay.

Firstly, we demonstrated that in the absence of external stimuli, the cells from 14/25 cases underwent progressive maturation in culture. The intrinsic mechanisms that brought about this spontaneous maturation most likely involve the autocrine production of factors that can stimulate blast cell maturation. Such an endogenous stimulus could originate either from AML progenitor cells, as proposed by others (Furukawa *et al*, 1986; Young & Griffin, 1986; Young *et al*, 1987) or from accessory cells. Candidate cells for endogenous growth factor production are the monocytic cells that are often found among the AML mass. Monocytes have been shown to actively release a variety of factors; e.g. GM-CSF (Thorens *et al*, 1987), G-CSF and M-CSF (Clark & Kamen, 1987; Rambaldi *et al*, 1987; Sieff, 1987a), interleukin-1 (Dinarello, 1984) and tumour necrosis factor (Beutler *et al*, 1985). The latter two monokines can in turn modulate the production of CSFs in a variety of cell types (Fibbe *et al*, 1986; Munker *et al*, 1986; Koeffler *et al*, 1987; Old, 1987; Sieff *et al*, 1987b; Zucali *et al*, 1987). Support for a role of endogenous factors would also be in accordance with the observation that 'spontaneous' maturation was noted most frequently in the AML cases with higher numbers of monocytic cells in their preculture materials (Fig 1). Most of those cases also exhibited 'spontaneous' DNA synthesis in non-stimulated cultures. However, after plastic adherence and depletion of mature monocytic elements the spontaneous maturation and proliferation were not reduced (data not shown). This indicates that the endogenous stimulus for these events mainly originated from AML blasts.

In a limited number of cases, IL-3, GM-CSF and G-CSF could enhance the formation of mature granulocytes or macrophages *in vitro*, while M-CSF only stimulated macrophage development. In several cases, one or more of these CSFs were extremely active in stimulating AML cell growth *in vitro*, nevertheless the same CSFs failed to induce a significant degree of maturation in most of these instances. For instance, among 13 proliferation responders to G-CSF, the blast cells of only two cases (nos. 7 and 18) matured in response to G-CSF. Thus, while the AML cells were apparently susceptible to these factors, the cells failed to mature. Only by supplying combined CSFs in culture, in a fraction of these cases (e.g. no. 11) maturation was achieved to some extent (Figs 3 and 4). These findings would indicate that the cells in AML are essentially responsive to the growth factors (activation of DNA synthesis) but the transduction of the signal and the intracellular cascade that results in proper myeloid cell maturation are abnormal (Sachs, 1986). This was most remarkable in those cases (e.g. nos. 1, 3, 4, 6 and 9) where a high proliferative response was achieved but no maturation was demonstrable even after the exposure of the AML cells to a cocktail of the four CSFs plus Epo. The variability in the degree of maturation and the response to different growth

factors among the patient group is a notable feature of the biological heterogeneity of the disease. Apparently it is an expression of the residual abilities of the cells to respond to these stimuli and to mature subsequently. It remains to be seen whether the cases that did not mature in the presence of CSFs, are really maturation defective clones with an absolute block of differentiation or whether they can still be successfully manipulated to proceed along the maturation lineage when other differentiation inducers are presented, e.g. DMSO (Collins *et al*, 1978) or vitamin D3 analogues (Abe *et al*, 1981).

Maturation along the granulocytic versus monocytic lineage, when expressed *in vitro*, could not usually be predicted by the type of CSF added to culture. For instance, IL-3, GM-CSF and G-CSF induced terminally differentiated granulocytic or monocytic elements at approximately equivalent frequencies. The results with M-CSF, on the other hand, showed a trend of preferential induction of monocytic maturation. It was also notable that no relationship was evident between the type of mature cells that appeared in stimulated or unstimulated cultures and the FAB classes of AML. Monoblastic (M5) leukaemia (e.g. case 23) gave rise in culture to mature granulocytes; and in several M1, M2 and M3 cases, monocytic maturation took place. This indicates that some leukaemias *in vivo* express mainly the cytological features of a certain lineage, but under certain *in vitro* conditions, preferentially differentiate into another direction.

In the example of erythroleukaemia (M6), different growth factors could promote the cellular DNA synthesis while leading to divergent differentiation events. Epo permitted the production of erythroid elements whereas IL-3 stimulated the development of cells with myelomonocytic morphology (Table I).

Active cell proliferation was apparent during maturation of AML cells in certain cases; e.g. in M3, M4 and M5 cases in the absence of CSFs (Fig 1) and in rare cases after exposure to single CSFs (Fig 2). In other instances, however, AML cells were able to mature without activation of DNA synthesis (e.g. cases 2, 7 and 8 with no added CSFs; Fig 1, and cases 2, 13 and 23 with M-CSF; Fig 2). In one case (no. 5) induction of maturation by GM-CSF or M-CSF was associated with a suppression of the leukaemic cell proliferation (Fig 2). This reveals that there is no strict linkage between maturation and proliferation responses in AML, and significant maturation of cells may appear in the presence or absence of activated DNA synthesis.

The findings reported here raise doubts about the therapeutic utility of CSFs for induction of maturation *in vivo* and thus for treating patients with AML. First, these factors are potent stimuli for AML cell proliferation in most cases which could include the risks of boosting the progressive outgrowth of the leukaemia. Secondly, AML cell maturation is only rarely potentiated by these factors, more often towards monocytes, and if so, to a limited extent only. It has also been shown that these CSFs can promote the self-renewal of AML cells (Vellenga *et al*, 1987b). Therefore, we believe that it is unlikely that therapy of patients with CSFs would have a general applicability. *In vivo* administration of CSFs, if intended, should benefit from prescreening of leukaemic cells from individual patients *in vitro* to predict their maturation and proliferation responses and to select appropriate CSFs.

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REFERENCES

Abe, E., Miyaura, C., Sakagami, H., Takeda, M., Konno, K., Yamazaki, T., Yoshiki, S. & Suda, T. (1981) Differentiation of mouse myeloid leukemia cells induced by 1,25-dihydroxyvitamin D3. *Proceedings of the National Academy of Sciences of the United States of America*, **78**, 4990–1994.

Beutler, B., Greenwald, D., Hulmes, J.D., Chang, M., Pan, Y.-C.E., Mathison, J., Ulevitch, R. & Cerami, A. (1985) Identity of tumour necrosis factor and the macrophage-secreted factor cachectin. *Nature*, **316**, 552–554.

Böyum, A. (1968) Separation of leukocytes from blood and bone marrow. *Scandinavian Journal of Clinical and Laboratory Investigation*, **21**, 77–89.

Clark, S.C. & Kamen, R. (1987) The human hematopoietic colony-stimulating factors. *Science*, **236**, 1229–1237.

Collins, S.J., Ruscetti, F.W., Gallagher, R.E. & Gallo, R.C. (1978) Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl-sulfoxide and other polar compounds. *Proceedings of the National Academy of Sciences of the United States of America*, **75**, 2458–2462.

Delwel, R., Dorssers, L., Touw, I., Wagemaker, G. & Löwenberg, B. (1987) Human recombinant multilineage colony stimulating factor (Interleukin-3): stimulator of acute myelocytic leukemia progenitor cells in vitro. *Blood*, **70**, 333–336.

Delwel, R., Salem, M., Pellens, C., Dorssers, L., Wagemaker, G., Clark, S. & Löwenberg, B. (1988) Growth regulation of human acute myeloid leukemia: effects of five recombinant hematopoietic factors in a serum free culture system. *Blood* (in press).

Dinarello, C.A. (1984) Interleukin-1 and the pathogenesis of the acute-phase response. *New England Journal of Medicine*, **311**, 1413–1418.

Fibbe, W.E., Damme, J. van, Billiau, A., Voogt, J., Duinkerken, N., Kluck, P.M.C. & Falkenburg, J.H.F. (1986) Interleukin-1 (22-K factor) induces release of granulocyte-macrophage colony-stimulating activity from human mononuclear phagocytes. *Blood*, **68**, 1316–1321.

French–American–British (FAB) Cooperative Group (1976) Proposals for the classification of the acute leukaemias. *British Journal of Haematology*, **32**, 451–458.

French–American–British (FAB) Cooperative Group (1985) Proposed revised criteria for the classification of acute myeloid leukemia. *Annals of Internal Medicine*, **103**, 626–629.

Furukawa, Y., Ohta, M., Miura, Y. & Saito, N. (1986) Interleukin-1 producing ability of leukaemia cells and its relationship to morphological diagnosis. *British Journal of Haematology*, **65**, 11–15.

Grabstein, K.H., Urdal, D.L., Tushinski, R.J., Mochizuki, D.Y., Price, V.L., Cantrell, M.A., Gillis, S. & Conlon, P.J. (1986) Induction of macrophage tumoricidal activity by granulocyte-macrophage colony-stimulating factor. *Science*, **232**, 506–508.

Griffin, J.D., Young, D., Herrmann, F., Wiper, D., Wagner, K. & Sabbath, K.D. (1986) Effects of recombinant human GM-CSF on

the proliferation of clonogenic cells in acute myeloblastic leukemia. *Blood*, **67**, 1448–1453.

Hoang, T., Nara, N., Wong, G., Clark, S., Minden, M.D. & McCulloch, E.A. (1986) Effects of recombinant GM-CSF on the blast cells of acute myeloblastic leukemia. *Blood*, **68**, 313–316.

Jacobs, K., Shoemaker, C., Rudersdorf, R., Neill, S.D., Kaufman, R.J., Mufson, A., Sechra, J., Jones, S.S., Hewick, R., Fritsch, E.F., Kawakita, M., Shimizu, T. & Miyake, P. (1985) Isolation and characterization of genomic and cDNA clones of human erythropoietin. *Nature*, **313**, 806–810.

Kawasaki, E.S., Ladner, M.B., Wang, A.M., Arsdell, J. van, Warren, M.R., Coyne, M.Y., Schweickart, V.L., Lee, M., Wilson, K.J., Bossman, A., Stanley, E.R., Ralph, P. & Mark, D.F. (1985) Molecular cloning of a complementary DNA encoding human macrophage specific colony-stimulating factor (CSF-1). *Science*, **230**, 291–230.

Kelleher, C., Miyauchi, J., Wong, G., Clark, S., Minden, M.D. & McCulloch, E.A. (1987) Synergism between recombinant growth factors, GM-CSF and G-CSF acting on blast cells of acute myeloblastic leukemia. *Blood*, **69**, 1498–1503.

Koeffler, H.P., Gasson, J., Ranyard, J., Souza, L., Shepard, M. & Munker, R. (1987) Recombinant human TNFalpha stimulates production of granulocyte colony-stimulating factor. *Blood*, **70**, 55–59.

Lin, F.K., Suggs, S., Lin, C.-H., Browne, J.F., Smalling, R., Egrie, J.C., Chen, K.K., Fox, G.M., Martin, F., Stabinsky, Z., Baddrowi, S.M., Lai, P.-H. & Golowasser, E. (1985) Cloning and expression of the human erythropoietin gene. *Proceedings of the National Academy of Sciences of the United States of America*, **82**, 7580–7584.

Lotem, J. & Sachs, L. (1974) Different blocks in the differentiation of myeloid leukemic cells. *Proceedings of the National Academy of Sciences of the United States of America*, **71**, 3507–3511.

Löwenberg, B., Hagemeyer, A. & Swart, K. (1982) Karyotypically distinct subpopulations in acute leukemia with specific growth requirements. *Blood*, **59**, 641–645.

Metcalf, D. (1984) *The Hematopoietic Colony Stimulating Factors*. Elsevier, Amsterdam.

Metcalf, D. (1986) The molecular biology and functions of the granulocyte-macrophage colony stimulating factor. *Blood*, **67**, 257–267.

Metcalf, D. & Nicola, N.A. (1985) Role of the colony stimulating factors in the emergence and suppression of myeloid leukemia populations. *Molecular Biology of Tumor Cells* (ed. by B. Wahren, G. Holm, S. Hammarstrom and P. Perlmann), pp. 215–232. Raven Press, New York.

Miyauchi, J., Kelleher, C.A., Yang, Y., Wong, G.G., Clark, S.C., Minden, M.D., Minkin, S. & McCulloch, E.A. (1987a) The effects of three recombinant growth factors, IL-3, GM-CSF and G-CSF on the blast cells of acute myeloblastic leukemia maintained in short-term suspension culture. *Blood*, **70**, 657–663.

Miyauchi, J., Minden, M.D. & McCulloch, E.A. (1987b) The effect of recombinant human CSF-1 on acute myeloblastic leukemia (AML) cells in culture. *Blood*, **70** (Suppl.), 264a.

Munker, R., Gasson, J., Ogawa, M. & Koeffler, H.P. (1986) Recombinant human TNF induces production of granulocyte-monocyte colony-stimulating factor. *Nature*, **323**, 79–82.

Nagata, S., Tsuchiya, M., Asano, S., Kaziro, Y., Yamazaki, T., Yamamoto, O., Hirata, Y., Kubota, N., Oheda, M., Nomura, H. & Ono, M. (1986) Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor. *Nature*, **319**, 415–418.

Old, L.J. (1987) Tumour necrosis factor: polypeptide mediator network, *Nature*, **326**, 330–331.

Perrussia, B., Trinchicri, G., Lebman, D., Jankiewicz, J., Lange, B. & Rovera, G. (1982) Monoclonal antibodies that detect differentiation surface antigens on human myelomonocytic cells. *Blood*, **59**, 382–392.

Rambaldi, A., Young, D.C. & Griffin, J.D. (1987) Expression of M-CSF (CSF-1) gene by human monocytes. *Blood*, **69**, 1409–1413.

Reyden, H.J. van der, Rhenen, D.J. van, Lansdorp, P.M., Veer, M.B. van 't, Langenhuijsen, M.M.A.C., Engelfriet, C.P. & Borne, A.E.G.Kr. von dem (1983) A comparison of surface marker analysis and FAB classification in acute myeloid leukemia. *Blood*, **61**, 443–448.

Sachs, L. (1986) Hematopoietic growth and differentiation factors and the reversibility of malignancy: cell differentiation and bypassing of genetic defects in leukemia. *Medical Oncology and Tumor Pharmacotherapy*, **3**, 165–176.

Salem, M., Delwel, R., Touw, I., Mahmoud, L. & Löwenberg, B. (1988) Human AML colony growth in serum-free culture. *Leukemia Research*, **12**, 157–165.

Sieff, C.A. (1987a) Hematopoietic growth factors. *Journal of Clinical Investigation*, **79**, 1549–1557.

Sieff, C.A., Tsai, S. & Faller, D.V. (1987b) Interleukin-1 induces cultured human endothelial cell production of granulocyte macrophage colony-stimulating factor. *Journal of Clinical Investigation*, **79**, 48–51.

Suda, T., Sato, Y., Furakawa, Y., Suda, I., Mituoki, E., Saito, M. & Miura, Y. (1986) Myeloid and erythroid lineage expression of haematopoietic progenitors derived from an abnormal clone in erythroleukaemia. *British Journal of Haematology*, **64**, 647–656.

Thorens, B., Mermod, J. & Vassalli, P. (1987) Phagocytosis and inflammatory stimuli induce GM-CSF mRNA in macrophages through posttranscriptional regulation. *Cell*, **48**, 671–679.

Touw, I. & Löwenberg, B. (1985) Variable differentiation of human acute myeloid leukemia during colony formation in vitro: a membrane marker analysis with monoclonal antibodies. *British Journal of Haematology*, **59**, 37–44.

Vellenga, E., Young, D.C., Wagner, K., Wiper, D., Ostapovicz, D. & Griffin, J.D. (1987a) The effects of GM-CSF and G-CSF in promoting growth of clonogenic cells in acute myeloblastic leukemia. *Blood*, **69**, 1771–1776.

Vellenga, E., Ostapovicz, D., O'Rourke, B. & Griffin, J.D. (1987b) Effects of recombinant IL-3, GM-CSF and G-CSF on proliferation of leukemic clonogenic cells in short-term and long-term cultures. *Leukemia*, **1**, 584–589.

Weisbart, R.H., Golde, D.W. & Gasson, J.C. (1986) Biosynthetic human GM-CSF modulates the number and affinity of neutrophil f-Met-Leu-Phe receptors. *Journal of Immunology*, **137**, 3584–3587.

Wong, G.G., Witek, J.S., Temple, P.A., Wilkens, K.M., Leary, A.C., Luxenberg, D.P., Jones, S.S., Brown, E.L., Kay, R.M., Orr, E.C., Shoemaker, C., Golde, D.W., Kaufman, R.J., Hewick, R.M., Wang, E.A. & Clark, S.C. (1985) Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. *Science*, **228**, 810–815.

Yam, L.T., Li, C.Y. & Crosby, W.H. (1971) Cytochemical identification of monocytes and granulocytes. *American Journal of Clinical Pathology*, **55**, 283–290.

Yang, Y.C., Ciarletta, A.B., Temple, P.A., Chung, M.P., Kovacic, S., Witek-Gianotti, J.S., Leary, A.C., Kriz, R., Donahue, R.E., Wong, G.G. & Clark, S.C. (1986) Human IL3 (Multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL3. *Cell*, **47**, 3–10.

Young, D.C. & Griffin, J.D. (1986) Autocrine secretion of GM-CSF in acute myeloblastic leukemia. *Blood*, **68**, 1178–1181.

Young, D.C., Wagner, R. & Griffin, J.D. (1987) Constitutive expression of the granulocyte-macrophage colony-stimulating factor gene in acute myeloblastic leukemia. *Journal of Clinical Investigation*, **79**, 100–106.

Zucali, J.R., Broxmeyer, H.E., Dinarello, C.H., Gross, M.A. & Weiner, R.S. (1987) Regulation of early human hematopoietic (BFU-E and CFU-GEMM) progenitor cells in vitro by interleukin 1-induced fibroblast-conditioned medium. *Blood*, **69**, 33–37.

CHAPTER 7

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Modulation of Colony Stimulating Factor-(CSF) Dependent Growth of Acute Myeloid Leukemia by Tumor Necrosis Factor

Modulation of Colony Stimulating Factor-(CSF) Dependent Growth of Acute Myeloid Leukemia by Tumor Necrosis Factor

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In this study we demonstrate that tumor necrosis factors (TNFα and TNFβ) are potent modulators of the in vitro proliferation of human AML cells. Blast cells from 11 cases of acute myeloblastic leukemia (AML) were incubated with recombinant TNFα or TNFβ in serum-free ^{3}H-TdR uptake and colony culture systems in the presence or absence of recombinant interleukin-3 (IL-3), granulocyte macrophage colony-stimulating factor (GM-CSF), G-CSF, or M-CSF. Depending on the supplemented CSF, TNF could upregulate or suppress AML blast proliferation. Enhancement of AML growth by TNF was observed in the presence of IL-3 (in 9 of 11 cases in ^{3}H-TdR assay; 6 of 9 cases in colony assay) and GM-CSF (in 8 of 11 cases in ^{3}H-TdR assay; 4 of 9 cases in colony assay). In certain cases in which IL-3 or GM-CSF alone was unable to induce proliferative responses of AML cells, the simultaneous addition of TNF elicited colony growth and DNA synthesis suggesting a synergistic action between TNF and IL-3 or GM-CSF. In contrast, TNF suppressed G-CSF-induced growth (9 of 10 cases in ^{3}H-TdR assay; 5 of 6 cases in colony assay). TNF could also stimulate DNA synthesis (in 2 of 11 cases) or colony formation (in 2 of 9 cases) in AML cultures without the addition of other growth factors. Experiments with neutralizing antibodies and specific radioimmunoassays for individual CSFs showed that the synergistic and antagonistic effects of TNF on AML growth could not be attributed to a release of one of these CSFs by the AML cells. The opposing consequences of exposure of AML blasts to TNF are of interest in view of our understanding of the pathophysiology of AML growth and the in vivo application of recombinant cytokines in AML patients.

INTRODUCTION

TUMOR necrosis factor (TNFα) and lymphotoxin (TNFβ) have been initially defined by their cytotoxic or cytostatic potential against an array of experimental tumors (1–4). Further studies, however, revealed that TNFs belong to a complex network of cytokines that regulate cell proliferation and function as well as the production of growth factors (5, 6). The growth of certain cells, e.g., normal human fibroblasts (7) and B lymphocytes (8), is efficiently promoted by TNFs. A role in modulating hematopoiesis has been attributed to the ability of TNF to induce the release of certain hematopoietic regulators from diverse cells, e.g. granulocyte macrophage colony-stimulating factor (GM-CSF) (9) and G-CSF (10) from human lung fibroblasts, M-CSF from human monocytes (11), and interleukin-1 (IL-1) from endothelial cells (12).

The colony stimulating factors (CSFs) IL-3, GM-CSF, G-CSF, and M-CSF have a major role in stimulating human acute myeloblastic leukemia (AML) cells in culture (13–18). Sometimes, the AML cells endogenously produce CSFs and stimulate their own growth (autocrine mechanism) (19–23). How TNF interacts with CSFs in regulating human AML growth is as yet unclear.

Our present studies had the objective to determine the contributing role of TNF in human AML blast proliferation under exactly defined serum-free in vitro conditions. The data reveal a typical profile of reactivity to TNFs that depends on the particular CSF stimulating AML cells in vitro.

MATERIALS AND METHODS

Patients and Preparation of AML Cells. Eleven adult untreated cases of AML were diagnosed according to the criteria of French-American-British Committee (FAB) (24, 25) as M1 (cases 1–3), M2 (cases 4–6), and M4 (cases 7–11). Bone marrow or peripheral blood (in cases 3, 5, 7, and 8) were obtained after permission. The cells were recovered from the interface following Ficoll-Isopaque centrifugation, depleted of T cells by AET-E rosette sedimentation, and cryopreserved (26, 27). The percentages of T cells in all AML specimens were less than 1%.

Morphological examination of cytospin slides of the cells after thawing revealed that ≥95% of the cells were blasts in cases 1–8, while in cases 9–11 (M4 cytology) the percentages of monocytes admixed with the blasts were 13%, 33%, and 18%, respectively. After incubation of 10^7 AML cells from the latter three cases in 2 ml serum-free medium in 35-mm plastic dishes (Greiner, Nürtingen, F.R.G.) for 75 min, about 50% of the cells adhered, resulting in a reduction of the monocytes to less than 5% of nonadherent cells.

Preparation of Normal Bone Marrow Cells. Normal marrow mononuclear cells were isolated on Ficoll-Isopaque (1.077 g/ml; Nycomed, Oslo, Norway). T cells, monocytes, and mature granulocytes were depleted after complement mediated cytolysis (28). The cells after Ficoll (20×10^6/ml) were incubated for 30 min on ice with a cocktail of three monoclonal antibodies at optimal concentrations: CD3 (OKT3; IgG2a), CD14 (B44-1; IgM) (29), and CD15 (B4.3; IgM) (30). Rabbit complement (40% v/v) was then added for another 30 min at 25°C, and the cells were finally washed twice in cold Hanks' balanced salt solution.

Culture Medium. The serum-free medium that was recently proposed for the growth of human AML blasts in vitro (31) was used throughout the experiments.

Purified Recombinant Colony Stimulating Factors (CSFs), Tumor Necrosis Factors (TNFs), and Neutralizing Antibodies. Purified recombinant IL-3 (Gist Brocades, Delft, The Netherlands) was used at 3 ng/ml, M-CSF (Cetus Corporation, Emmeryville, CA) at a dilution of 1,000 U/ml, G-CSF (Immunex, Seattle, WA) at a 10 ng/ml dilution, and GM-CSF at 200 U/ml (25 ng/ml) as these concentrations were found to maximally support AML cell proliferation under serum-free conditions (17, 18, 32–35). Recombinant human TNFα (6×10^7 U/mg), and TNFβ (1.2×10^8 U/mg) were provided by Dr. G. R. Adolf (Boehringer Institute, Vienna, Austria) (36, 37). TNFs were tested at various concentrations (up to 10^4 U/ml) in the presence or absence of CSFs and finally used at a concentration of 1,000 U/ml.

Rabbit anti-human IL-3 and sheep anti-human GM-CSF (Genetics Institute, Cambridge, MA) were used at optimal neutralizing concentrations (i.e., 0.3% v/v and 0.2% v/v, respectively) in DNA synthesis

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assay of AML blasts. Murine monoclonal antibody (IgG1) to recombinant human TNFα (neutralizing capacity: 1.0×10^7 U TNFα/ml) and rabbit antiserum to recombinant human TNFβ (neutralizing capacity: 2.9×10^7 U TNFβ/ml) were gifts from Boehringer Institute.

^{3}H-thymidine (^{3}H-TdR) Incorporation Assay. DNA synthesis of AML cells was measured in the presence or absence of CSFs or TNFs for 90 hr in 96-well microtiter plates as described (31). The results of triplicate incubations of 10^4 AML cells are expressed as means ± standard deviations (SD). Preirradiated (30 Gy) AML cells were included in each experiment to assess the background thymidine incorporation. Statistical significance ($p < 0.05$) between values of TNF supplemented and TNF-free cultures was estimated by Student *t*-test. ^{3}H-TdR uptake of normal human bone marrow progenitors was assessed similarly.

AML Clonogenic Assay. Based on the data of ^{3}H-TdR incorporation, the effect of adding 10^3 U/ml TNFα on the clonogenic cells of nine AML cases (nos. 1–6 and 9–11) was tested in the presence or absence of individual CSFs. In these experiments 10^5 AML cells were plated in 1 ml serum-free medium that contained 0.9% methylcellulose, and cultured in humidified 5% CO_2 atmosphere at 37°C. All aggregates of 10 cells or more were scored between days 7–10, and the numbers of colonies in Tables 1–3 refer to means of duplicate cultures.

Preparation of AML Conditioned Media. Serum-free media were conditioned by 0.5×10^6/ml AML cells from cases 2, 4, 6, 9, and 10. TNFα (10^3 U/ml) or IL-3 (1:1,000 v/v) or both were added to AML cell cultures as well as to cell-free media running in parallel. Control cultures with no added factors were also included. Supernatants were collected after 4–5 day incubation. Another panel of conditioned media was prepared by pulse stimulating AML cells overnight with 10^4 U/ml TNFα alone (cases 4 and 6) or in combination with IL-3 (case 2), washing the cells then further incubating them for 3 days in fresh medium.

Table 1. The Effect of TNFs on AML Cell Proliferation in the Absence of Added CSFs

Case No.	^{3}H-TdR Assay[a]			Colony Assay[b]	
	No TNF	TNFα (10^3 U/ml)	Irradiated Control	No TNF	TNFα (10^3 U/ml)
1	149 ± 15	178 ± 87	70 ± 13	0	0
2	351 ± 47	101 ± 36	46 ± 29	2	0
3	1,059 ± 54	637 ± 95[d]	518 ± 42	0	0
4	168 ± 42	345 ± 86	40 ± 8	0	SC
5	4,432 ± 134[d]	4,476 ± 484[c]	—	55	159[f]
6	755 ± 143	6,004 ± 492[f]	92 ± 20	0	27[f]
7	199 ± 32	252 ± 45	47 ± 6	—[e]	—
8	1,740 ± 52	2,418 ± 77[f]	1,162 ± 90	—	—
9	992 ± 199	633 ± 433	140 ± 35	220	16[d]
10	6,691 ± 287	1,376 ± 19[d]	105 ± 34	88	3[d]
11	1,712 ± 18	172 ± 3[d]	62 ± 23	273	142[d]

Proliferation of AML cells was assessed in the absence of exogenous CSFs in ^{3}H-TdR and colony assays. In control cultures with no TNF, the blasts of seven cases (i.e., nos. 3, 5, 6, 8–11) exhibited spontaneous DNA synthesis (as compared to ^{3}H-TdR uptake of preirradiated cells which was always less than 290 dpm). Four cases (i.e., nos. 5, 9–11) showed spontaneous colony formation.

[a]Values refer to mean dpm per 10^4 AML cells ± SD.

[b]Values refer to mean number of colonies per 10^5 AML cells.

[c]100 U/ml TNFα was used in cultures with cells from case 5.

[d]Indicates significant inhibition of DNA synthesis or colony formation following the addition of TNF. (SC) refers to small clusters (less than 10 cells) that were not found in control cultures without TNFα.

[e]— = not done.

[f]Indicates significant enhancement of DNA synthesis or colony numbers (as compared to cultures without TNF).

Parallel experiments were carried out with TNFβ with similar results (not shown).

Table 2. The Effect of TNF on AML Cell Proliferation Induced by IL-3 or GM-CSF

Case No.	^{3}H-TdR Assay		Colony Assay	
	No TNF	TNFα (10^3 U/ml)	No TNF	TNFα (10^3 U/ml)
IL-3				
1	116 ± 35	106 ± 34	3	2
2	535 ± 71	1,127 ± 14[a]	3	79[a]
3	1,611 ± 72	5,411 ± 557[a]	2	181[a]
4	2,232 ± 87	4,955 ± 148[a]	758	885
5	9,238 ± 1479	22,395 ± 1084[a]	245	1,014[a]
6	6,931 ± 609	7,078 ± 2194	260	281
7	214 ± 29	740 ± 73[a]	—	—
8	3,700 ± 129	5,968 ± 447[a]	—	—
9	1,778 ± 218	4,987 ± 369[a]	248	358[a]
10	6,144 ± 294	11,465 ± 332[a]	190	584[a]
11	2,043 ± 292	4,803 ± 85[a]	339	537[a]
GM-CSF				
1	110 ± 10	249 ± 101	4	37[a]
2	647 ± 85	1,820 ± 46[a]	6	10
3	2,518 ± 60	4,136 ± 405[a]	11	31[a]
4	13,187 ± 619	23,136 ± 1226[a]	882	953
5	8,196 ± 33	21,678 ± 1342[a]	473	838[a]
6	23,173 ± 850	10,980 ± 340[b]	570	277[b]
7	637 ± 56	3,141 ± 62[a]	—	—
8	5,478 ± 251	8,238 ± 535[a]	—	—
9	2,791 ± 524	6,525 ± 202[a]	275	179[b]
10	7,419 ± 348	6,654 ± 439	268	1,196[a]
11	2,455 ± 78	4,064 ± 215[a]	496	504

The effects of TNFα were determined in AML cultures stimulated by IL-3 or GM-CSF.

[a]Indicates positive effects of TNF.

[b]Indicates negative effects of TNF.

For further explanation see Table 1.

Table 3. The Effect of TNFs on AML Cell Proliferation in the Presence of G-CSF or M-CSF

Case No.	^{3}H-TdR Assay		Colony Assay	
	No TNF	TNFα (10^3 U/ml)	No TNF	TNFα (10^3 U/ml)
G-CSF				
1	93 ± 35	124 ± 55	6	1
2	551 ± 57	154 ± 96[a]	2	0
3	4,175 ± 187	1,433 ± 103[a]	5	0
4	1,120 ± 43	750 ± 121[a]	42	11[a]
5	14,819 ± 942	8,380 ± 670[a]	698	260[a]
6	16,186 ± 307	13,957 ± 1465	36	30
7	501 ± 107	154 ± 15[a]	—	—
8	3,443 ± 96	2,606 ± 281[a]	—	—
9	17,893 ± 572	2,637 ± 350[a]	263	110[a]
10	24,460 ± 1639	7,414 ± 437[a]	1,102	29[a]
11	4,064 ± 215	1,630 ± 161[a]	339	214[a]
M-CSF				
1	300 ± 18	293 ± 98	4	2
2	359 ± 29	143 ± 104	0	SC
3	954 ± 48	581 ± 124	1	0
4	172 ± 15	396 ± 21	0	SC
5	4,513 ± 440	6,068 ± 574	192	754
6	2,138 ± 230	6,195 ± 408	0	31
7	90 ± 107	172 ± 52	—	—
8	2,119 ± 201	2,538 ± 195	—	—
9	2,423 ± 264	416 ± 44[a]	255	230
10	—	—	256	118
11	—	—	273	161

The effects of TNFα on AML cultures were determined in the presence of G-CSF or M-CSF; ([a]) indicates negative effects. For further explanations see Table 1.

Testing AML Conditioned Media for Colony Stimulating Activity. As potential sources of CSF activities, conditioned media of TNF stimulated AML cells were tested (in concentrations ranging from 3–30% v/v) to stimulate:

A. DNA synthesis of CD3, CD14, and CD15 negative normal human bone marrow progenitors.

B. DNA synthesis of AML cells from cases 4 and 6. Cells of both cases were selected as they were CSF dependent, i.e., they showed no or limited baseline ^{3}H-TdR uptake in the absence of growth stimuli (Table 1) but were stimulable by IL-3, GM-CSF (Table 2), as well as G-CSF (Table 3).

Specific Immunoassay for GM-CSF, G-CSF, and M-CSF. Medium conditioned by AML cells stimulated by TNFα alone (in case 6) or by TNFα plus IL-3 (in case 10) were assayed for GM-CSF, G-CSF, and M-CSF (37–39). The monospecific sandwich radioimmunoassay for GM-CSF (developed by K. Kaushansky) employs on two non-crossreacting murine monoclonal antibodies raised by immunization with recombinant GM-CSF. The assay has a sensitivity of 10 U/ml and is specific for human GM-CSF. It does not recognize G-CSF, M-CSF, or IL-3. G-CSF was quantitated in a similar immunoassay employing human recombinant G-CSF as a standard (D. Chang, M. Hockman, and B. W. Altrock; manuscript in preparation). The limit of sensitivity of the assay is 2 ng/ml. The assay is negative for GM-CSF, M-CSF, or IL-3. Radioimmunoassay for M-CSF (Cetus Corp.) has a sensitivity of 6.1 ng/ml and is not influenced by G-CSF, GM-CSF, IL-3, nor denatured M-CSF.

RESULTS

Effects of TNF on AML Cell Proliferation in the Absence of Exogenous CSFs. In 7 of 11 cases, AML cells cultured without exogenous CSFs exhibited baseline DNA synthesis that significantly exceeded the ^{3}H-TdR uptake of irradiated control cells. This "spontaneous" DNA synthesis was inhibited by TNF in three of these cases (nos. 3, 10, and 11). Spontaneous colony formation observed in four cases was inhibited by TNF in three of them (nos. 9–11) but enhanced in case 5.

On the other hand, of five cases without spontaneous DNA synthesis, TNF stimulated ^{3}H-TdR incorporation in two (nos. 6 and 8). Cells of case 6 formed some small-sized colonies (10–20 cells), and those of case 4 tiny clusters (less than 10 cells) in response to TNFα as a single stimulus.

TNF Promotes the Proliferative Response of AML Blasts to IL-3 or GM-CSF. In many instances, TNF enhanced blast cell proliferation initiated by IL-3 (in 9 of 11 cases in ^{3}H-TdR assay, and in 6 of 9 cases in colony assay) or by GM-CSF (in 8 of 11 cases in ^{3}H-TdR assay, and in 4 of 9 cases in colony assay) (Table 2). The relationship between TNF stimulation of AML thymidine uptake and TNF concentration is shown in Figure 1 (panel A), indicating that the maximal effect is expressed at 3×10^2 U/ml. The magnitude of the stimulative effects of the combinations TNF plus IL-3 or TNF plus GM-CSF was often greater than the sum of the effects of the individual factors suggesting synergism (Table 1 vs. Table 2). Moreover, in the situations where TNF, as a single agent, had suppressed spontaneous DNA synthesis (cases 3, 10, and 11) and spontaneous colony formation (cases 9–11), IL-3 or GM-CSF, when combined with TNF, entirely reversed that negative influence of TNF and permitted remarkable enhancement of AML growth (e.g., compare Fig. 2A with B or C). Large colonies (40 cells or more) predominated in cultures when TNFα was added along with IL-3 or GM-CSF.

In all the experiments reported herein, we included a set of incubations to which TNFβ, instead of TNFα, had been added. We do not give the complete data on TNFβ, since we observed that the effects of TNFα and TNFβ on AML cultures with or without CSFs were very much alike (e.g. see Figs. 2 and 3).

TNF Suppresses the Proliferative Response of AML Blasts to G-CSF. TNF inhibited the proliferation of cells of 9 of 10 cases responding to G-CSF in ^{3}H-TdR assay and in 5 of 6 cases in colony culture (Table 3 and Fig. 2D). Suppression of G-CSF-induced growth of AML was TNF dose dependent (Fig. 1, panel B). Only small colonies (10–20 cells) appeared when TNFα was added to G-CSF-containing cultures.

M-CSF only occasionally activated DNA synthesis or colony formation of AML blasts. The effects of TNF on M-CSF containing cultures were therefore not really different from those

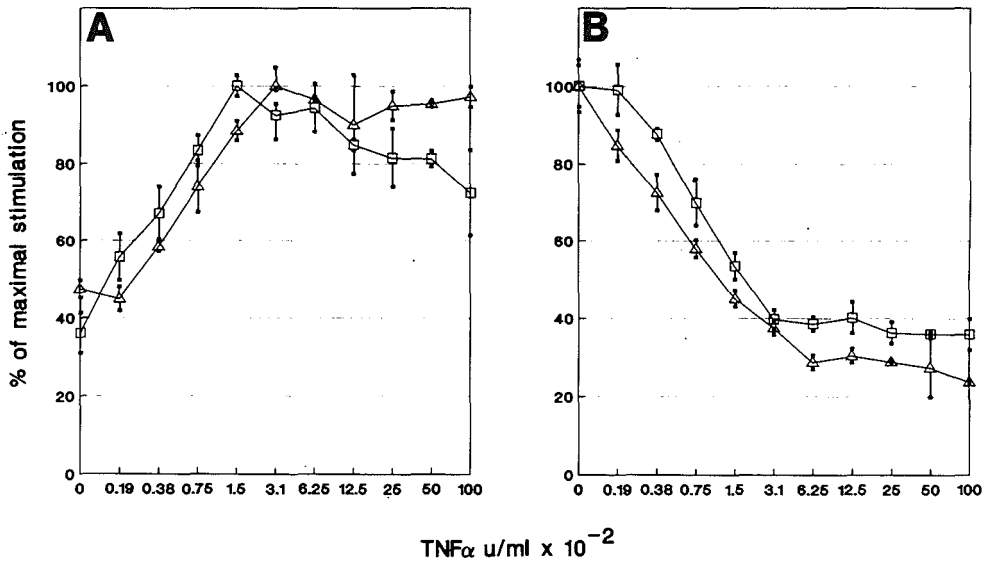


Figure 1. Modulation of IL-3 and G-CSF induced DNA synthesis of AML cells by TNFα: dose effect relationships. Effects of titrated TNF concentrations on tritiated thymidine uptake of AML cells (from cases 10 and 11) induced by IL-3 (section A, enhancement by TNF) or G-CSF (section B, inhibition by TNF). Results are expressed as percentages of maximal stimulation (100%). Maximal thymidine incorporation in the IL-3 and TNF cultures was 12, 979 ± 597 dpm (case 10) and 5,670 ± 153 dpm (case 11). Maximal values of thymidine uptake in the G-CSF cultures were 24,460 ± 1639 (case 10) and 4,069 ± 215 (case 11). (▲—▲ cells from case 10; □—□ cells from case 11.)

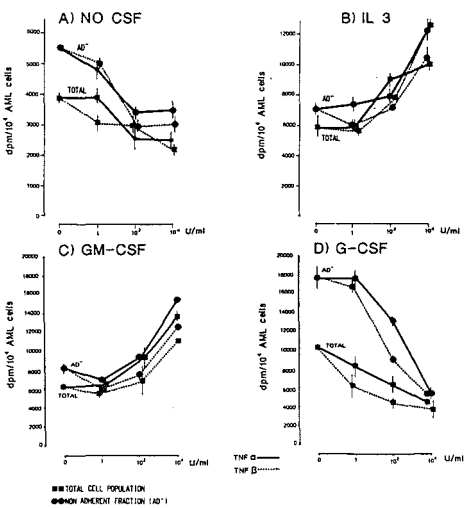
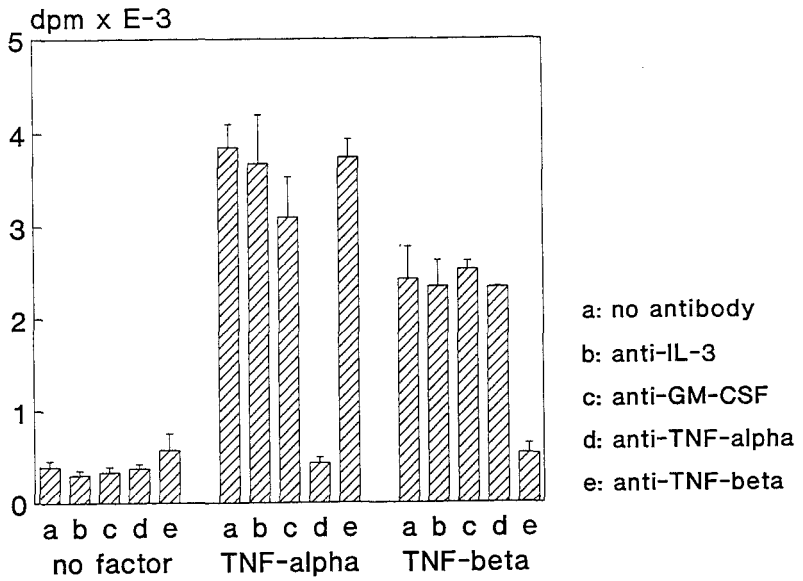


Figure 2. Interaction between TNF and CSFs on DNA synthesis of AML cells (before and after plastic adherence). The effect of TNFα (continuous lines) and TNFβ (interrupted lines) on DNA synthesis of AML cells from case 10 (M4 cytology) was compared before (■) and after plastic adherence (●). A, The dose response data show inhibition of spontaneous DNA synthesis by TNF, B, synergism between TNF and IL-3, C, synergism between TNF and GM-CSF, and D, antagonism between TNF and G-CSF.

observed in the absence of CSFs (Table 1). Rarely (in 1 of 9 cases in colony assay), TNF potentiated M-CSF induced proliferation of AML. In one case (no. 9), TNF suppressed DNA synthesis of M-CSF stimulated AML cells.

Role of Auxillary Cells. It is unlikely that TNF effects on AML growth were mediated through T cells or monocytes. All AML specimens in this study had been depleted of T cells. When the in vitro responses to TNFα and TNFβ were compared in ^{3}H-TdR assay before and after plastic adherence (patients 9–11—M4 cytology), the results were identical (e.g., see Fig. 2).

Stimulatory Effects of TNF on AML Cell Cultures are not the Result of Induction of CSF Production. Since TNF can trigger in a variety of cell types the production of CSFs (e.g., GM-CSF, G-CSF, and M-CSF) (9–11), we examined whether AML cell cycle activation by TNF was caused by CSF release. This was checked in a case where TNF as a single agent stimulated the cells (i.e., no. 6) as well as in some instances where TNF synergized with IL-3 or GM-CSF. Stimulation of DNA synthesis by TNFα in case 6 could not be blocked with anti-IL-3 or anti-GM-CSF antibody (Fig. 3). Supernatants of the cells incubated with TNFα could not support the DNA synthesis of cells from the same patients, nor that of enriched human normal bone marrow progenitors (data not shown). Elaboration of CSFs into the medium following incubation of cells of case 6 with TNF could not be demonstrated with monospecific immunoassays for GM-CSF, G-CSF, and M-CSF. In the latter assay, significant quantities of GM-CSF (160 pM), G-CSF (3.3 ng/ml), and M-CSF (10.3 ng/mol) were detected in a medium



* Figure 3. Effect of anti-IL-3, anti-GM-CSF, anti-TNFα, and anti-TNFβ on TNF-induced DNA synthesis of AML cells. The increase of ^{3}H-TdR uptake of cells from patient 6 following exposure to TNFα or TNFβ could not be reversed by anti-IL-3 or anti-GM-CSF.

conditioned by phytohemagglutinin-stimulated peripheral blood leukocytes.

In addition, anti-IL-3 could not block the enhancing potential of TNF upon cultures containing GM-CSF, nor could anti-GM-CSF inhibit the synergistic effect of TNF plus IL-3 (experiments performed on cells of cases 2, 9–11; data not shown). To ensure that the AML cells had not produced another critical factor during the 4–5 day incubation, supernatants from AML cell cultures with no stimuli, supernatants from AML cell cultures costimulated with IL-3 and TNF, and medium from cell-free incubations with TNF and IL-3 were directly compared as regards their stimulating activity on AML cells from case 6 and purified normal marrow, but no differences were noted. The enhanced growth in response to TNF plus IL-3—in case 10—could not be attributed to the generation of GM-CSF, G-CSF, or M-CSF as evaluated by specific immunoassays.

DISCUSSION

The data of this report disclose contrasting responses of AML precursors to TNFs. DNA synthesis and colony growth of AML cells following stimulation by IL-3 or GM-CSF was frequently enhanced by TNF, even in certain cases in which AML cells hardly formed any colonies in response to IL-3 or GM-CSF alone. In marked contrast, TNFs exerted a dose-dependent suppression of G-CSF stimulated AML cell proliferation. Thus, proliferation of AML cells from the same patient could be positively or negatively modified following the exposure to TNF depending on the type of CSF supplemented to culture (IL-3 or GM-CSF vs. G-CSF).

How TNFs accomplish their stimulatory or inhibitory influences upon AML cell proliferation remains elusive. The ability of TNF to stimulate the production of CSFs in cells of several tissues (9–11) is now well established. Thus, one could hypothesize that the growth promoting capacities of TNF result from CSF release from AML cells following induction by TNF. However, testing of AML conditioned media for CSF activities with CSF neutralizing antibodies and specific radioimmunoassays did not provide positive indications for the production by AML cells of any of the known CSFs as a result of exposure to TNF as a single molecule nor in conjunction with IL-3 or GM-CSF.

An explanation for the suppressive effects of TNFs in cultures stimulated with G-CSF or cultures with spontaneous growth of AML cells would be their differentiation inducing capacity (5, 6). We consider this possibility unlikely since cytological examination of cells from colony and suspension cultures revealed only a moderate increase in monocytes/macrophages in cultures with TNFα or TNFβ and an infrequent effect on the appearance of mature granulocytic elements (data not shown).

Finally, a third possible mechanism underlying both synergistic and antagonistic effects of TNFs is that TNFs modulate the expression of surface receptors of CSFs on AML cells in a differential fashion, thus upregulating the receptors for GM-CSF and IL-3 and downregulating those for G-CSF, for instance. Investigations to explore this hypothesis are in progress.

In a separate study we have investigated whether the features of responsiveness to TNF described here are unique to AML blasts or reflect common properties of normal myeloid pre-

* Figure modified from original

cursors. Very similar to the TNF responses in AML, we observed that GM-CSF and IL-3 stimulated myeloid colony formation from highly purified ($CD34^+$, $CD14^-$) normal marrow samples was significantly enhanced by TNFs, whereas G-CSF induced colony growth was strongly suppressed by TNF (manuscript in preparation). This is in agreement with earlier studies showing that normal marrow progenitors responding in vitro to GM-CSF stimulation are more resistant to the cytotoxic effects of TNFs than those responding to G-CSF (40, 41). TNFα has also been shown to predominantly suppress colony growth of neutrophils with no significant effect on the formation of eosinophilic colonies in vitro (42). This is of interest because it is now obvious that eosinophilic colonies are mainly triggered by IL-3 or GM-CSF, while G-CSF acts as a selective stimulus for neutrophilic colonies from highly enriched progenitors (28, 43).

Recombinant CSFs and TNFs are being introduced in clinical medicine (44, 45). Therefore, it is crucial to understand their spectrum of effects and interactions on normal and neoplastic cells. The paradox of stimulation and inhibition of human AML growth by TNF may have an important bearing on the in vivo utility of this agent in patients with AML. Insight into the interplay of these hematopoietic activities may assist in selecting appropriate combinations of recombinant cytokines for in vivo administration.

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REFERENCES

1. Old LJ. Tumor necrosis factor. Clin Bull **1976**;6:118–120.
2. Old LJ. Tumor necrosis factor. Science **1985**;230:630–632.
3. Ruff MR, Gifford GE. Tumor necrosis factor. In: Pick E, ed. Lymphokines, vol 2. New York: Academic Press, **1981**;235–275.
4. Nauts HC. The beneficial effects of bacterial infections on host resistance to cancer. End results in 449 cases. Cancer Res Inst Monogr **1980**;8:3.
5. Munker R, Koeffler HPh. Tumor necrosis factor: recent advances. Klin Wochenschr **1987**;65:345–352.
6. Old LJ. Tumour necrosis factor: polypeptide mediator network. Nature **1987**;326:330–331.
7. Vilcek J, Palombella NJ, Henriksen-de Stefano D, Swenson C, Feinman R, Hirai M, Tsujimoto M. Fibroblast growth enhancing activity of tumor necrosis factor and its relationship to other polypeptide growth factors. J Exp Med **1986**;163:632–643.
8. Kehrl JH, Alvarez-Mon M, Delsing GA, Fauci AS. Lymphotoxin is an important T cell-derived growth factor for human B cells. Science **1987**;238:1144–1146.
9. Munker R, Gassen J, Ogawa M, Koeffler HP. Recombinant human TNF induces production of granulocyte-monocyte colony-stimulating factor. Nature **1986**;323:79–82.
10. Koeffler HP, Gasson J, Ranyard J, Sonza L, Shepard M, Munker R. Recombinant human TNFα stimulates production of granulocyte colony-stimulating factor. Blood **1987**;70:55–59.
11. Oster W, Lindermann A, Hor S, Mertelsmann R, Herrmann F. Tumor necrosis factor (TNF)-alpha but not TNF-beta induces secretion of colony stimulating factor for macrophages (CSF-1) by human monocytes. Blood **1987**;70:1700–1703.
12. Nawroth PP, Stern DM. Modulation of endothelial cell homostatic properties by tumor necrosis factor. J Exp Med **1986**;163:1363–1375.
13. Kelleher C, Miyauchi J, Wong G, Clark S, Minden MD, McCulloch EA. Synergism between recombinant growth factors, GM-CSF and G-CSF acting on blast cells of acute myeloblastic leukemia. Blood **1987**;69:1498–1503.
14. Vellenga E, Young DC, Wagner K, Wiper D, Ostapovicz D, Griffin JD. The effects of GM-CSF and G-CSF in promoting growth of clonogenic cells in acute myeloblastic leukemia. Blood **1987**;69:1771–1776.
15. Vellenga E, Ostapovicz D, O'Rourke B, Griffin JD. Effects of recombinant IL-3, GM-CSF and G-CSF on proliferation of leukemic cells in short-term and long-term cultures. Leukemia **1987**;1:584–589.
16. Miyauchi J, Kelleher CA, Yang Y, Wong GG, Clark SC, Minden MD, Minkin S, McCulloch EA. The effects of three recombinant growth factors, IL-3, GM-CSF and G-CSF on the blast cells of acute myeloblastic leukemia maintained in short-term suspension culture. Blood **1987**;70:657–663.
17. Salem M, Delwel R, Mahmoud LA, Clark S, Elbasousy EM, Löwenberg B. Maturation of human acute myeloid leukemia in vitro: the response to five recombinant hematopoietic factors in serum free system. Br J Haematol **1989**;71:363–370.
18. Delwel R, Salem M, Pellens C, Dorssers L, Wagemaker G, Clark S, Löwenberg B. Growth regulation of human acute myeloid leukemia: effects of five recombinant hematopoietic factors in a serum free culture system. Blood **1988**;72:1944–1949.
19. Young DC, Griffin JD. Autocrine secretion of GM-CSF in acute myeloblastic leukemia. Blood **1986**;68:1178–1181.
20. Young DC, Wagner K, Griffin JD. Constitutive expression of the GM-CSF gene in acute myeloblastic leukemia. J Clin Invest **1987**;79:100–106.
21. Cheng GYM, Kelleher CA, Miyauchi J, Wang C, Wong G, Clark SC, McCulloch EA, Minden MD. Structure and expression of genes of GM-CSF and G-CSF in blast cells from patients with acute myeloblastic leukemia. Blood **1988**;71:204–208.
22. Mannoni P, Mueller T, Torres H, Dubreuil P, Waal R de, Birg F. Expression of IL3, GM-CSF and G-CSF genes in myeloid leukemia cells. Blood **1987**;70:263a.
23. Oster W, Lindemann A, Horn S, Mertellsmann R, Herrmann F. Constitutive expression of genes for hematopoietic growth factors in acute myeloblastic leukemia. Blood **1987**;70:266a.
24. French-American-British (FAB) Cooperative Group. Proposals for the classification of the acute leukemia. Br J Haematol **1976**;32:451–458.
25. French-American-British (FAB) Cooperative Group. Proposed revised criteria for the classification of acute myeloid leukemia. Ann Intern Med **1985**;103:620–625.
26. Swart K, Hagemeyer A, Löwenberg B. Acute myeloid leukemia colony growth in vitro: differences of colony forming cells in PHA supplemented and standard leukocyte feeder cultures. Blood **1982**;59:816–821.
27. Löwenberg B, Hagemeyer A, Swart K. Karyotypically distinct subpopulations in acute leukemia with specific growth requirements. Blood **1982**;59:641–645.
28. Bot FJ, Dorssers L, Wagemaker G, Löwenberg B. Stimulating activity of human recombinant multi-CSF on human marrow precursors: importance of accessory cells. Blood **1988**;71:1609–1614.
29. Perrussia B, Trinchieri G, Lebman D, Jankiewicz J, Lange B, Rovera G. Monoclonal antibodies that detect differentiation sur-

face antigens on human myelomonocytic cells. Blood **1982**;59:382–392.
30. Van der Reijden HJ, Van Rhenen DJ, Lansdorp PM, Van 't Veer MD, Langenhuijsen MMAC, Engelgriet CP, Von dem Borne AEGKr. A comparison of surface marker analysis and FAB classification in acute myeloid leukemia. Blood **1983**;61:443–448.
31. Salem M, Delwel R, Touw I, Mahmoud L, Löwenberg B. Human AML colony growth in serum-free culture. Leuk Res **1988**;12:157–165.
32. Yang YC, Ciarletta AB, Temple PA, Chung MP, Kovacic S, Witek-Gianotti JS, Leary AC, Kriz R, Donahue RE, Wong GG, Clark SC. Human IL3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL3. Cell **1986**;47:3–10.
33. Wong GG, Witek JS, Temple PA, Wilkens KM, Leary AC, Luxenberg DP, Jones SS, Brown EL, Kay RM, Orr EC, Shoemaker C, Golde DW, Kaufman RJ, Hewick RM, Wang EA, Clark SC. Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science **1985**;228:810–815.
34. Nagata S, Tsuchiya M, Asano S, Kaziro Y, Yamazaki T, Yamamoto O, Hirata Y, Kubota N, Oheda M, Nomura H, Ono M. Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor. Nature **1986**;319:415–418.
35. Kawasaki ES, Ladner MB, Wang AM, Arsdell J van, Warren MR, Coyne MY, Schweickart VL, Lee M, Wilson KJ, Boosman A, Stanley ER, Ralph P, Mark DF. Molecular cloning of a complementary DNA encoding human macrophage specific colony-stimulating factor (CSF-1). Science **1985**;230:291–296.
36. Pennica D, Nedwin GE, Hayflick JS, Seeburg PH, Derynck R, Palladino MA, Kohr WJ, Aggarwal BB, Goeddel DV. Human tumour necrosis factor: precursor structure, expression, and homology to lymphotoxin. Nature **1984**;312:724–729.
37. Gray PB, Aggarwal BB, Benton CV, Bringman TS, Hensel WJ, Jarrett JA, Leung DW, Moffar B, Ng P, Svedersky LP, Palladino MA, Nedwin GE. Cloning and expression of cDNA for lymphotoxin, a lymphokine with tumour necrosis activity. Nature **1984**;312:721–724.
38. Fibbe WE, Damme J van, Billiau A, Goselink HM, Voogt PJ, Eeden G van, Ralph P, Altrock BW, Falkenburg JHF. Interleukin-1 induces human marrow stromal cells in long-term culture to produce G-CSF and M-CSF. Blood **1988**;71:430–435.
39. Fibbe WE, Damme J van, Billiau A, Duinkerken N, Larvink E, Ralph P, Altrock BW, Raushansky R, Willemze R, Falkenburg JHF. Human fibroblasts produce G-CSF, M-CSF and GM-CSF following stimulation by interleukin-1 and poly (rI)-poly (rC). Blood **1988**;72:860–866.
40. Barber KE, Crosier PS, Watson JD. The differential inhibition of hemopoietic growth factor activity by cytotoxins and interferon-γ. J Immunol **1987**;139:1108–1112.
41. Barber KE, Crosier PS, Gillis S, Watson JD. Human granulocyte-macrophage progenitors and their sensitivity to cytotoxins: analysis by limiting dilution. Blood **1987**;70:1773–1776.
42. Murase T, Hotta T, Ohno R, Saito H. Predominant suppression of neutrophil colony growth by recombinant human tumor necrosis factor. Proc Soc Exp Biol Med **1987**;186:188–191.
43. Sonoda Y, Yang YC, Wong GG, Clark S, Ogawa M. Analysis is serum-free of the targets of recombinant human hemopoietic factors: demonstration that interleukin-3 and granulocyte/macrophage colony stimulating factors are specific for early developmental stages. Blood **1987**;70:185a.
44. Kolata G. Clinical promise with new hormones. Science **1987**;236:517–519.
45. Greaven PJ, Plager JE, Dupere S, Huben RP, Takita H, Mittelman A, Proefrock A. Phase I clinical trial of recombinant human tumor necrosis factor. Cancer Chemother Pharmacol **1987**;20:137–144.

CHAPTER 8

Blood, Vol 74: pp 586-593, 1989.

Interleukin-1 Stimulates Proliferation of Acute Myeloblastic Leukemia Cells by Induction of Granulocyte-Macrophage Colony-Stimulating Factor Release

Interleukin-1 Stimulates Proliferation of Acute Myeloblastic Leukemia Cells by Induction of Granulocyte-Macrophage Colony-Stimulating Factor Release

By Ruud Delwel, Carin van Buitenen, Mohammed Salem, Freek Bot, Steven Gillis, Kenneth Kaushansky, Bruce Altrock, and Bob Löwenberg

In this study, we further established the role of interleukin-1 (IL-1)α and IL-1β as regulators of proliferation of acute myeloid leukemia (AML) cells. IL-1 stimulated tritiated thymidine (^{3}H-TdR) uptake of AML cells in 13 of 28 cases. Cytogenetic analysis confirmed the leukemic clonality of the IL-1–stimulated cells. Most likely, IL-1 exerted these stimulative effects directly on AML blast cells because IL-1 effectively induced ^{3}H-TdR uptake of CD34-positive AML blasts (separated following cell sorting). Furthermore, adherent cell-depleted AML samples of three patients were more effectively stimulated than nondepleted AML fractions. Cluster and colony formation from adherent cell depleted AML samples could also be stimulated with IL-1, ie, in seven of ten cases analyzed. Subsequent experiments indicated that IL-1 stimulation depended on the release of GM-CSF because (1) induction of DNA synthesis of AML cells by IL-1 could be abrogated with antigranulocyte-macrophage colony-stimulating factor (GM-CSF) antibody, (2) conditioned media (CM) prepared from IL-1 stimulated AML blasts (adherent cell depleted) could stimulate the proliferation of purified normal bone marrow progenitors whereas supernatants from nonstimulated AML blasts did not, and (3) GM-CSF was demonstrated in IL-1/AML-CM with a specific radioimmunoassay, while GM-CSF was not detectable in nonstimulated supernatants. In one case of AML showing significant ^{3}H-TdR uptake in the absence of CSFs, this spontaneous DNA synthesis was found to depend on autocrine IL-1β release as it could be suppressed with anti-IL-1β antibody or anti-GM-CSF. The blockade by anti-IL-1β could be overcome by the addition of high concentrations of IL-1β as well as GM-CSF. Thus, in this particular case, endogenously produced IL-1β had stimulated the release of GM-CSF which resulted in GM-CSF-dependent proliferation. The results indicate that GM-CSF production by AML blasts is often regulated by IL-1 rather than being constitutive.

IN VITRO GROWTH of human acute myeloid leukemia (AML) is regulated by several colony stimulating factors (CSFs) and interleukins (ILs), in particular IL3, granulocyte-macrophage-CSF (GM-CSF), granulocyte-CSF (G-CSF) and macrophage-CSF (M-CSF).[1-4] The molecule IL-1 is a pleiotropic factor, which exhibits various effects on different target cells, eg, it induces proliferation of fibroblasts and epithelial cells and it stimulates cytokine production in various cell types.[5] IL-1 has a clear regulatory role in hematopoiesis. In human endothelial cells and fibroblasts, IL-1 stimulates the release of GM-CSF and G-CSF.[6-8] A possible role of IL-1 in the regulation of AML blast cell proliferation has recently been suggested as IL-1 was found to synergize with GM-CSF and G-CSF.[9] In the present study, we further investigated how IL-1 may stimulate the proliferation of human AML cells in vitro. We show that IL-1 stimulates proliferation of AML cells in 13 of 28 cases and that in four of five cases activation of growth is the result of autocrine GM-CSF production induced by IL-1.

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MATERIALS AND METHODS

Patients and preparations of AML cells. AML was diagnosed according to the French-American-British (FAB) Committee (Table 1).[10] The leukemic cells from 28 patients were separated following bovine serum albumin (BSA) density gradient[11,12] or Ficoll-isopaque (Nycomed AS, Oslo) centrifugation (cases 7, 13, 16, 19, 21, 23, 24, and 27) and subsequent removal of E rosette forming cells as described.[11,12] The cells were cryopreserved in 7.5% dimethyl sulfoxide (DMSO) and 20% fetal calf serum (FCS: Flow Laboratories; Isrike, UK) in liquid nitrogen using a controlled freezing method.[13] AML cells from bone marrow or blood as well as cells from healthy donors were obtained after informed consent.

Hematopoietic growth factors and neutralizing polyclonal antibodies. Human purified recombinant IL-1α and β were obtained from Immunex Corp (Seattle). Recombinant human GM-CSF and G-CSF were prepared at the Genetics Institute (Cambridge, MA). The structural and biological characteristics of these human compounds have been reported in detail.[14-20] IL-3 was obtained following expression of the human IL-3 cDNA[21] in bacteria (Dorssers et al, manuscript in preparation). Polyclonal sheep anti-GM-CSF and rabbit anti-IL-3 were also gifts from Genetics Institute. Anti-GM-CSF was used in a 0.25% concentration. DNA synthesis of AML cells stimulated with 200 U/mL GM-CSF showed a 50% inhibition in the presence of 0.06% anti-GM-CSF. This preparation did not inhibit IL-3-induced DNA synthesis. Anti-IL-3 was used at a 0.5% concentration. A 50% reduction of IL-3 (1:1,000)-induced DNA synthesis of AML cells was observed with 0.06% anti-IL-3. Anti-IL-3 did not inhibit GM-CSF induced AML cell proliferation. Anti-IL-1α and anti-IL-1β were obtained from Immunex Corp. The antisera were specific, ie, anti-IL-1α did not inhibit IL-1β-induced DNA synthesis, or vice versa. These antisera did not suppress GM-CSF or IL-3-induced DNA synthesis. Anti-IL-1α was used at a 0.5% concentration. Anti-IL-1α at a 0.015% dilution completely inhibited ^{3}H-TdR incorporation of AML cells induced with 25 U/mL IL-1α. Anti-ILβ was used at a 0.5% dilution. Fifty percent inhibition of DNA synthesis of AML cells stimulated with 25 U/mL IL-1β was seen when this antiserum was added at a 0.06% concentration.

Table 1. Effect of IL-1α and β on DNA Synthesis of AML Cells

FAB	Patient No.	No. of Factors	IL-1α (U/mL)			IL-1β (U/mL)			Irradiated Control
			10	100	1,000	10	100	1,000	
M1	1	1.2*	1.3	1.2	1.0				0.3
	2	2.3	*11.7*	*11.5*	*9.5*	*13.4*		*9.9*	0.4
	3	1.5	1.2	1.1	1.1		*11.8*		0.5
	4	8.4	8.9	*21.4*	*20.0*				2.3
	5	107.9	45.6†	30.8†	23.6†				0.4
	6	1.1	2.1	*9.0*	*13.0*	2.6		*13.7*	0.5
	7	0.5		*37.5*			*7.3*		0.5
	8	1.8		*20.0*					1.0
M2	9	0.5	0.6	0.5	0.4	0.3	0.5	0.5	0.2
	10	5.4	7.5		8.1				4.5
	11	2.1	3.2	*18.6*	*14.1*				0.9
	12	6.1	6.0	5.7	10.6				0.3
	13	22.1	27.1	33.9	*39.2*	31.7	*36.9*	*41.0*	0.7
	14	9.7	*73.2*	*154.9*	*192.0*				1.1
M3	15	47.5	55.5	51.6	42.2				1.6
	16	1.6	2.9	*11.5*	*13.1*				1.4
M4	17	5.2	8.9	*16.1*	*19.6*				1.4
	18	24.2	21.3	32.2	*36.6*				ND
	19	9.5		9.6	7.0				1.2
	20	4.6	5.3	3.9	3.4	4.1	3.9	2.6	0.5
	21	18.2	25.0	28.5	24.4	21.8	27.5	24.2	1.7
	22	30.3	31.8	39.3	*40.9*	33.0	37.9	33.3	1.7
	23	44.1	37.1†	24.8†	21.4†				0.7
	24	14.1	*21.3*	*30.5*	*30.6*	*22.0*	*28.7*	*26.7*	0.6
	25	22.0	22.7	23.7	19.2				0.6
M5	26	3.7	3.7	2.5	2.3	1.5	0.9	1.0	1.3
	27	8.2	9.8	14.2	13.1				0.
M6	28	1.4	1.4	0.6	1.6				0.8

Italics indicate that the results from IL-1–stimulated cultures were significantly different from those of unstimulated cultures (Student's t test; $P < .05$).

Abbreviations: FAB, French-American-British nomenclature; ND, not done.

*^{3}H-TdR uptake expressed as mean dpm $\times 10^{-2}$.

†In these cases, DNA synthesis was significantly inhibited by IL-1.

Tritiated thymidine uptake in vitro and colony cultures of AML cells. DNA synthesis was measured by tritiated thymidine uptake in serum-free culture.[4,22,23] Preirradiated (30 Gy) AML cells were run as controls to assess background ^{3}H-TdR incorporation. Data from cultures not supplemented with factors were compared with background control values to assess whether in certain cases AML cells showed spontaneous DNA synthesis. Experiments were performed in triplicate and data are expressed as means ± SD. The Student's t test ($P < .05$) was performed to verify whether differences were significant. The effects of IL-1 were also determined in colony culture: 1×10^5 cells were plated in 1 mL of serum-free medium containing 0.9% methyl cellulose and cultured in a humidified 5% CO_2 atmosphere at 37°C. Clusters of ten to 50 cells and colonies of 50 cells or more were scored at day 12 to 14 of culture. Colony numbers represent means of duplicate experiments.

Cell adherence and preparation of AML-conditioned medium. Adherent cells were separated from the AML specimens following incubation of 10^7 cells in 5 mL serum-free medium in 6 cm Petri dishes (Greiner, Nurtingen, FRG) for one hour. Nonadherent AML cells were cultured in serum-free medium (1×10^6 cells/mL) in 6 cm Petri dishes (5 mL) with or without 1,000 U IL-1α/mL. After four days of culture, the conditioned media (CM) were harvested.

Fluorescence-activated cell sorting. Analysis and cell sorting were performed after labeling with the monoclonal antibody CD34 (BI-3C5; Sera Lab, Crawly Down, UK, concentration 1:1,000)[24] and goat antimouse-FITC (GAM-FITC; Nordic, Tilburg, The Netherlands) fluorescence-activated cell sorter (FACS) 440 under sterile conditions as described.[25,26]

Isolation of normal bone marrow progenitors and culture in the CFU-GEMM assay. The presence of colony-stimulating activity in IL–1-stimulated AML-CM was tested on purified normal bone marrow progenitors. These precursors were purified following two subsequent separation steps, ie, complement lysis and cell sorting. T cells, granulocytic cells, and monocytes were removed from Ficoll-isopaque-sedimented marrow by cytolysis using a cocktail of monoclonal antibodies CD3 (IgG2, T-3), CD15 (IgM B4.3),[27] and CD14 (IgM, B44.1),[28] and rabbit complement. Cells were then labeled with CD34 and positive cells were sorted by FACS. The final progenitor cell fraction was cultured in the CFU-GEMM assay[25,29] with AML-CM (15%) to determine stimulating activity. GM–CSF- and G–CSF-stimulated cultures with or without the addition of IL-1 were run for comparison as well as cultures containing 15% phytohemagglutinin-stimulated leukocyte conditioned medium (PHA-LCM).

Radioimmunoassay for GM-CSF, G-CSF, and M-CSF. GM-CSF was assessed using a monospecific sandwich radioimmunoassay developed by K. Kaushansky (University of Washington, Seattle). The assay relies on two noncrossreacting murine monoclonal antibodies raised by immunization with recombinant GM-CSF. The

antibodies are specific for human GM-CSF and are purified by *Staphylococcus Aureus* affinity chromatography. The first antibody was added to microtiter wells and then nonspecific protein adherence was blocked by the addition of 2% albumin. Culture supernatants were added at a 10% final volume, incubated, and after washing ^{125}I-labeled second antibody was added. After washing the excess labeled antibody, individual wells were counted. The results were compared with standards containing recombinant GM-CSF.[30] The assay is specific for human GM-CSF; it does not recognize G-CSF, M-CSF, or IL-3 and has a sensitivity of 10 U/mL. G-CSF was assayed in a similar monospecific sandwich immunoassay using human recombinant G-CSF as a standard (D. Chang, M. Hockman, and B.W. Altrock; manuscript in preparation) that does not react with GM-CSF, M-CSF, and IL-3. The limit of sensitivity of the assay is 2 ng/mL. The sensitivity of the specific radioimmunoassay for M-CSF that was developed by J. Allen and M. Geier (Cetus Corp, Emmeryville, CA) is 6.1 ng/mL. The assay is specific for M-CSF and does not recognize G-CSF, GM-CSF, IL-3, or denaturated M-CSF.

Cytogenetic analysis. Cytogenetic analysis of the cells was done after five days of culture according to standard techniques.[31] The spread metaphases were stained with 1 mg Atebrin (BDH Chemicals; Poole, UK) in 100 mL ethanol.

RESULTS

Stimulation of AML proliferation by IL-1α or IL-1β. The stimulative effects of IL-1 on AML cell proliferation were determined according to ^{3}H-TdR incorporation and colony formation in vitro. In 17 of 28 cases, AML cells showed spontaneous DNA synthesis. DNA synthesis was enhanced or stimulated by IL-1α in 13 of 28 cases (Table 1). In three cases (ie, cases 7, 8, and 14), it resulted in an increase of ^{3}H-TdR incorporation of 2,000 dpm or more. In the other ten cases, IL-1 induced a moderate but significant stimulation of DNA synthesis of AML cells (ie, 1,000 to 1,500 dpm). A direct comparison between the stimulative activities of IL-1α and IL-1β revealed similar results for both molecules (n = 9 cases, Table 1). Polyclonal antisera towards IL-1α or β neutralized the stimulative effects of the corresponding type of IL-1, confirming that activation of DNA synthesis had been IL-1 dependent (data not shown). In two cases, cytogenetic analysis was performed to confirm the leukemic origin of the IL-1–stimulated AML cells. In case 7, five metaphases were analyzed following IL-1 incubation of the cells, which all exhibited the cytogenetic abnormality, ie, 48, X, Yp^{+}, 1p^{-}, 9q^{-}, 12q^{+}, 13q^{-}, +14p^{+}, 18q^{-}, 20q^{+}, +21. All 16 metaphases analyzed from case 18 revealed the AML karyotype as well (46, −3, 5q^{-}, 7q^{-}, 8?, 8p^{-}, +8p^{+}, −12, +13, 17p^{+}). CD34-positive cells were sorted (FACS) from the T-cell depleted AML samples of case 14 to exclude the possibility of residual assessory cells being stimulated by IL-1. CD34-positive cells (100% blast cells) were efficiently stimulated by IL-1 (Fig 1). We also examined the effect of IL-1 on ^{3}H-TdR uptake of the cells of three cases (cases 2, 4, and 24) after removal of adherent cells (Fig 2). It appeared that IL-1 induced DNA synthesis increased following elimination of adherent cells in those cases. This indicates that AML cell stimulation did not depend on an intermediate effect of adherent monocytic cells in the cell preparations but resulted from direct effects of IL-1 upon AML blasts.

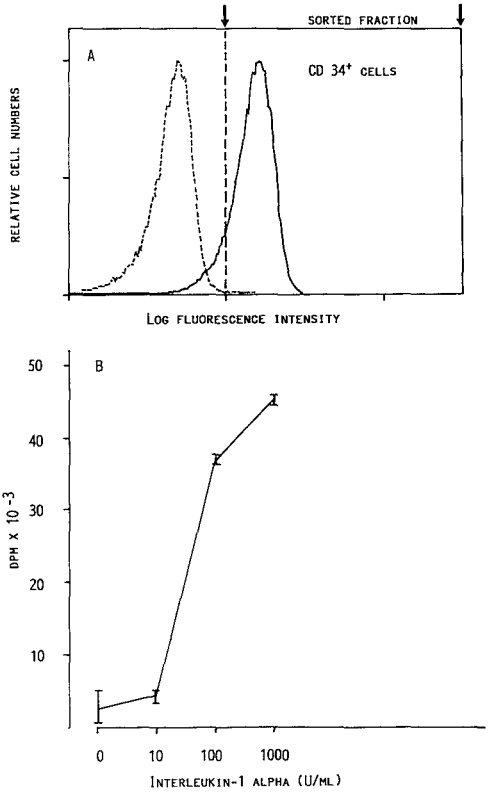

Fig 1. Effect of IL-1 on DNA synthesis of CD34-positive AML cells. The upper panel shows the fluorescence histogram of CD34-labeled AML cells (case 14) and the nonlabeled control cells, ie, cells incubated with GAM-FITC only (stippled graph). CD34-positive cells were sorted as indicated (fraction between arrows). Morphologic analysis of this fraction revealed that 100% of the cells were blasts. The lower panel shows DNA synthesis of CD34-positive AML cells stimulated with IL-1α. Two × 10^{4} cells were plated per well.

The data of colony and cluster formation from adherent cell-depleted samples of ten cases of AML following stimulation with IL-1 (Table 2) are consistent with those of ^{3}H-TdR uptake. IL-1 stimulated cluster formation in cases 2, 4, 7, 8, and 24. In cases 13 and 22, clusters and colonies were spontaneously formed but following IL-1 stimulation colony numbers increased significantly. In three cases (ie, cases 3, 10, and 20) IL-1 had no effect on colony or cluster growth.

Stimulation of AML Growth by IL-1: induction of CSF release. Because IL-1 acts as a modulator of CSF production in endothelial and stromal cells,[6-8] we investigated whether activation of DNA synthesis of AML cells was caused by growth factor release induced by IL-1 and subsequent autocrine stimulation. AML cells from five cases (cases 2, 4, 7, 14, and 24) were cultured with IL-1α and polyclonal antisera that specifically neutralized GM-CSF or

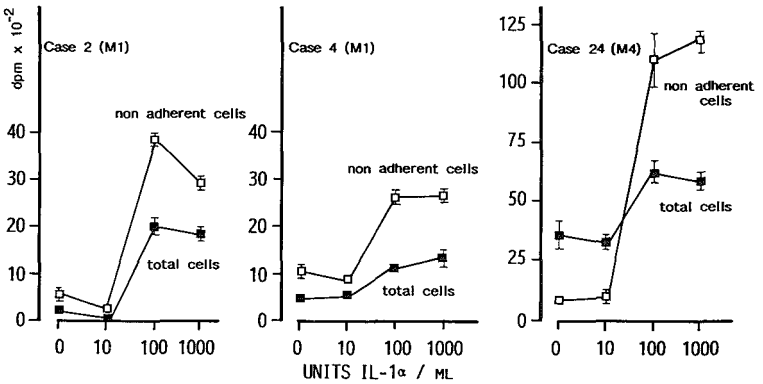

Fig 2. Effects of titrated concentrations of IL-1 on DNA synthesis of adherent cell-depleted (nonadherent) AML cells. Cells without prior adherence were examined for comparison (total cells). Two × 10^4 cells were plated per well.

IL-3 activity (Table 3). It appeared that the IL-1-stimulated ^{3}H-TdR incorporation could be abrogated by anti-GM-CSF in four of five cases. Blocking of IL-1 stimulation by anti-GM-CSF in case 7 could be restored by the simultaneous addition of GM-CSF to culture. Anti-IL-3 did not suppress IL-1-activated DNA synthesis. In three AML cases in which IL-1 mediated cluster formation (adherent cell depleted samples), cluster numbers could be reduced by anti-GM-CSF from 476 (case 2), 227 (case 7), and >500 (case 24) to 8, 4, and 12 clusters, respectively. Thus, it appears that IL-1 frequently stimulates proliferation of AML cells through the activation of GM-CSF production in these cells that subsequently stimulate AML growth.

To obtain further evidence for CSF production by AML blasts in the culture media, nonadherent AML cells from seven cases were incubated with IL-1α and AML-CM prepared. The stimulating activity of these AML-CM were examined upon purified ($CD34^+$, $CD3^-$, $CD14^-$, $CD15^-$) normal bone marrow progenitors (Table 4). AML-CM of IL-1-stimulated AML blasts of cases 2, 7, 8, and 24 demonstrated colony stimulating activity whereas control AML-CM (prepared without IL-1) did not reveal any stimulatory activity. The data are compatible with GM-CSF-like activity in AML-CM of cases 2, 7, and 24 because these AML-CM stimulated BFU-E, CFU-Eo, and CFU-GEMM.[32] G-CSF-like activity was found in AML-CM of cases 2 and 7. The colony data suggest M-CSF-like activity in AML-CM of cases 2, 7, 8, and 24. AML-CM from cases 4, 13, and 14 did not induce colony growth. Radioimmunoassays were performed in those seven cases (cases 2, 4, 7, 8, 13, 14, and 24) to estimate CSF concentrations in AML-CM. GM-CSF was detected in AML-CM of cases 2, 7, and 24 (ie, 290, 175, and 220 pmol/L, respectively) following IL-1 stimulation and not in AML-CM of cases 4, 8, 13, and 14. In control supernatants of AML cells (ie, not induced with IL-1), no GM-CSF was detectable. In PHA-stimulated leukocyte conditioned medium (PHA-LCM) 160 pM GM-CSF was demonstrated. G-CSF and M-CSF were not detectable in any of the IL-1-stimulated cultures, whereas significant amounts of G-CSF (3.3 ng/mL) and M-CSF (10.2 ng/mL) were found in PHA-LCM. Thus, although G-CSF- and M-CSF-like activity had been suggested to have been released into the AML-CM, these factors were not detectable with the radioimmunoassay.

Spontaneous proliferation as the consequence of constitutional IL-1 release by AML cells. Others have shown that AML cells are able to produce IL-1β.[33] It appears from the experiments presented above that IL-1 can stimulate DNA synthesis of AML cells. Therefore, we investigated the hypothesis that spontaneous DNA synthesis in certain AML

Table 2. Stimulation of AML Cluster and Colony Formation by IL-1α

Patient No.	No Factor		IL-1α (1,000 U/mL)	
	Clusters	Colonies	Clusters	Colonies
2	9* (9,8)	0 (0,0)	476 (425,527)	0 (0,0)
3	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)
4	0 (0,0)	0 (0,0)	>500† (>500,>500)	0 (0,0)
7	4 (0,7)	0 (0,0)	227 (217,237)	0 (0,0)
8	0 (0,0)	0 (0,0)	24 (28,21)	0 (0,0)
10	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)
13	>500 (>500,>500)	313 (331,295)	>500 (>500,>500)	>500 (>500,>500)
20	>500 (>500,>500)	0 (0,0)	>500 (>500,>500)	0 (0,0)
22	>500 (>500,>500)	157 (152,162)	>500 (>500,>500)	308 (308,308)
24	5 (9,1)	0 (0,0)	>500 (>500,>500)	0 (0,0)

Cluster and colony formation were determined in adherent cell depleted AML samples.

*Mean values per 10^5 cells plated as well as colony and cluster numbers of duplicate experiments (between brackets) are indicated.

†In this patient, >500 clusters of four to ten cells per dish appeared following IL-1 stimulation.

Table 3. The Effects of Anti-GM-CSF and Anti-IL-3 on IL-1-Induced DNA Synthesis in AML Cells

Patient No.	No. of Factor	IL-1α	IL-1α + Anti-IL-3	IL-1α + Anti-GM-CSF	IL-1α + Anti-GM-CSF + GM-CSF (10^3 U/mL)	Irradiated Control
2	7.5 ± 0.4	34.7 ± 3.2	34.8 ± 1.3	*5.6 ± 0.9*	ND	0.5 ± 0.2
4	14.1 ± 1.0	31.6 ± 4.4	31.6 ± 3.8	*10.5 ± 0.7*	ND	4.9 ± 0.1
7	0.5 ± 0.1	37.5 ± 2.4	39.7 ± 0.1	*5.6 ± 2.0*	28.5 ± 1.2	0.4 ± 0.1
14	2.9 ± 2.1	45.1 ± 7.8	54.2 ± 9.0	42.6 ± 4.1	ND	1.6 ± 0.2
24	6.6 ± 0.8	13.5 ± 1.4	14.0 ± 1.1	*1.9 ± 4.1*	ND	1.4 ± 0.8

Values of ^{3}H-TdR uptake are expressed as dpm × 10^{-2} (means ± SD). IL-1α concentration, 100 U/mL. In case 7, the inhibition by anti-GM-CSF was overcome by the addition of 10^3 U/mL GM-CSF. Stimulation of ^{3}H-TdR uptake with the same concentration of GM-CSF alone was 38.6 ± 2.5 dpm × 10^{-2}. Italics indicate significant inhibition following the addition of anti-GM-CSF.

Abbreviation: ND, not determined.

cases was the result of endogenously produced IL-1β and subsequent CSF release. In one of nine cases (ie, case 24) anti-IL-1β inhibited the spontaneous DNA synthesis, whereas anti-IL-1α did not (Fig 3). Inhibition by anti-IL-1β could be overcome not only by the addition of IL-1β (10^3 U/mL) but also GM-CSF (10^4 U/mL) to the cultures. Spontaneous DNA synthesis could also be abrogated with anti-GM-CSF. This inhibition could only be competed with high concentrations of GM-CSF and not with IL-1β. Thus, it appears that in this particular case AML cell cycling resulted from stimulation by GM-CSF released as the consequence of endogenously produced IL-1β.

DISCUSSION

The results of this study show that human IL-1α and β frequently stimulate DNA synthesis of AML cells (13 of 28 cases). Sorting of CD34 (a marker for primitive hematopoietic cells) positive AML cells in one case and removal of adherent cells in three other cases did not result in loss of stimulating activity. Instead, enrichment of blasts increased the stimulatory effects of IL-1. Analysis of cluster and colony formation in adherent cell-depleted AML samples showed a clear stimulative effect of IL-1 as well (ie, in seven of ten cases). These observations are consistent with a stimulative effect of IL-1 directly on AML blast cells and not on the monocytic component of the neoplasm or a normal contaminating cell population.

IL-1 has shown to be a stimulus of cytokine production in several cell types, such as endothelial cells and fibroblasts.[6-8] In mice IL-1 or hemopoietin-1 acts synergistically with M-CSF as well as with IL-3, ie, IL-3 or M-CSF-dependent colony formation increases considerably in the presence of IL-1.[34,35] Hoang et al[9] demonstrated a synergistic effect of

Table 4. Effects of Conditioned Medium of AML Blasts Stimulated With or Without IL-1α on Colony Formation of Purified Normal Marrow Progenitors

	Experiment No. 1						Experiment No. 2					
Stimulus	G	Eo	M	G/M	Mix	E	G	Eo	M	G/M*	Mix	E
AML-CM												
2−IL-1	—	—	—	—	—	—	0	0	0	0	0	31
+IL-1	—	—	—	—	—	—	62	14	54	9	13	122
4−IL-1	0	0	0	0	0	15	—	—	—	—	—	—
+IL-1	0	0	0	0	0	39	—	—	—	—	—	—
7−IL-1	0	0	0	0	0	55	0	0	0	0	0	49
+IL-1	16	25	7	4	2	78	11	9	34	6	16	109
8−IL-1	0	0	0	0	0	22	0	0	0	0	0	41
+IL-1	0	0	16	0	0	55	3	1	38	3	1	76
13−IL-1	—	—	—	—	—	—	0	0	0	0	0	50
+IL-1	—	—	—	—	—	—	0	0	1	0	0	57
14−IL-1	0	0	0	0	0	—	—	—	—	—	—	—
+IL-1	0	0	0	0	0	—	—	—	—	—	—	—
24−IL-1	—	—	—	—	—	—	0	1	0	0	0	54
+IL-1	—	—	—	—	—	—	5	3	89	4	6	82
No factor	0	0	0	0	0	55	0	0	0	0	0	38
PHA-LCM	25	27	50	12	3	104	86	17	64	21	7	136
GM-CSF	0	13	2	0	2	63	2	12	9	0	15	114
G-CSF	19	0	0	0	0	51	42	0	0	0	0	45
GM-CSF + G-CSF	112	19	3	3	3	70	162	18	14	7	14	115

The stimulative effects of 15% AML-CM on CD34-positive, CD3-negative, CD14-negative, CD15-negative bone marrow progenitor cells were examined in the CFU-GEMM assay. The numbers of granulocytic (G), eosinophylic (Eo), macrophage (M), granulocyte/macrophage (M/G), mixed (Mix) and erythroid (E) colonies were determined at day 15 of culture. Colony numbers are absolute values derived from the growth of 10^4 bone marrow cells. Cultures containing mixtures of GM-CSF or G-CSF plus IL-1 were run in parallel and the results were identical to the cultures with GM-CSF or G-CSF but without IL-1. IL-1 alone had no effect on colony formation.

Abbreviation: —, not done.

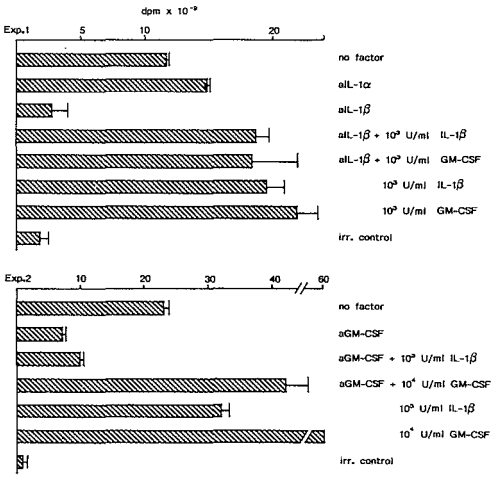

Fig 3. The effects of anti-IL-1α (aIL-1α), anti-IL-1β (a/L-1β), and anti-GM-CSF (aGM-CSF) on spontaneous DNA synthesis of AML cells (case 24). The results of two separate experiments are shown.

IL-1 and GM-CSF as well as IL-1 and G-CSF on AML colony formation. They indicated that no distinction had been made between direct and indirect effects and suggested the possibility that the IL-1 stimulation might be explained by the induction of growth factor release. The experiments presented here disclose that the stimulation of AML growth by IL-1 was mediated by induction of GM-CSF production. In four of five cases, a neutralizing antiserum against GM-CSF interfered with IL-1 induced DNA synthesis (Table 3). The addition of high concentrations of GM-CSF could overcome the blockade imposed by anti-GM-CSF on IL-1-dependent DNA synthesis. In addition, in three cases tested IL-1-induced cluster formation was abrogated by anti-GM-CSF as well. Thus IL-1-dependent growth of AML cells appears to be a two-stage process: (1) elaboration of GM-CSF by IL-1, followed by (2) activation of DNA synthesis by GM-CSF. It is possible that in the one case where we could not inhibit DNA synthesis with anti-GM-CSF, IL-1 induced the release of another factor.

The production of colony-stimulating activity by nonadherent AML cells in response to IL-1 was further supported by results obtained from the specific radioimmunoassay for GM-CSF and by the ability of supernatants of AML blasts incubated with IL-1, to induce colony growth from purified ($CD34^+$, $CD3^-$, $CD14^-$, $CD15^-$) normal bone marrow progenitors. Although G-CSF and M-CSF were not detected by specific radioimmunoassays in conditioned media of IL-1-stimulated AML blasts, the results from the in vitro stimulation of highly purified normal marrow progenitors may suggest that other CSFs were produced by AML cells as well following IL-1 stimulation.[32]

Recently it has been shown by Griffin et al[33] that in ten of 17 AML cases, the blast cells were able to produce IL-1β. Because AML cells frequently produce GM-CSF, G-CSF, or M-CSF as well and are able to proliferate in response to the latter factors,[36-41] one wonders whether these phenomena may have been cytokine dependent rather than constitutive. We addressed this question and investigated whether spontaneous DNA synthesis of AML blast cells was provoked by endogenously produced IL-1. In one of nine cases tested, anti-IL-1β antiserum could suppress spontaneous DNA synthesis. Blockade of DNA synthesis of the cells of this patient by anti-IL-1β could be overridden with high concentrations of IL-1β or GM-CSF. Obviously these data fit with a model of endogenously produced IL-1β stimulating the production of GM-CSF in AML cells leading to activation of DNA synthesis. Most likely IL-1β was released by the monocytic compartment of the leukemia (M4), because after adherence, the cells did not show spontaneous DNA synthesis (Fig 2C). The expression of mRNAs for GM-CSF, G-CSF, and M-CSF, as reported by others, may not have been constitutive in those instances but has been the result of activation by monokines released during the one hour incubation to deplete adherent cells.[36,37,40,41]

Because IL-1 is an inducer of CSF production in several normal cell types, eg, fibroblasts, endothelial cells,[6-8] it is possible that the GM-CSF production by AML cells following IL-1 stimulation represents a feature of normal cells. However, whether IL-1 is a stimulator of CSF production by normal marrow blast cells, considered as the normal counterparts of the leukemic cells, remains a pertinent question to address.

In summary, IL-1α and β modify proliferation in AML cells by stimulating the release of GM-CSF. As IL-1 is produced by various cell types including AML cells, it is conceivable that progression of AML in vivo is influenced by conditions that elevate tissue concentrations of IL-1 in vivo.

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REFERENCES

1. Delwel R, Dorssers L, Touw I, Wagemaker G, Löwenberg B: Human recombinant multilineage colony stimulating factor (interleukin 3): Stimulator of acute myelocytic cells in vitro. Blood 70:333, 1987

2. Vellenga E, Ostapovicz D, O'Rourke B, Griffin JD: Effects of recombinant IL-3, GM-CSF and G-CSF on the proliferation of clonogenic cells in short term and long term cultures. Leukemia 1:584, 1987

3. Miyauchi J, Kelleher CA, Yang Y, Wong GG, Clark SC, Minden MD, Minkin S, McCulloch EA: The effects of three growth factors, IL-3, GM-CSF and G-CSF on the blast cells of acute

myeloblastic leukemia maintained in short term suspension culture. Blood 70:657, 1987

4. Delwel R, Salem M, Pellens C, Dorssers L, Wagemaker G, Clark S, Löwenberg B: Growth regulation of human acute myeloid leukemia: Effects of five recombinant hematopoietic factors in a serum free culture system. Blood 72:1944, 1988

5. Oppenheim JJ, Kovacks EJ, Matsushima K, Darum SK: There is more than one interleukin-1. Immunol Today 7:45, 1986

6. Bagby GC, Dinarello CA, Wallace P, Wagner C, Hefeneider S, McCall E: Interleukin-1 stimulates granulocyte macrophage colony stimulating activity release by vascular endothelial cells. J Clin Invest 78:1316, 1986

7. Broudy VC, Kaushansky K, Harlan JM, Adamson JW: Interleukin-1 stimulates human endothelial cells to produce granulocyte macrophage colony stimulating factor and granulocyte colony stimulating factor. J Immunol 139:464, 1987

8. Kaushansky K, Lin N, Adamson JW: Interleukin-1 stimulates fibroblasts to synthesize granulocyte-macrophage and granulocyte colony stimulating factors. J Clin Invest 81:92, 1988

9. Hoang T, Haman A, Goncalves O, Letendre F, Mathieu M, Wong GG, Clark SC: Interleukin-1 enhances growth factor dependent proliferation of the clonogenic cells in acute myeloblastic leukemia and of normal human haematopoietic precursors. J Exp Med 168:463, 1988

10. French American British (FAB) Cooperative Group: Proposed revised criteria for the classification of acute myeloid leukemia. Ann Intern Med 103:620, 1985

11. Swart K, Hagemeyer A, Löwenberg B: Acute myeloid leukemia colony growth in vitro: Differences of colony forming cells in PHA supplemented and standard leukocyte feeder cultures. Blood 59:816, 1982

12. Löwenberg B, Hagemeyer A, Swart K: Karyotypically distinct subpopulations in acute leukemia with specific growth requirements. Blood 59:641, 1982

13. Schaeffer UW, Dicke KA, van Bekkum DW: Recovery of haematopoiesis in lethally irradiated monkeys by frozen allogeneic bone marrow grafts. Rev Eur Etud Clin Biol 17:443, 1972

14. March CJ, Mosley B, Larsen A, Cerretti DP, Braedt G, Price V, Gillis S, Henny CS, Kronheim SR, Grabstein K, Conlon PJ, Hopp TP, Cossman D: Cloning, sequence and expression of two distinct human interleukin-1 complementary DNA's. Nature 315:641, 1985

15. Wong GG, Witek JS, Temple PA, Wilkins KM, Leary AC, Luxenberg DP, Jones SS, Brown EL, Kay RM, Orr EC, Shoemaker C, Golde DW, Kaufman RJ, Hewick RM, Wang EA, Clarck SC: Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science 228:810, 1985

16. Sieff CA, Emerson SG, Donahue RE, Nathan DG, Wang DE, Wong GG, Clarck SC: Human recombinant granulocyte-macrophage colony stimulating factor: A multi-lineage hematopoietin. Science 230:1171, 1985

17. Souza LM, Boone TC, Gabrilove J, Lai PH, Zsebo KM, Murdock DC, Chazin VR, Bruszewski J, Lu H, Chen KK, Barendt J, Platzer E, Moore MAS, Mertelsmann R, Welte K: Recombinant human granulocyte colony stimulating factor: Effects on normal and leukemic myeloid cells. Science 232:61, 1986

18. Nagata S, Tsuchiya M, Asano S, Kaziro Y, Yamazaki T, Yamamoto O, Hirata Y, Kubota N, Obeda M, Nomura H, Ono M: Molecular cloning and expression of cDNA for human granulocyte colony stimulating factor. Nature 319:415, 1986

19. Kawasaki ES, Ladner MB, Wang AM, van Arsdell J, Warren MK, Coyne MY, Schweickart VL, Lee MT, Wilson KJ, Boosman A, Stanley ER, Ralph P, Mark DF: Molecular cloning of a complementary DNA encoding human macrophage specific colony stimulating factor (CSF-1). Science 230:291, 1985

20. Wong GG, Temple PA, Leary AC, Witeck-Giannotti JS, Yang YC, Ciarletta AB, Chung M, Murtha P, Kriz R, Kaufman RJ, Ferenz CR, Sibley BS, Turner KJ, Herwich RM, Clarck SC, Yanai N, Yokota H, Yamata M, Saito M, Motoyoshi K, Takaku F: Human CSF-1: Molecular cloning and expression of 4 kb cDNA encoding the human urinary protein. Science 235:1504, 1987

21. Dorssers L, Burger H, Bot F, Delwel R, Geurts van Kessel A, Löwenberg B, Wagemaker G: Characterization of a human multi-CSF cDNA clone identified by a conserved noncoding sequence in mouse interleukin-3. Gene 55:115, 1987

22. Touw I, Delwel R, van Zanen G, Löwenberg B: Acute lymphoblastic leukemia and non-Hodgkin's lymphoma of T lineage: Colony forming cells retain growth factor (interleukin-2) dependence. Blood 68:1088, 1986

23. Salem M, Delwel R, Touw I, Mahmoud L, Löwenberg B: Human AML colony growth in serum free culture. Leuk Res 12:157, 1988

24. Katz FE, Tindle R, Sutherland DR, Greaves MF: Identification of a membrane glycoprotein associated with haemopoietic progenitor cells. Leuk Res 9:191, 1985

25. Delwel R, Touw I, Bot F, Löwenberg B: Fucose binding lectin for characterizing acute myeloid leukemia progenitor cells. Blood 68:41, 1986

26. Delwel R, Touw I, Löwenberg B: Towards detection of minimal residual disease: Discrimination of AML precursors from normal myeloid precursors using a combination of surface markers, in Hagenbeek A, Löwenberg B (eds): Minimal Residual Disease in Acute Leukemia, Dordrecht/Boston, Nijhoff, 1986, p 68

27. van der Reyden HJ, van Rhenen DJ, Lansdorp PM, van't Veer MB, Langenhuijsen MMAC, Engelfriet CP, von Borne AEGKr: A comparison of surface marker analysis and FAB classification in acute myeloid leukemia. Blood 61:443, 1983

28. Perussia B, Trinchieri G, Lebman D, Janiewicz J, Lange B, Rovera G: Monoclonal antibodies that detect differentiation surface antigens on human myelomonocytic cells. Blood 59:382, 1982

29. Bot FJ, Dorssers L, Wagemaker G, Löwenberg B: Stimulating activity of human recombinant multi-CSF (IL-3) on human marrow precursors: Importance of assessory cells. Blood 71:1609, 1988

30. Fibbe WE, Damme I van, Billiau A, Duinkerken N, Larvink E, Ralph P, Altrock BW, Kaushansky K, Willemze R, Falkenburg JHF: Human fibroblasts produce granulocyte-CSF, macrophage CSF and granulocyte-macrophage-CSF following stimulation by Interleukin-1 and Poly(rI), Poly (rC). Blood 72:860, 1988

31. Hagemeyer A, van Dongen JJM, Slater RM, van't Veer MB, Behrendt H, Hählen K, Sizoo W, Abels J: Characterization of the blast cells in acute leukemia with translocation (4, 11): Report of eight additional cases and one case with a variant translocation. Leukemia 1:24, 1987

32. Bot FJ, van Eijk LI, Schipper PJ, Löwenberg B: Stimulating activity of human IL-3 compared to human GM-CSF and relation to accessory cells. Exp Hematol 16:505, 1988 (abstr)

33. Griffin JD, Rambaldi A, Vellenga E, Young DC, Ostapovicz D, Cannistra SA: Secretion of interleukin-1 by acute myeloblastic leukemia cells in vitro induces endothelial cells to secrete colony stimulating factors. Blood 70:1218, 1987

34. Bartelmez SH, Stanley ER: Synergism between hemopoietic growth factors (HGFs) detected by their effects on cells bearing receptors for a lineage specific HGF: assay for hemopoietin-1. J Cell Physiol 122:370, 1985

35. Stanley ER, Bartocci A, Patinkin D, Rosendaal M, Bradley TR: Regulation of very primitive, multipotent, hemopoietic cells by hemopoietin-1. Cell 45:667, 1986

36. Young DC, Griffin JD: Autocrine secretion of GM-CSF in acute myeloblastic leukemia. Blood 68:1178, 1986

37. Young DC, Wagner K, Griffin JD: Constitutive expression of the GM-CSF gene in acute myeloblastic leukemia. J Clin Invest 79:100, 1987

38. Mannoni P, Mueller T, Torres H, Dubreuil P, Waal R, Birg F: Expression of IL-3 GM-CSF and G-CSF genes in myeloid leukemia cells. Blood 70:263a, 1987

39. Oster W, Lindemann A, Horn S, Mertellsmann R, Herrman F: Constitutive expression of genes for hematopoietic growth factors in acute myeloblastic leukemia. Blood 70:266a, 1987

40. Rambaldi A, Wakamiya N, Vellenga E, Horiguchi J, Warren MK, Kufe DW, Griffin JD: Expression of the M-CSF and cfms gene in human acute myeloblastic leukemia cells. J Clin Invest 81:1030, 1988

41. Young DC, Demetri GD, Ernst TJ, Cannistra SA, Griffin JD: In vitro expression of colony-stimulating factor genes by human acute myeloblastic leukemia cells. Exp Hematol 16:378, 1988

CHAPTER 9

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Hemopoietin-1 Activity of Interleukin-1 (IL-1) on Acute Myeloid Leukemia Colony Forming Cells (AML-CFU) in Vitro: IL-1 Induces Production of Tumor Necrosis Factor-Alpha which Synergizes with IL-3 and Granulocyte-Macrophage Colony Stimulating Factor

Hemopoietin-1 Activity of Interleukin-1 (IL-1) on Acute Myeloid Leukemia Colony-Forming Cells (AML-CFU) in Vitro: IL-1 Induces Production of Tumor Necrosis Factor-α Which Synergizes with IL-3 or Granulocyte-Macrophage Colony-Stimulating Factor

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Interleukin-1 (IL-1) has hemopoietin-1 (H-1) activity, i.e., it synergizes with macrophage-colony stimulating factor (M-CSF), granulocyte-macrophage-CSF (GM-CSF) and interleukin-3 (IL-3) in stimulating in vitro colony formation of hematopoietic progenitor cells. In this study the synergistic activity of IL-1 was investigated on IL-3 and GM-CSF induced growth of acute myeloid leukemia colony forming cells (AML-CFU) in vitro. Among 12 cases of human AML, IL-1 significantly elevated IL-3 stimulated colony numbers in eight instances and enhanced GM-CSF induced colony growth in five cases. As IL-1 is an inducer of cytokine production and since tumor necrosis factor (TNF) elevates IL-3 or GM-CSF induced proliferation of AML-CFU, we examined whether IL-1 enhanced AML-CFU growth via the induction of TNF production. Neutralizing anti-TNF-α antibodies significantly decreased IL-1/IL-3 or IL-1/GM-CSF stimulated colony numbers in six of seven cases studied, whereas anti-TNF-β had no effect, indicating that endogenously produced TNF-α costimulated the growth of AML-CFU. Furthermore, AML blast cells stimulated by IL-1 released increased amounts of TNF-α (between 25 and 533 pg/ml; median 255 pg/ml) into the culture medium (TNF-α specific radioimmunoassay) as compared with noninduced AML cells (<1 to 149 pg TNF-α/ml; median 31 pg/ml). Thus, the effect of IL-1 on AML-CFU proliferation is not the result of direct activation of AML progenitors, but IL-1 stimulates the release of TNF-α by AML cells and endogenous TNF subsequently synergizes with IL-3 or GM-CSF.

INTRODUCTION

INTERLEUKIN-1 (IL-1), of which two different forms exist (i.e. IL-1-α and β), is a factor which exerts multiple effects on a number of cell types (1). It can induce the release of cytokines from endothelial cells, fibroblasts, T lymphocytes, and as was recently shown, also from acute myeloblastic leukemia (AML) cells (2–5). IL-1 is a growth stimulator of fibroblasts and epithelial cells (1). Furthermore, IL-1 has been identified as hemopoietin-1 (H-1) (6, 7), a stimulus that synergizes with colony-stimulating factors (CSFs) (6, 8, 9). For instance, it significantly elevates the number and size of macrophage-CSF (M-CSF) stimulated colonies in murine bone marrow in vitro (8). IL-1 also synergizes with granulocyte-macrophage CSF (GM-CSF) or interleukin-3 (IL-3) in the stimulation of proliferation of primitive murine stem cells (6, 9). Similar effects of IL-1 have been described on proliferation of AML progenitor cells (10), i.e., IL-1 synergized with GM-CSF or G-CSF in the stimulation of growth of AML colony forming cells (AML-CFU) in vitro. In this study we show that IL-1 may also elevate IL-3 dependent growth of AML-CFU. We examined the mechanism by which IL-1 costimulates AML cell proliferation with IL-3 and GM-CSF. The results of these experiments disclose that IL-1 activates AML cells to produce tumor necrosis factor (TNF)-α, which subsequently synergizes with IL-3 or GM-CSF.

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MATERIALS AND METHODS

Patients and Preparation of AML Blasts. Cells from 12 patients with AML were obtained from bone marrow or blood after informed consent. Blast cells were purified from the marrow or blood by Ficoll-isopaque (11) or bovine serum albumin centrifugation and subsequent depletion of T cells by E rosette sedimentation (12). The cells were then cryopreserved as described (13). After thawing, monocytic cells were removed by plastic adherence: 10^7 cells in 5-ml serum free medium (14) were incubated for 1 hr in 6-cm petridishes (Greiner, Nurtingen, F.R.G.) at 37°C. Following this procedure, cell fractions were obtained that contained 97% or more blast cells.

Purified Recombinant Growth Factors and Neutralizing Antibodies. IL-3 (Gist Brocades, Delft, The Netherlands) (15) was used at a concentration of 3 ng/ml, GM-CSF (a gift from Dr. S. Clark from Genetics Institute, Cambridge, MA) (16) at 200 U/ml (25 ng/ml) and IL-1-α (donated by Dr. S. Gillis from Immunex, Seattle, WA) (17) at 100 or 1,000 U/ml (1 or 10 ng/ml). Both antibodies, anti-TNF-α and anti TNF-β (Dr. Adolf, Boehringer Institute, Vienna, Austria) were used at concentrations of 1:1,000 and completely inhibited the proliferation of AML blasts in response to TNF (TNF-α or TNF-β; 1,000 U/ml). Anti-TNF-α did not neutralize the TNF-β stimulation nor did anti-TNF-β interfere with TNF-α stimulated growth of AML cells. Conditioned medium from phytohaemagglutinin stimulated leukocytes (PHA-LCM) was prepared from serum free cultures of peripheral blood mononuclear cells (10^6 cells per ml; obtained after Ficoll separation) stimulated with PHA (1%). Media were harvested after 4 days of culture.

In Vitro Culture of Human AML Blasts. Growth of AML colony forming cells (AML-CFU) was determined in serum free methylcellulose cultures exactly as described (18). Colonies (>50 cells) and clusters (>10 cells) were counted at day 14 of culture. Numbers represent means of duplicate experiments.

Radioimmunoassay for Tumor Necrosis Factor-α. Nonadherent AML cells (10^6 cells per ml) were cultured in 6-cm petri dishes Greiner) in serum free medium in the presence or absence of 1,000 U/ml IL-1-α. Conditioned media were harvested after 5 days of culture at 37°C. The concentration of TNF-α in these media were determined with a specific radioimmunoassay according to the methods described by the manufacturer (IRE-Medgenix, Fleurus, Belgium). The assay relies on several monoclonal antibodies directed to distinct epitopes of TNF-α and shows no cross reactivity with TNF-β, IL-1, IL-2 and interferon α, β, and γ. The mean amount of TNF-α in cell free culture media determined in 10 separate experiments was 2.4 pg/ml ranging from 1–7.5 pg/ml, and the interassay coefficient of variation at a level of 131 pg/ml was 7.2% (n = 10).

RESULTS

Synergistic Effect Between IL-1 and IL-3 or IL-1 and GM-CSF on AML Growth. We investigated whether IL-1 synergized with IL-3 and GM-CSF in stimulating AML colony formation (Table 1). In eight of 12 cases of AML, IL-1 significantly elevated colony or cluster numbers in the presence of IL-3. IL-1 synergized with GM-CSF in stimulating AML-CFU proliferation in five of those eight cases.

Inhibition of IL-1/IL-3 or IL-1/GM-CSF Stimulated Growth of AML-CFU by Anti-TNF-α Antibodies. It has recently been shown that TNF may synergize with GM-CSF (19, 20) and with IL-3 (20) in stimulating AML-CFU. In addition, under certain conditions TNF-α can be produced by AML blast cells (21, 22). In anti-TNF-α blocking experiments we addressed the question whether the enhancement of IL-3 or GM-CSF stimulated growth of AML-CFU by IL-1 is caused by endogenously produced TNF-α. In three cases where IL-1 synergized with IL-3 as well as with GM-CSF (nos. 6, 10, and 11), the increase of AML colony numbers by IL-1 was reversed when neutralizing antibodies against TNF-α were supplemented to the cultures (Fig. 1, A–C). In cases 4, 5, and 7, where IL-1 synergized with IL-3 only (Table 1), anti TNF-α suppressed colony numbers as well (Fig. 1, D–F). These results suggest that the positive effect of IL-1 was the result of endogenous TNF-α released into the cultures following IL-1 stimulation. In one case (#8) anti-TNF-α could not abrogate the synergistic effect provoked by IL-1 (data not shown). Anti TNF-α did not inhibit the growth of AML-CFU from cases 2, 3, 9, and 12, in which IL-1 did not enhance IL-3 or GM-CSF stimulated growth. Neither did the anti-TNF-α antibody affect control cultures of AML cells with IL-3 or GM-CSF alone (data not shown). Addition of anti-TNF-β did not inhibit AML colony growth in any of the experiments (example see Fig. 1F).

In three cases (nos. 6, 8, and 10) IL-1 as the sole factor added to culture stimulated considerable numbers of AML colonies (Table 1). In two of those (nos. 6 and 10) anti-TNF-α reduced IL-1 stimulated colony numbers from 126 to 20 and from 161 to 34, respectively. IL-1 induced AML colony formation from case 8 was not affected by anti-TNF-α (312 vs. 299 colonies).

Production of TNF-α by AML Blasts. To further demonstrate that AML blasts produce TNF-α following IL-1 stimulation, the concentrations of TNF-α in culture media of AML cells were assessed in the 12 cases (Table 2). Noninduced blast cells released variable concentrations of TNF-α, i.e., between not detectable (<1 pg/ml) and 149 pg/ml (median 31 pg/ml). Significantly higher concentrations of TNF-α were apparent in the supernatants of IL-1 stimulated AML cells, i.e., between 25 and 533 pg/ml TNF-α (median 255 pg/ml).

DISCUSSION

In murine systems IL-1 increases the number of colonies formed by hematopoietic progenitor cells in vitro following stimulation with IL-3 or GM-CSF (6, 7, 9). We have shown in this report that IL-1 elevates IL-3 induced AML colony formation and confirmed the data reported by others that IL-1 enhances GM-CSF stimulated growth of AML-CFU (10). Our results demonstrate that the synergistic action of IL-1 on AML colony growth frequently depends on the induction of TNF-α that is released by the AML blasts. Endogenously produced TNF-α then costimulates AML cell proliferation with IL-3 or GM-CSF. This is in agreement with recent evidence indicating that TNF-α strongly synergizes with IL-3 and GM-CSF in stimulation of AML growth in vitro (19, 20).

In a few cases (three of 12) IL-1 did not synergize with IL-3 or GM-CSF although a clear production of TNF-α was apparent following IL-1 stimulation (Tables 1 and 2). These heterogenous responses among AML cases remain presently unexplained, but could for instance be related to variations of the maturation arrest of AML-CFUs. Likewise, when exogenous TNF is added to IL-3 or GM-CSF supplemented cultures of AML blasts synergism is not always apparent (20). In one case (no. 8) the AML cells elaborated TNF-α (Table 2), but anti TNF-α did not inhibit the synergistic effect of IL-1 upon AML-CFU growth. In this case the positive effect of IL-1 is apparently not mediated through TNF-α, but may be explained by the release of another cytokine. Previous studies have shown that AML blast cells express TNF-α mRNA under certain conditions, i.e., after incubation for 1 hr with the purpose to remove cells adherent to plastic (monocytes) (21, 22). TNF-α transcripts were not demonstrated in fresh AML samples (22). Similarly, in certain cases of AML the blast cells express the RNA message for GM-CSF (23), but usually only after (1 hr) culture (24). A plausible explanation for the appearance of TNF-α and GM-CSF mRNAs in AML blasts is that a factor is released by monocytes (activated following adherence), which subsequently induces the expression of mRNAs for these cytokines. This factor could be IL-1, since IL-1 is a monokine that stimulates the production of GM-CSF by AML blast cells (5) and as shown here stimulates the release of TNF-α by AML

Table 1. Effects of IL-1 on IL-3 and GM-CSF Induced AML Colony Formation

Case No.	FAB	No Factor	IL-1	IL-3	GM-CSF	IL-1 + IL-3	IL-1 + GM-CSF
*1	M1	14	10	18	80	237	517
2	M1	1	0	35	5	12	21
3	M1	0	1	306	7	361	18
*4	M1	0	0	0	0	44	0
*5	M1	0	0	0	0	45	0
*6	M1	0	126	34	17	>1,000	501
*7	M2	0	0	36	12	99	9
8	M2	0	312	360	113	>1,000	>1,000
9	M2	0	0	21	82	62	92
10	M4	0	161	54	210	269	697
*11	M4	0	0	21	0	340	410
12	M4	0	0	9	32	5	31

Numbers indicate colonies or clusters per 10^5 cells plated.
*Indicates that in those cases only clusters of less than 50 cells were grown.
FAB French-American-British nomenclature (25).

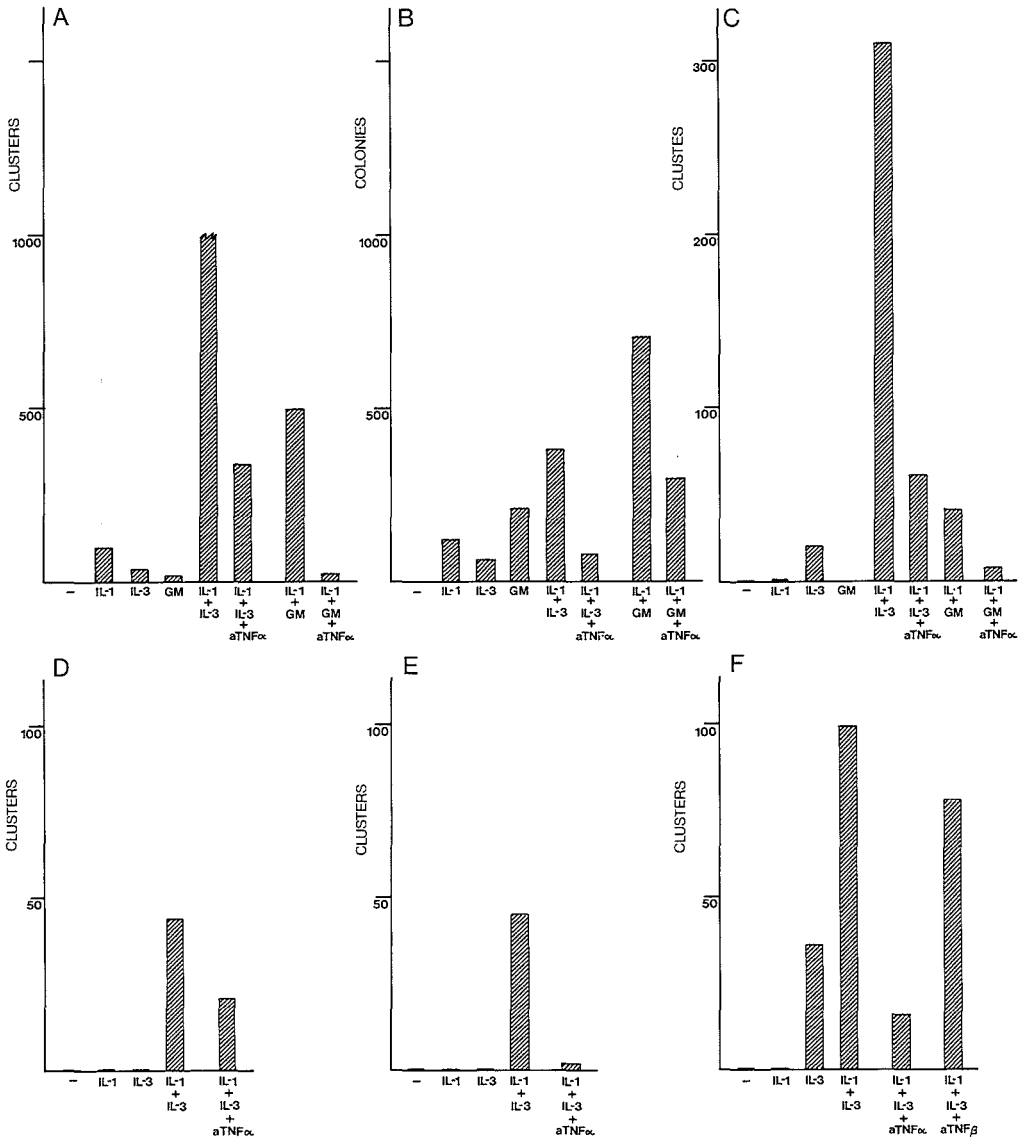

Figure 1. Anti TNF-α antibodies suppress colony formation stimulated by the combinations of IL-1 and IL-3, or IL-1 and GM-CSF. AML blasts were cultured serum free with optimal concentrations of IL-1 and/or IL-3 or GM-CSF in methylcellulose (cases 6(A), 10(B), and 11(C)). Cells from cases 4(D), 5(E), and 7(F) were cultured with IL-1 and/or IL-3. AML cells from those cases did not form colonies with GM-CSF plus IL-1 (see Table 1). To verify whether the synergistic effect of IL-1 on IL-3 or GM-CSF induced colony growth was the result of endogenously produced TNF-α, neutralizing antibodies against TNF-α (aTNFα) were added to the cultures. In control experiments anti TNF-β neutralizing antibodies were added to the cultures as well. An example is shown (F). GM: GM-CSF.

cells as well. Thus, the production of at least two cytokines, i.e., GM-CSF and TNF-α, by AML blast cells is under the control of IL-1.

Since AML blast cells may produce GM-CSF in addition to TNF-α, colony formation stimulated by IL-1 alone may be the result of the synergistic action of endogenously produced GM-CSF and TNF-α. We recently showed that AML colony formation stimulated by IL-1 could be inhibited with anti-GM-CSF antibodies. Here we show that in two cases where IL-1 alone stimulated AML colony growth, anti-TNF-α was inhibitory as well. As TNF does not stimulate AML colony formation by itself (20), it is most likely that AML colony growth in

Table 2. TNF-α Concentrations in Culture Media of AML Blasts Incubated in the Presence or Absence of IL-1

FAB	AML Case No.	pg/ml TNF-α in Culture Medium	
		Stimulus	
		None	IL-1
M1	1	20	208
	2	21	101
	3	10	485
	4	14	533
	5	33	40
	6	6	372
M2	7	7	107
	8	<1	25
	9	15	96
M4	10	59	499
	11	37	284
	12	149	277
PHA-LCM		>5,000	

AML blasts (10^6 cells per ml) were cultured for 5 days in serum free medium in the presence or absence of 1,000 U/ml IL-1-α. The concentration of TNF-α in the medium produced by the AML blasts was determined with a specific radioimmunoassay.
PHA-LCM = phytohemagglutinin stimulated leukocyte conditioned medium.

those instances was synergistically stimulated by endogenous GM-CSF and TNF-α.

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REFERENCES

1. Oppenheim JJ, Kovacs EJ, Matsushima K, Durum SK. There is more than one interleukin-1. Immunol Today **1986**;7:45–56.
2. Broudy VC, Kaushansky K, Harlan JM, Adamson JW. Interleukin-1 stimulates human endothelial cells to produce granulocyte macrophage colony stimulating factor and granulocyte colony stimulating factor. J Immunol **1987**;139:464–468.
3. Kaushansky K, Lin N, Adamson JW. Interleukin-1 stimulates fibroblasts to synthesize granulocyte macrophage and granulocyte colony stimulating factors. J Clin Invest **1988**;81:92–97.
4. Herrmann F, Oster W, Meuer SC, Lindemann A, Mertelsmann RH. Interleukin-1 stimulates T lymphocytes to produce granulocyte-monocyte colony-stimulating factor. J Clin Invest **1988**;81:1415–1418.
5. Delwel R, van Buitenen C, Salem M, Bot F, Gillis S, Kaushansky K, Altrock B, Löwenberg B. Interleukin-1 stimulates proliferation of acute myeloblastic leukemia cells by induction of granulocyte-macrophage colony-stimulating factor release. Blood **1989**;74:586–593.
6. Mochizuki DY, Eisenman JR, Conlon PJ, Larsen AD, Tushinski RJ. Interleukin-1 regulates hematopoietic activity, a role previously ascribed to hemopoietin-1. Proc Natl Acad Sci USA **1987**;84:5267–5271.
7. Zsebo KM, Wypych J, Yuschenkoff VN, Lu H, Hunt P, Dukes PP, Langley KE. Effects of hematopoietin-1 and interleukin-1 on early hematopoietic cells of the bone marrow. Blood **1988**;71:962–968.
8. Bartelmez SH, Stanely ER. Synergism between hemopoietic growth factors (HGFs) detected by their effects on cells bearing receptors for a lineage specific HGF: assay of hemopoietin-1. J Cellular Physiol **1985**;122:370–378.
9. Stanley ER, Bartocci A, Patinkin D, Rosendaal M, Bradley TR. Regulation of very primitive, multipotent, hemopoietic cells by hemopoietin-1. Cell **1986**;45:667–674.
10. Hoang T, Haman A, Goncalves O, Letendre F, Mathieu M, Wong GG, Clark SC. Interleukin-1 enhances growth factor dependent proliferation of the clonogenic cells in acute myeloblastic leukemia and of normal human primitive hemopoietic precursors. J Exp Med **1988**;168:463–474.
11. Boyum A. Separation of leukocytes from blood and bone marrow. Scan J Clin Lab Invest **1968**;21:77–89.
12. Löwenberg B, Hagemeyer A, Swart K. Karyotypically distinct subpopulations in acute leukemia with specific growth requirements. Blood **1982**;59:641–645.
13. Schaeffer UW, Dicke KA, van Bekkum DW. Recovery of haematopoiesis in lethally irradiated monkeys by frozen allogeneic bone marrow grafts. Rev Eur Etud Clin Biol **1972**;17:443–488.
14. Salem M, Delwel R, Touw I, Mamoud L, Löwenberg B. Human AML Colony growth in a serum free system. Leuk Res **1988**;12:157–165.
15. Dorssers L, Burger H, Bot F, Delwel R, Geurts van Kessel A, Löwenberg B, Wagemaker G. Characterization of a human multi-CSF cDNA clone identified by a conserved noncoding sequence in mouse interleukin-3. Gene **1987**;55:115–124.
16. Wong GG, Witek JS, Temple PA, Wilkins KM, Leary AC, Luxenberg DP, Jones SS, Brown EL, Kay RM, Orr EC, Shoemaker C, Golde DW, Kaufman RJ, Hewick RM, Wang EA, Clark SC. Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science **1985**;230:810–815.
17. March CJ, Mosley B, Larsen A, Cerretti DP, Braedt G, Price V, Gillis S, Henny CS, Kronheim SR, Grabstein K, Conlon PJ, Hopp TP, Cossman D. Cloning, sequence, and expression of two distinct human interleukin-1 complementary DNAs. Nature **1985**;315:641–648.
18. Delwel R, Salem M, Pellens C, Dorssers L, Wagemaker G, Clark S, Löwenberg B. Growth regulation of human acute myeloid leukemia: effects of five recombinant hematopoietic factors in a serum free culture system. Blood **1988**;72:1944–1949.
19. Hoang T, Levy B, Onetto N, Haman A, Rodriquez-Cimadevilla JC. Tumor necrosis factor alpha stimulates the growth of the clonogenic cells of acute myeloblastic leukemia in synergy with granulocyte/macrophage colony-stimulating factor. J Exp Med **1989**;170:15–26.
20. Salem M, Delwel R, Touw I, Mahmoud LA, Elbasousy EM, Löwenberg B. Modulation of colony stimulating factor (CSF)-dependent growth of acute myeloid leukemia by tumor necrosis factor. Leukemia **1990**;4:37–43.
21. Oster W, Cicco NA, Klein H, Hirano T, Kishimoto T, Lindemann A, Mertelsmann RH, Herrmann F. Participation of the cytokines IL-6, TNF-alpha, and IL-1-beta secreted by acute myelogenous leukemia blasts in autocrine and paracrine leukemia growth control. J Clin Invest **1989**;84:451–457.
22. Wakamiya N, Stone R, Takeyama H, Spriggs D, Kufe D. Detection of tumor necrosis factor gene expression at a cellular level in human acute myeloid leukemias. Leukemia **1989**;3:51–56.
23. Young DC, Wagner K, Griffin JD. Constitutive expression of the GM-CSF gene in acute myeloblastic leukemia. J Clin Invest **1987**;79:100–106.
24. Kaufman DC, Baer MR, Gao XZ, Wang Z, Preisler HD. Enhanced expression of the granulocyte-macrophage colony stimulating factor gene in acute myelocytic leukemia cells following in vitro blast cell enrichment. Blood **1988**;72:1329–1332.
25. French American British (FAB) cooperative group. Proposed revised criteria for the classification of acute myeloid leukemia. Ann Intern Med **1985**;103:620–629.

CHAPTER 10

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Comparative Analysis of IL-1 Regulated and Spontaneous Growth of Acute Myeloid Leukemia in Vitro

Comparative analysis of IL-1 regulated and spontaneous growth of acute myeloid leukemia *in vitro*

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INTRODUCTION

In vitro cultures of AML cells may have diagnostic applications, e.g. for the classification of AML cells based on growth factor responses (1) or for monitoring residual leukemic cells in remission marrow (2). Furthermore, culture assays are important tools for the analysis of leukemic growth characteristics (3). Two characteristic growth types of primary AML cells which may be of use in the diagnosis of AML and which should be investigated in more detail for a better understanding of the mechanisms of AML growth are a) the spontaneous growth and b) the IL-1 stimulated proliferation of AML blasts (1,4). In several studies it has been suggested that AML cells may produce their own growth factors constitutively, i.e. nonregulated. Thus GM-CSF, G-CSF, M-CSF, IL-6, TNFα and IL-1β may stimulate AML growth in an autocrine fashion (5-7). On the other hand, it has also been demonstrated that GM-CSF and TNFα may be produced by AML blast cells under the control of IL-1 (4,8). Since these results may appear contradictory we investigated in more detail: a) which HGFs are produced by IL-1 responding AML cells and AML blasts that grow spontaneously, and b) whether spontaneous growth of AML blasts can always be attributed to autocrine stimulation or whether in certain cases the cells may grow independent of any regulator.

MATERIALS AND METHODS.

Patients and preparation of AML cells
Cells from AML patients were obtained after informed consent. Blast cells were purified from the marrow or blood by a ficoll-isopaque centrifugation (9) and subsequent depletion of T-cells by E-rosette sedimentation (10). The cells were cryopreserved as described (11). After thawing monocytic cells were removed by plastic adherence: 10^7 cells in 5 ml serum free medium (12) were incubated for 1 hour in 6 cm petridishes (Greiner, Nurtingen,FRG) at 37 °C. Following this procedure cell fractions were obtained that contained 97% or more blast cells.

3H-Thymidine (3H-TdR) incorporation assay
DNA synthesis of AML cells was measured in 96 well microtiter plates (Greiner) as described (1). Thymidine uptake was determined at day 3 or 7 of culture. Eighteen hours before harvesting the cells, tritiated thymidine (0.1 uCi ^{3}H-TdR, specific activity 2 Ci/mmol; Amersham International, Amersham, UK) was added. Cells were harvested on nitrocellulose paper using a Titerteck harvester 550 (Flow Laboratories, Isrike, UK). Radioactivity was determined with a Beckman LS 1701 scintillation counter (Beckman Instruments, Fullerton, Ca). The results of triplicate experiments of $2x10^4$ cells per well are expressed as mean ± standard deviations (SD). Preirradiated (30Gy) AML cells were always run in parallel to assess background thymidine incorporation.

Purified recombinant hematopoietic growth factors and neutralizing antibodies
Interleukin-3 (IL-3, Gist Brocades, Delft, The Netherlands) was used at concentrations of 10 ng/ml. GM-CSF (Genetics Institute, Cambridge,MA) was supplied to the cultures at a concentration of 25 ng/ml. G-CSF (Immunex, Seattle,WA) was used at 10 ng/ml and M-CSF (Cetus Corporation, Emeryville, CA) at 2000 U/ml and human IL-6 (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service [CLB], Amsterdam) at 2000 U/ml. Plateau concentration of IL-1α (Immunex) was 100 U/ml (1 ng/ml). Polyclonal rabbit anti IL-3, sheep anti GM-CSF (both from Genetics Institute), rabbit anti IL-1α, rabbit anti IL-1β, rabbit anti G-CSF (from Immunex) and monoclonal (IgG1) anti M-CSF (Cetus) were used in dilutions that completely blocked the effects of the corresponding growth factors at the indicated concentrations. Optimal concentrations of IL-6 and anti IL-6 (CLB) were determined on a murine IL-6 dependent plasmacytoma cell line.

Preparation of AML conditioned media
Adherent cell depleted AML samples (10^6 cells per ml) were cultured serum free in 6 cm petri dishes. After 3 days of culture media were harvested and tested on HGF activity on factor responsive AML cells and an IL-6 dependent murine plasmacytoma cell line.

Northern blotting
AML cells were cultured for 18 hours in serum free medium in the presence or absence of 10 ng/ml IL-1α. Total cellular mRNA was isolated as described (13). Electrophoresis of mRNA and northern blotting was performed according to standard procedures (14). The cDNA probes for GM-CSF, G-CSF, M-CSF, IL-3, IL-6, IL-1α and IL-1β were kindly provided by Genetics Institute. Control blots were hybridized with a cDNA probe of the gene coding for human glyceraldehyde -3- phosphate dehydrogenase (GAPDH)(15).

RESULTS

IL-1 stimulated growth of AML cells
Human AML cells may produce their own growth factors (5-7). However, release of GM-CSF and TNF by AML cells is frequently regulated by IL-1 (4,8). To investigate whether IL-1 may stimulate the release of other cytokines by AML blasts as well, the expression of mRNA for GM-CSF, G-CSF, M-CSF, IL-6 and IL-1β was studied in five IL-1 responsive (both in thymidine uptake and colony assay) cases (Figure 1). GM-CSF mRNA expression was induced in three cases (#1-3) and enhanced in two patients (#4 and 5) by IL-1. G-CSF mRNA expression was elevated by IL-1 in two cases (#3

and 5) and the amounts of M-CSF mRNA increased in three AML patients (#1-3) after exposure to IL-1. In the other two patients baseline M-CSF mRNA levels did not increase further. In two of three cases (#2 and 3) IL-6 mRNA was expressed only after IL-1 induction. Interestingly, IL-1β mRNA expression could be influenced in three cases (#1-3) by IL-1 as well. IL-3 and IL-1α mRNA were not expressed in AML cells. Hybridization with the GAPDH cDNA probe indicated similar amounts of mRNA in all lanes (data not shown).
Using cells from the same five cases and an additional patient with AML the effects of HGF neutralizing antibodies on IL-1 stimulated DNA synthesis were investigated (TABLE 1). Anti GM-CSF was highly inhibitory in all cases. Anti G-CSF partly abrogated IL-1 stimulated DNA synthesis in case #1. In cases #3, 4 and 6 anti M-CSF partly suppressed thymidine uptake elicited by IL-1. In cases #3 and 6 neutralizing antibodies against IL-6 partially inhibited the IL-1 dependent growth. In the presence of combinations of antibodies the IL-1 stimulated growth was always brought back to the baseline level of proliferation, i.e DNA synthesis in the absence of IL-1.

Spontaneous growth of AML cells
In approximately 50% of the cases AML cells show a considerable baseline DNA synthesis in the absence of HGFs (1). To study how frequently this spontaneous growth is the result of autocrine stimulation, anti HGF neutralizing antibodies were added to the cultures (TABLE 2). Anti GM-CSF was inhibitory in five out of twelve cases (#10,13 and #15-17). However, abrogation due to anti GM-CSF was not as effective as in IL-1 stimulated AMLs. Anti G-CSF was partly inhibitory in three cases, i.e. # 10, 11 and 16. Anti M-CSF was not at all inhibitory and anti

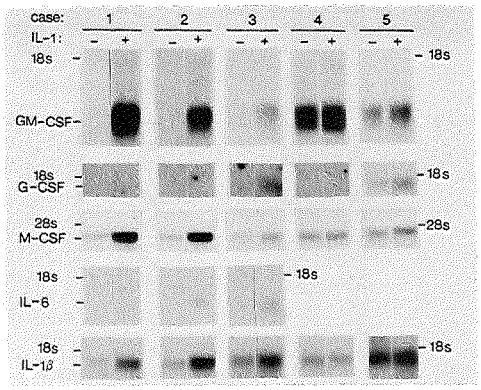

FIGURE 1. EXPRESSION OF HGF mRNA IN IL-1 RESPONSIVE AML CELLS.
AML cells from five cases were cultured for 18 hours with (+) or without (-) IL-1 and the expression of the HGF mRNAs was determined by northern blotting. HGF mRNA analysis included GM-CSF, G-CSF, M-CSF, IL-6 and IL-1β.

IL-6 suppressed spontaneous thymidine uptake to some extent in two cases (#13 and 14).
In a recent study we described an AML case that growed spontaneously as the result of endogenous IL-1β release which stimulated the production of GM-CSF by AML blast cells. This endogenously released GM-CSF in a subsequent step induced AML growth (1). In the cases presented here we investigated as well whether autocrine

TABLE 1. IL-1 STIMULATED DNA SYNTHESIS OF AML BLASTS. Effects of anti HGF neutralizing antibodies.

Case	no IL-1	IL-1 plus: no ab.	aGM-CSF	aG-CSF	aM-CSF	aIL-6	Comb.	irr. contr.
1	3.6±0.3	78.2±12.6	*5.6±0.6*	*52.4±1.8*	69.9±4.8	62.7±9.4	*1.6±0.3*	0.7±0.3
2	3.8±0.6	38.3±2.1	*11.1±2.2*	nd	nd	nd	nd	0.6±0.1
3	5.0±1.0	91.8±1.3	*16.9±2.4*	90.2±14.1	*28.2±1.6*	*53.4±12.2*	*4.4±0.5*	0.8±0.4
4	5.0±0.4	41.2±3.7	*4.8±0.1*	40.2±3.0	*32.5±1.2*	46.6±1.3	*7.1±1.0*	1.0±0.2
5	14.7±3.7	56.9±3.4	*8.1±1.1*	82.9±11.3	53.9±7.3	62.4±2.0	*9.5±0.7*	0.5±0.1
6	1.9±0.4	14.0±3.5	*1.5±0.6*	14.5±3.6	*2.8±0.6*	*6.2±0.1*	*1.0±0.2*	0.4±0.4

Values represent dpm x 10^{-2} (± SD)
In italics significant inhibition of IL-1 stimulated thymidine uptake is indicated.
no ab. : no antibodies were supplied.
aHGF : anti HGF antibodies were added to the cultures.
Comb. : combinations of all four antisera were added to the cultures.
irr. contr.: AML cells were preirradiated.

TABLE 2 SPONTANEOUS DNA SYNTHESIS OF AML BLASTS
Effects of anti HGF neutralizing antibodies

Case	no ab.	aGM-CSF	aG-CSF	aM-CSF	aIL-6	Comb.	irr. contr.
7	103.6±7.2	100.6±1.4	105.9±6.7	103.8±4.3	108.6±6.2	105.7±2.8	0.5±0.1
8	127.3±3.3	120.1±9.4	122.7±3.0	124.0±14.9	129.7±3.5	*98.1±9.3*	1.2±0.4
9	38.8±1.8	42.6±0.8	52.6±2.0	36.9±0.5	34.9±2.1	41.7±3.2	0.8±0.2
10	143.6±13.2	*117.1±1.2*	*88.3±11.7*	149.9±14.2	163.9±0.8	*106.7±0.8*	1.4±0.4
11	55.9±5.8	48.3±4.1	*35.9±0.8*	55.8±6.8	68.1±4.2	*6.3±0.5*	3.8±0.3
12	19.8±1.7	20.5±1.2	23.7±3.1	16.4±0.9	16.7±0.7	*7.5±0.9*	1.0±0.3
13	54.7±0.6	*44.1±2.6*	54.1±4.7	49.0±1.7	*34.3±2.0*	*31.4±1.4*	1.0±0.3
14	9.1±1.7	11.5±0.6	10.2±2.4	8.2±1.4	*3.5±0.4*	*3.4±0.9*	0.7±0.1
15	12.7±2.6	*2.5±0.5*	9.5±4.2	11.9±2.3	9.6±1.7	*2.7±0.6*	1.7±0.1
16	53.7±7.8	*35.6±5.7*	*25.9±2.4*	48.9±7.2	53.4±1.9	*29.0±0.8*	0.7±0.1
17	70.8±6.1	*24.0±0.9*	72.0±0.9	69.8±7.7	80.0±7.3	*15.8±1.0*	0.8±0.2

In italics significant inhibition of spontaneous DNA synthesis is indicated.
For further explanation see TABLE 1.

proliferation was regulated by IL-1 that was released by the AML blasts in culture. Except for one case (#15) anti IL-1(β) did not abrogate spontaneous growth of AML cells (data not shown). Thus spontaneous/autocrine growth of AML cells is in general not regulated by endogenous IL-1.

Interestingly, in most cases the neutralizing antibodies only partially abrogated spontaneous DNA synthesis. The complete combination of antibodies was usually more effective to suppress spontaneous growth of AML (n=9) than were the individual anti HGF antibodies. Nevertheless, in most of the cases even the cocktail of antibodies did not entirely suppress spontaneous DNA synthesis. In two cases (# 7 and 9) the complete antibody supplement did not suppress the spontaneous DNA synthesis of AML cells at all. Addition of antibodies against IL-3, TNFα or TNFβ neither affected the spontaneous proliferation. The lack of suppression by these anti cytokine antibodies may suggest that spontaneous growth in those cases is not due to autocrine stimulation but that the cells proliferate independent of any regulator. Northern blot analysis in a selection of four of those cases (#7-10) demonstrated that GM-CSF, G-CSF and IL-6 mRNA were not expressed in AML cells that had been cultured for 18 hours without a stimulus (FIGURE 2). Only low amounts of M-CSF mRNA were expressed in cases # 9 and 10. When stimulated with IL-1 limited amounts of mRNA for all cytokines were observed in cells from case #7. Low expression of M-CSF mRNA and IL1β mRNA was observed in the other three cases following IL-1 exposure. Thus even following IL-1 stimulation the blasts from those spontaneous growers show limited or no HGF mRNA expression (FIGURE 2). In contrast, control cells from case #11, which growed spontaneously as the result of autocrine stimulation, expressed high levels of the mRNAs for all cytokines following culture without a stimulus (18 hours).

Finally, conditioned media were prepared from nonstimulated cells of these five spontaneously growing cell specimens of AML and tested for HGF activity on factor dependent AML cells and also on an IL-6 dependent cell line (TABLE 3). No activity that stimulated AML growth was evident in the conditioned media of four cases (#7-10). Conditioned medium from case #10 contained some IL-6 stimulatory activity. It was calculated that the concentration of IL-6 in this preparation (250 U/ml) was 8 times less than the optimal stimulatory concentration of recombinant IL-6 (2000 U/ml). Since the concentration of anti IL-6 used in the blocking experiments (Table 2), which completely abrogates the effect of 2000 U/ml IL-6, did not abrogate the spontaneous growth of case #10, endogenously produced IL-6 cannot explain the autonomous growth in this particular case. Thus, in those cases AML cells proliferate spontaneously, without an apparent role of endogenous IL-3, GM-CSF, G-CSF, M-CSF, IL-6, IL-1 or TNF. Conditioned medium prepared from cells of case #11 (autocrine grower) stimulated the growth of HGF responsive cells in all instances.

DISCUSSION

In recent studies contradicting results have been generated concerning autocrine growth of AML cells in vitro. Some studies suggest that AML cells may produce their own growth factor spontaneously (5-7), whereas others have implied that HGF production by AML cells is regulated by IL-1 (4,8). Furthermore, one cannot exclude the possibility of spontaneous growth not founded on

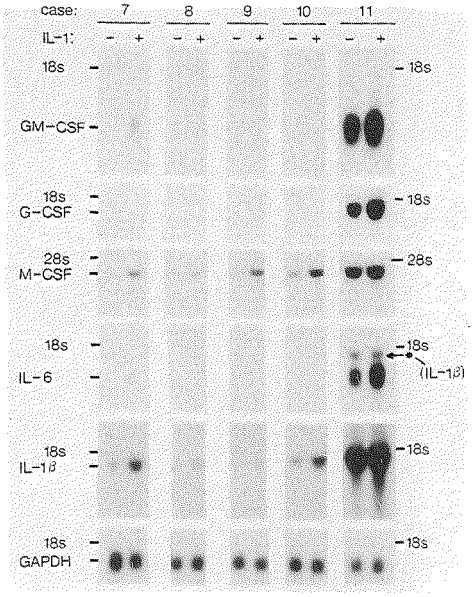

FIGURE 2. EXPRESSION OF HGF mRNA IN SPONTANEOUSLY GROWING AML CELLS.

autocrine stimulation. Here we show that all these mechanisms may occur. First, expression of mRNA for GM-CSF, G-CSF, M-CSF, IL-6, IL-1β may be induced by IL-1, whereas in other cases the expression of these HGF mRNAs appears nonregulated. Blocking studies revealed that anti HGF neutralizing antibodies may abrogate IL-1 induced and spontaneous DNA synthesis. Thus, IL-1 mediated and IL-1 independent autocrine growth of AML cells may both occur. Finally, blocking studies as well as northern blotting suggest that in certain AML cases spontaneous growth may not be the result of autocrine stimulation. In those situations AML cells may grow truly spontaneous.
Northern blot analysis of IL-1 responsive cases and blocking studies with HGF neutralizing antibodies of IL-1 stimulated AML cells do not strictly correspond. For instance, sometimes the RNA message of a certain HGF was expressed following IL-1 stimulation whereas antibodies against this particular factor could not abrogate the IL-1 induced DNA synthesis or vice versa. The reasons for these lacks of correlations can be conceived. Expression of HGF mRNA by AML cells does not necessarily imply that the peptide is produced. Neither will it be a rule that the AML cells always respond to the produced cytokine. On the other hand, when HGF mRNA is not detected in the northern blot, but IL-1 stimulation can be inhibited with the neutralizing antibodies, the amount of mRNA is probably below the detection level of the assay.

In addition to autocrine growth whether or not inducible by IL-1 a mechanism of autonomous growth may be involved as well. It is of interest to note that blocking of thymidine uptake of AML cells by neutralizing antibodies was always much more efficient in IL-1 stimulated cells than in spontaneously growing blasts. In fact in certain cases spontaneous growth could not be inhibited with neutralizing antibodies at all. An extended analysis in those latter cases neither revealed the mRNAs of most of these cytokines. Examination of the conditioned media of these AML samples for stimulating activity indicated that in general none of the studied regulators of AML growth had been released by the AML cells. One possibility to explain these findings is that these cells produce an as yet unknown growth factor that stimulates the cells in an autocrine fashion. Future studies should reveal whether factors such as IL-2, IL-4, IL-5, IL-7, IL-9 or other cytokines are involved in the growth of these cells. A second possibility is that the neoplastically transformed cells have really

TABLE 3. HGF ACTIVITY IN AML CONDITIONED MEDIA.

AML-CM from case#:	Target cells: AML #18	AML #4	IL-6 dependent cell line
7	27.0±2.9	15.3±5.2	n.d.
8	14.1±0.6	10.2±1.9	1.9±0.2
9	22.5±2.6	14.8±4.8	5.7±0.2
10	14.4±1.3	12.1±0.8	*43.6±5.0*
11	*47.3±3.8*	*69.1±6.0*	*139.9±2.0*
HGF:			
none	27.0±2.9	25.5±5.2	6.1±0.4
IL-3	*65.2±5.0*	*97.8±13.4*	-
GM-CSF	*73.5±7.6*	24.6±4.0	-
G-CSF	*58.5±2.3*	*66.7±16.0*	-
M-CSF	*63.3±0.5*	21.6±3.9	-
IL-6	-	17.7±4.6	*143.3±5.1*
IL-1	*60.9±2.2*	-	-

HGF activity in AML-CM was examined on factor responsive AML cells and on an IL-6 dependent murine plasmacytoma cell line. Conditioned medium (50%) was tested in the cultures. In control experiments the effects of recombinant HGFs were investigated. Values represent dpm x 10^{-2} (± SD). In italics significant stimulation is indicated.

become independent of HGFs.
These three groups of AML, i.e. 1) the IL-1 responding cases, 2) the IL-1 independent autocrine growers and 3) cases that proliferate independent of HGFs could be of importance in the diagnosis and prognosis of AML since these distinct groups of leukemia may have a different in vivo biology as well. In particular the latter two groups of leukemias may behave differently in vivo since their growth may not require any exogenous regulator. Finally it may be possible to make use of these specific growth characteristics of AML cells for monitoring residual AML cells in remission bone marrow in combination with cytogenetics since these patterns of growth may be rare in normal marrow.

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REFERENCES

1. Delwel R., Salem M., Pellens C. et al. Blood 1988, 72:1944.
2. Delwel R., Gurp R. van, Bot F. et al. Leukemia 1988, 2:814.
3. Griffin J.D. and Lowenberg B. Blood 1986, 68:1185.
4. Delwel R., Buitenen C. van, Salem M. et al. Blood 1989, 74:586.
5. Young D.C., Wagner K. and Griffin J.D. J Clin Invest 1987, 79:100.
6. Young D.C., Demetri G.D., Ernst T.J. et al. Exp Hematol 1988, 16:378.
7. Oster W., Cicco N.A., Klein H. et al. J Clin Invest 1989, 84: 451.
8. Delwel R., Buitenen C. van, Salem M. et al. Submitted.
9. Boyum A. Scan J Clin Lab Invest 1968, 21:77.
10. Lowenberg B., Hagemeyer A. and Swart K. Blood 1982, 59:641.
11. Schaeffer U.W., Dicke K.A. and Bekkum D.W. van. Rev Eur Etud Clin Biol 1972, 17:443.
12. Salem M, Delwel R, Touw I.P. et al. Leuk Res 1988, 12:157.
13. Chomczynski P. and Sacchi N. Anal Biochem 1987, 162:156.
14. Maniatis T., Fritsch E. and Sambrook J. Cold Spring Harbor Laboratory publication, 1982.
15. Benham F.J., Hodgkinson S. and Davies K.E. EMBO J 1984, 3:2635.

CHAPTER 11

General Discussion

11.1 *Heterogeneity of growth factor responses*

In vitro studies presented in this thesis show that AML blasts have usually retained responsiveness to hematopoietic growth factors (HGFs) (Table 1). Comparison of the growth factor responses among a large number of cases reveals a considerable variability. The most important stimulators of AML proliferation are IL-3, GM-CSF and G-CSF (Chapters 4 and 5). M-CSF stimulates AML growth in about 20% of the cases only (Chapter 5). TNF often costimulates AML proliferation with IL-3 and GM-CSF, whereas it inhibits G-CSF induced proliferation of AML cells (Chapter 7). IL-1 induces autocrine growth in approximately 40% of AML cases (Chapters 8 and 9). Furthermore, analysis of growth regulation in more than hundred cases of AML disclosed that infrequently AML growth may be stimulated by other cytokines, e.g. erythropoietin (Epo), IL-5, IL-6, IL-2 or IL-7 (data not presented). These results indicate that regulation of AML growth is complex and may involve a multitude of cytokines.

TABLE 1. Mechanisms of AML growth control.

		Chapters	Ref.
- Stimulation by exogenous growth factors	IL-3	4,5,7,9	1,2
	GM-CSF	4,5,7,9	1-5
	G-CSF	4,5,7	1,4,6,7
	M-CSF	5	8
	IL-6		9
	TNF	7	10
- Stimulation by endogenous growth factors (autocrine growth)	IL-1 dependent	8,9,10	11-13
	IL-1 independent	10	14-17
- Factor independence		10	

Heterogeneity among AML cases has previously been established by morphologic and immunophenotypic criteria (18-20). Morphologic and immunologic differences have been explained as variations of maturation stages at which the leukemic cells have been arrested and form the basis for current morphological (FAB) and immunological classifications of leukemias (18-20). Similarly, phenotypes of AML-CFUs may vary as well among different AML cases. These dissimilarities indicate that AML precursors

represent distinct normal marrow precursor cells at different stages of maturation (Chapters 2 and 3). In analogy, the heterogeneity of growth factor responses of AML precursors may reflect HGF reactivities of normal marrow precursors at various differentiation stages. Thus, AML cells that respond to G-CSF, but which are less responsive to IL-3 or GM-CSF, may be classified as cases with relatively mature subsets of precursors. On the other hand, AML cells that respond to IL-3 and GM-CSF but not to G-CSF may be representative of more immature precursor cell subsets. Of particular interest are recent observations that AML cells from certain cases respond to the lymphoid growth factor IL-7 (21). Thus in those AML cases the cells respond to a lymphoid growth factor as well as to myeloid proliferation inducers. Cells in those instances frequently coexpress the stem cell antigen CD34, the myeloid markers CD13 and CD33 and the B-lymphoid marker CD19. These AML cases may therefore be considered as biphenotypic leukemias with progenitors that express myeloid and primitive B lymphoid differentiation features. Based upon these results we assume that leukemias may be classified on basis of growth factor responses. A growth factor response related classification could be of importance for the diagnosis of leukemias, because it is based on the biology of the proliferating cell subsets. The most important aspects of the role of growth factors in AML are discussed below.

11.2 *IL-3 and GM-CSF stimulation*

An interesting aspect of the regulation of AML proliferation is that the mitogenic responses to IL-3 and GM-CSF frequently coincide (Chapter 5). On the other hand, there is no correlation between the responses of AML cells to G-CSF and IL-3 (or GM-CSF). Binding studies with radiolabeled IL-3 and GM-CSF revealed that high affinity receptors for those ligands are allways coexpressed on AML cells (22). Studies with purified normal bone marrow cells (CD34 positive) show closely associated effects of IL-3 and GM-CSF as well (23,24). Both IL-3 and GM-CSF stimulate the proliferation of CFU-GEMM, BFU-E and CFU-Eo in colony culture. When IL-3 and GM-CSF are supplemented simultaneously to cultures of AML cells (Chapter 5) or normal bone marrow precursors (23,24), no additive effect is generally apparent as compared to cultures supplemented with IL-3 or GM-CSF alone. These results suggest that IL-3 and GM-CSF stimulate identical target cell populations in AML and normal bone marrow.

A possible explanation for the association between IL-3 and GM-CSF stimulation of proliferation has recently been unveiled. Binding studies with radioactive ligands suggest a common high affinity binding structure for IL-3 and GM-CSF on AML blast cells and on monocytes (25,26). IL-3 and GM-CSF may reciprocally inhibit the binding of the other ligand to these cells. Based on these experiments, two types of high affinity receptors have been postulated on AML cells and on monocytes, i.e. one that binds IL-3 as well as GM-CSF and another type that uniquely binds IL-3. In addition, AML cells and monocytes also express low affinity GM-CSF receptors with no affinity for IL-3. Presently, the structure of these receptors remains unknown. Recently, two cDNA clones have been isolated that code for two different receptor structures. One of these codes for a low affinity IL-3 receptor (27) and the second cDNA clone encodes a low affinity GM-CSF receptor (28). These receptors are specific for the corresponding ligand and show no crossbinding. It is possible that in analogy with IL-2 receptors (29) these low affinity receptors represent subunits that in association with other proteins in the cell membrane, e.g. receptor structures with intermediate affinity for the corresponding ligands, constitute high affinity IL-3 or GM-CSF receptors. Common IL-3/GM-CSF binding structures may share a subunit that has affinity (possibly intermediate) for both ligands. When combined with the unique low affinity IL-3 receptor a high affinity IL-3 receptor may be formed, whereas in association with the low affinity GM-CSF receptor this common binding site may form a high affinity GM-CSF receptor. Another explanation for IL-3/GM-CSF cross competition could be that GM-CSF and IL-3 bind to distinct high affinity receptors (each comprised of different unique subunits), which are situated in a complex structure on the membranes of AML cells and monocytes. Steric hindrance or a change in the structure of this receptor complex following ligand binding may inhibit the binding of the second ligand (26).

The fact that IL-3 and GM-CSF compete for a common structure may explain the closely related **in vitro** responses of the two HGFs (26). This may also suggest that these molecules activate the same intracellular signal pathways in those cells. The question which pathways are triggered following receptor activation by IL-3 or GM-CSF in human cells is presently under investigation. Murine IL-3 and GM-CSF receptors as well as intracellular substrates are phosphorylated on tyrosine following ligand binding (30-32). By analogy it is likely to assume that the high affinity human IL-3 and GM-CSF receptors have intrinsic tyrosine kinase activity or

are associated with tyrosine kinases. Analysis of substrate phosphorylation in AML cells or monocytes after IL-3 or GM-CSF activation could give further insight into the signal pathways that follow IL-3 or GM-CSF stimulation.

11.3 *G-CSF stimulation*

Following stimulation with G-CSF normal marrow granulocyte precursors (CFU-G) are activated and give rise to colonies containing mature neutrophils (23,33). G-CSF is a stimulator of AML proliferation as well (Chapters 4 and 5). However, generally AML cells do not mature towards neutrophils in reaction to G-CSF (Chapter 6). Since AML blasts express high affinity G-CSF receptors at their surface membrane (34) and proliferation can be activated by G-CSF, one may assume that a defect in the signal transduction pathway, that follows receptor activation, is responsible for the disturbed maturation of AML cells. Specific questions to be addressed in future investigations are: 1) Which intracellular pathways are activated in myeloid cells following G-CSF stimulation? 2) Is the inability of AML cells to mature towards functional end cells related to deficiencies of specific pathways? 3) Is the maturation blockade of AML cells the result of inappropriate expression or activation of certain gene (proto-oncogene) products?

Since it is almost impossible to obtain sufficient precursor cells from normal bone marrow for a comparative analysis, it will be of interest to examine the effects of G-CSF in well defined models. These models could include G-CSF responsive myeloid cell lines that proliferate in response to G-CSF without maturation (e.g. NFS 60 cells) versus cell lines that mature towards neutrophils following G-CSF exposure (e.g. WEHI3B D^+ cells) (35). Results obtained from those cell lines may provide important clues for the disturbed maturation processes in primary AML. For example, about 20% of the HGF dependent murine cell lines contain common sites of viral integrations, resulting in the expression of the evi-1 gene (34,35). Interestingly, the evi-1 gene is rearranged in the G-CSF responsive nonmaturing NFS-60 cells but not in the WEHI3B D^+ cells that are able to mature towards neutrophils following G-CSF stimulation. Possibly, the evi-1 nuclear protein blocks transcription of genes that are required for maturation (35,36). Experiments to pursue the role of this gene in the transformation of cells may resolve whether this gene is involved in leukemic transformation in subgroups of AML (35,36). Several studies have

revealed a role of the nuclear proteins c-myb and/or c-myc in the maturation blockade of murine hematopoietic cell lines and the human HL-60 cell line (37-41). Whether these genes play a role in transformation of primary AML cells is uncertain.

Another issue that has been considered is whether the disordered maturation of AML cells can be restored by the addition of other (artificial or physiological) compounds to G-CSF supplemented cultures of AML cells (42,43). In an attempt to overcome the maturation blockade in AML, we recently examined the effects of several compounds that are able to induce differentiation in myeloid cell lines. In these studies it became evident that AML cells from certain cases, when stimulated in vitro with G-CSF plus retinoic acid (RA), could mature along the granulocytic lineage (41,42). Thus the maturation blockade may not always be absolute. In those cases where maturation is restored to a certain extent, experiments should be conducted to determine second messengers that are essential for granulocytic maturation.

11.4 *TNF stimulation*

As shown in Chapter 7, TNF may enhance GM-CSF or IL-3 induced proliferation whereas it often inhibits G-CSF induced growth of AML cells in vitro. How does TNF modulate proliferation of AML cells? While TNF is an inducer of growth factor production, e.g. in endothelial cells, fibroblasts or lymphocytes (44-46), the data presented in Chapter 7 indicate that the costimulatory effects of TNF cannot be explained by the release of IL-3, GM-CSF or M-CSF. Although it is possible that TNF stimulates the release of unknown growth factors that subsequently activate AML proliferation synergistically with IL-3 or GM-CSF, recent data suggest other explanations for the costimulatory effects of TNF in AML. TNF is a regulator of growth factor receptor expression. GM-CSF and G-CSF receptors are eliminated from human neutrophils following exposure to TNF (47,48). On murine macrophages TNF downregulates the expression M-CSF receptors (49). Most probably this effect of TNF is the result of stimulation of protein kinase-C which may activate function and release of proteolytic enzymes (47). Those enzymes may subsequently cleave the receptors from the membranes. Recent studies in our laboratory have suggested that the inhibitory effect of TNF on G-CSF stimulated proliferation of AML cells may be the consequence of the selective elimination of G-CSF receptors from the plasma membranes as well (47). In contrast, the number of high affinity receptors for IL-3 and

GM-CSF increase significantly on AML cells following exposure to TNF (48), which may explain the costimulatory effects of TNF with IL-3 or GM-CSF.

Are the stimulatory effects of TNF on AML colony growth leukemia specific? Experiments performed in our laboratory with CD34 positive (FACS separated) normal marrow precursors revealed that at a concentration of 100 U/ml, which is suboptimal (see Chapter 7), TNF inhibits G-CSF stimulated colony formation (CFU-G), but enhances IL-3 or GM-CSF stimulated colony growth (CFU-Eo, BFU-E and CFU-G) (50). Thus it appears that TNF exerts similar effects on normal bone marrow precursor cells. Normal bone marrow derived colonies contain mature cells (23,24,33) which need HGFs to survive **in vitro** (51). Since TNF downregulates HGF receptors from mature cells, one may assume that in the presence of optimal concentrations of TNF throughout colony culture, the mature cells that are produced will lose their HGF receptors, become insensitive to the HGFs and die. In the colony cultures containing 100 U/ml TNF, the amount of this cytokine appears to be insufficient to generate the downmodulating effects on the mature colony cells at the end stage of the colony cultures. To test this possibility the same experiments with higher TNF concentrations (1000 U/ml) were performed. Indeed with 1000 U/ml TNF, IL-3 or GM-CSF stimulated colonies contained degenerated cells (50). Thus, although proliferation and colony formation were initially stimulated, the colony cells degenerated in a later phase of the culture. These data are in agreement with a model in which TNF has stimulatory effects on immature IL-3 or GM-CSF responding cells but on the contrary hastens cell death of mature cells through the elimination of HGF receptors. Interestingly, a concentration of 1000 U/ml TNF synergizes with IL-3 or GM-CSF in the generation of AML colonies and those colonies do not contain degenerated cells (Chapter 7). An explanation for this finding is that AML cells do not mature following IL-3 or GM-CSF stimulation and thus escape the downregulating effects of TNF during culture.

11.5 *IL-1 stimulation*

AML cells may produce HGFs, e.g. GM-CSF, G-CSF, M-CSF, IL-6, IL-1-beta and TNF-alpha (14,17). In Chapters 8, 9 and 10 it has been shown that the expression of HGF mRNA and the release of these cytokines may be controled by IL-1, an inducer of growth factor production (Figure 1). In situ hybridization experiments using a GM-CSF cDNA probe confirmed that the GM-CSF transcripts are present in IL-1 stimulated AML blast cells and not in admixed normal cells, such as lymphocytes or monocytes (data not shown).

The mechanism by which IL-1 elevates the expression of HGF mRNA in AML cells has not been elucidated. However, studies with other cell types have suggested that IL-1 may either act on the stability of HGF mRNA in the cytoplasm or activate transcription of mRNA (52-54).

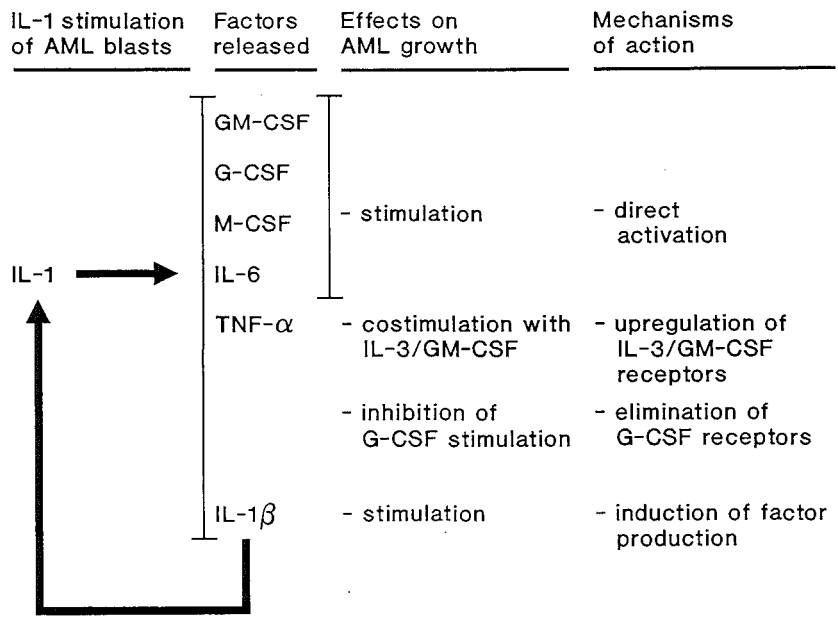

Figure 1: Autocrine growth control of AML. IL-1 stimulates the release of HGFs in AML blasts in 40% of the cases. The cells may either produce one or more HGFs. In certain cases AML blasts produce HGFs independent of exogenous IL-1.

One may ask the question whether IL-1 regulated HGF production and subsequent activation of growth of AML cells is a neoplastic feature or whether it represents a growth mechanism of normal marrow progenitor cells. CD34 expressing normal marrow cells have been collected by fluorescence activated cell sorting and cultured in the presence of IL-1. DNA synthesis experiments (thymidine uptake) revealed that those cells may respond (although weakly) to IL-1. This stimulation of normal marrow cells by IL-1 can be abrogated with neutralizing antibodies against GM-CSF and TNFα (55). IL-1 stimulated low numbers of colonies and clusters when $CD34^+$ normal marrow cells were placed in colony culture (unpublished observations).

These data suggest that $CD34^+$ normal marrow blasts that produce cytokines in response to IL-1 exist, but comprise a minor population among $CD34^+$ marrow cells. In fact, in situ hybridisation experiments using 35S labeled DNA probes of GM-CSF established that a subgroup of CD34 positive normal marrow blast cells indeed express GM-CSF mRNA following IL-1 stimulation (55).

11.6 *Spontaneous proliferation*

Proliferation of AML cells **in vitro** is often dependent on exogenous factors. However, in certain cases, AML blast cells proliferate **in vitro** independent of HGFs. Those spontaneously growing leukemias can be subdivided into two groups:

- AML cases that proliferate spontaneously as the result of autocrine stimulation. In those cases, AML cells may produce GM-CSF, G-CSF, M-CSF, IL-6, TNFα or IL-1β constitutively, i.e. not regulated by inducers.
- Leukemias that grow spontaneously without an apparent release of the known HGFs and subsequent activation of growth.

Whether constitutive factor production by AML cells is a neoplastic feature of the cells is uncertain and deserves further investigation. The second class of spontaneously growing AMLs is an interesting group as well. It is unlikely that in those cases other factors, yet to be identified, are produced and stimulate growth. Conditioned media, preirradiated cells or cytosolic fractions prepared from those AML samples could not stimulate the patients' own cells (data not shown). Thus in those cases AML cells may grow autonomously, independent of endogenous factors. Several mechanisms may be considered to explain factor independent growth:
1) overexpression of HGF receptors, 2) permanently activated receptors, 3) abnormal activation of intracellular (proto-oncogene) products that play a role in signal transduction. AML cells express normal numbers of IL-3, GM-CSF, G-CSF and M-CSF receptors (22,26,34,56), so that overexpression of HGF receptors cannot be the explanation for autonomous growth. Of interest, are recent studies showing that in some (2 out of 110) AML cases and patients with myelodysplastic syndrome (MDS) the M-CSF receptor gene has been mutated at codon 301 (57). Introduction of such mutated M-CSF receptors into HGF dependent cell lines renders them HGF independent and tumorigenic (58). A direct role of these mutated receptors in growth of primary AML cells has not been demonstrated. However, the possibility that in those cases mutated M-CSF receptors trigger AML proliferation

independent of their ligand, still needs verification. In murine cell lines second messenger abnormalities may play a role in neoplastic growth (Chapter 1). While AML cells in certain cases carry mutated RAS genes (59,60), a role of these genes in neoplastic growth of AML has not been established. Nevertheless, it is conceivable that defects in the receptor transduction pathways, are involved in spontaneous growth in certain cases of AML **in vitro**. Studies with HGF-dependent and independent AML cells could be of use to provide insight into abnormalities in post receptor events that may determine neoplastic growth in the latter group of AML cases.

11.7. *References*

1. Vellenga E, Ostapovicz D, O'Rourke B and Griffin JD. Effects of recombinant IL-3, GM-CSF, and G-CSF on proliferation of leukemic clonogenic cells in short-term and long-term cultures. Leukemia 1:584, 1987.
2. Miyauchi J, Kelleher CA, Yang Y-C, Wong GG, Clark SC, Minden MD, Minkin S and McCulloch EA. The effects of three recombinant growth factors, IL-3, GM-CSF, and G-CSF, on the blast cells of acute myeloblastic leukemia maintained in short-term suspension culture. Blood 70:657, 1987.
3. Kelleher C, Miyauchi J, Wong G, Clark S, Minden MD and McCulloch EA. Synergism between recombinant growth factors, GM-CSF and G-CSF, acting on the blast cells of acute myeloblastic leukemia. Blood 69:1498, 1987.
4. Vellenga E, Young DC, Wagner K, Wiper D, Ostapovicz D and Griffin JD. The effects of GM-CSF and G-CSF in promoting growth of clonogenic cells in acute myeloblastic leukemia. Blood 69:1771, 1987.
5. Hoang T, Nara N, Wong G, Clark S, Minden MD and McCulloch EA. Effects of recombinant GM-CSF on the blast cells of acute myeloblastic leukemia. Blood 68:313, 1986.
6. Nara N, Murohashi I, Suzuki T, Yamashita Y, Maruyama Y, Aoki N, Tanikawa S and Onozawa Y. Effects of recombinant human granulocyte colony-stimulating factor (G-CSF) on blast progenitors from acute myeloblastic leukaemia patients. Br J Cancer 56: 49, 1987.
7. Pébusque MJ, Fay C, Lafage M, Sempéré C, Saeland S, Caux C and Mannoni P. Recombinant human IL-3 and G-CSF act synergistically in stimulating the growth of acute myeloid leukemia cells. Leukemia 3:200, 1989.
8. Pébusque MJ, Lopez M, Torres H, Carotti A, Guilbert L and Mannoni P. Growth response of human myeloid leukemia cells to colony-stimulating factors. Exp Hematol 16:360, 1988.
9. Hoang T, Haman A, Goncalves O, Wong GG and Clark SC. Interleukin-6 enhances growth factor-dependent proliferation of the blast cells of acute myeloblastic leukemia. Blood 72:823, 1988.
10. Hoang T, Levy B, Onetto N, Haman A and Rodriguez-Cimadevilla JC. Tumor necrosis factor α stimulates the growth of the clonogenic cells of acute myeloblastic leukemia in synergy with granulocyte/macrophage colony-stimulating factor. J Exp Med 170:15, 1989.

11. Hoang T, Haman A, Goncalves O, Letendre F, Mathieu M, Wong GG and Clark SC. Interleukin 1 enhances growth factor-dependent proliferation of the clonogenic cells in acute myeloblastic leukemia and of normal human primitive hemopoietic precursors. J Exp Med 168:463, 1988.
12. Cozzolino F, Rubartelli A, Aldinucci D, Sitia R, Torcia M, Shaw A and Guglielmo RD. Interleukin 1 as an autocrine growth factor for acute myeloid leukemia cells. Proc Natl Acad Sci USA 86:2369, 1989.
13. Sakai K, Hattori T, Matsuoka M, Asou N, Yamamoto S, Sagawa K and Takatsuki K. Autocrine stimulation of interleukin 1β in acute myelogenous leukemia cells. J Exp Med 166:1597, 1987.
14. Young DC, Wagner K and Griffin JD. Constitutive expression of the GM-CSF gene in acute myeloblastic leukemia. J Clin Invest 79:100,1987.
15. Young DC, Demetri GD, Ernst TJ, Cannistra SA and Griffin JD. In vitro expression of colony-stimulating factor genes by human acute myeloblastic leukemia cells. Exp Hematol 16:378, 1988.
16. Oster W, Cicco NA, Klein H, Hirano T, Kishimoto T, Lindemann A, Mertelsmann RH and Herrmann F. Participation of the cytokines IL-6, TNF-alpha and IL-1-beta secreted by acute myelogeneous leukemia blasts in autocrine and paracrine leukemia growth control. J Clin Invest 84: 451, 1989.
17. Murohashi I, Tohda S, Suzuki T, Nagata K, Yamashita Y and Nara N. Autocrine growth mechanisms of the progenitors of blast cells in acute myeloblastic leukemia. Blood 74:35, 1989.
18. French-American-British (FAB) Cooperative Group: proposals for the classification of the acute leukemias. Br J Haematol 33:451, 1976.
19. French-American-British (FAB) Cooperative Group: proposed revised criteria for the classification of the acute myeloid leukemia. Ann Intern Med 103:620, 1985.
20. Dongen JJM van, Adriaansen HJ and Hooijkaas H. Immunophenotyping of leukaemias and non-Hodgkin's lymphomas. Immunologic markers and their CD codes. Neth J Med 33:298, 1988.
21. Touw I, Donath J, Pouwels K, Buitenen K van, Schipper P, Santini V, Hagemeijer A, Löwenberg B and Delwel R. Acute myeloid leukemias with the chromosomal translocation (8;21)(q22;q22) respond to interleukin-7 and express CD19. Submitted.
22. Budel LM, Touw IP, Delwel R, Clark S and Löwenberg B. IL-3 and GM-CSF receptors on human acute myelocytic leukemia cells and the relationship to the proliferative response. Blood 74:565, 1989.

23. Bot FJ, Dorssers L, Wagemaker G and Löwenberg B. Stimulating spectrum of human recombinant multi-CSF (IL-3) on human marrow precursors: importance of accessory cells. Blood 71:1609, 1988.
24. Bot FJ, Eijk L van, Schipper P and Löwenberg B. Human granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates immature marrow precursors but no CFU-GM, CFU-G, or CFU-M. Exp Hematol 17:292, 1989.
25. Elliot MJ, Vadas MA, Eglinton JM, Park LS, Bik To L, Cleland LG, Clark SC and Lopez AF. Recombinant human interleukin-3 and granulocyte-macrophage colony-stimulating factor show common biological effects and binding characteristics on human monocytes. Blood 74:2349, 1989.
26. Budel LM, Elbaz O, Hoogerbrugge H, Delwel R, Mahmoud LA, Löwenberg B and Touw IP. Common binding structure for granulocyte macrophage colony-stimulating factor and interleukin-3 on human acute myeloid leukemia cells and monocytes. Blood 75:1439, 1990.
27. Itoh N, Yonehara S, Schreurs J, Gorman DM, Maruyama K, Ishii A, Yahara I, Arai KI and Miyajima A. Cloning of an interleukin-3 receptor gene: A member of a distinct receptor gene family. Science 247:324, 1990.
28. Gearing DP, King JA, Gough NM and Nicola NA. Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor. EMBO J 8:3667, 1989.
29. Hatakeyama M, Tsudo M, Minamoto S, Kono T, Doi T, Miyata T, Miyasaka M and Taniguchi T. Interleukin-2 receptor β chain gene: Generation of the three receptor forms by cloned human α and β chain cDNA's. Science 244:551, 1989.
30. Morla AO, Schreurs J, Miyajima A and Wang JYJ. Hematopoietic growth factors activate the tyrosine phosphorylation of distinct sets of proteins in interleukin-3-dependent murine cell lines. Mol Cell Biol 8:2214, 1988.
31. Isfort RJ, Stevens D, May WS and Ihly JN. Interleukin-3 binds to a 140 kDa phosphotyrosine-containing cell surface protein. Proc Natl Acad Sci USA 85:7982, 1988.
32. Isfort RJ, Huhn RD, Frackelton AR and Ihly JN. Stimulation of factor-dependent myeloid cell lines with interleukin-3 induces tyrosine phosphorylation of several cellular substrates. J Biol Chem 263:19203, 1988.

33. Bot FJ, Eijk L van, Schipper P, Backx B and Löwenberg B. Synergistic effects between GM-CSF and G-CSF or M-CSF on highly enriched human marrow progenitor cells. Leukemia 4:325, 1990.
34. Budel LM, Touw IP, Delwel R and Löwenberg B. Granulocyte colony-stimulating factor receptors in human acute myelocytic leukemia. Blood 74:2668, 1989.
35. Morishita K, Parker DS, Mucenski ML, Jenkins NA, Copeland NG and Ihly JN. Retroviral activation of a novel gene encoding a zinc finger protein in IL-3 dependent myeloid leukemia cell lines. Cell 54:831, 1988.
36. Matsugi T, Morishita K and Ihle JN. Identification, nuclear localization, and DNA-binding activity of the zinc finger protein encoded by the evi-1 myeloid transforming gene. Mol Cell Biol 10:1259, 1990.
37. Dmitrovski E, Kuehl WM, Hollis GF, Kirsch IR, Bender TP and Segal S. Expression of a transfected human c-myc oncogene inhibits differentiation of a mouse erythroleukemia cell line. Nature 322:748, 1986.
38. Graf T, Weizsaecker F von, Grieser S, Coll J, Stehelin D, Patschinsky T, Bister K, Bechade C, Calothy G and Leutz A. v-mil induces autocrine growth and enhanced tumorigenicity in v-myc-transformed avian macrophages. Cell 45:357, 1986.
39. Clarke MF, Kukowska-Latallo JF, Westin E, Smith M and Prochownik EV. Constitutive expression of a c-myb cDNA blocks friend murine erythroleukemia cell differentiation. Mol Cell Biol 8:884, 1988.
40. Lüscher B, Christenson E, Litchfield DW, Krebs EG and Eisenman RN. Myb DNA binding inhibited by phosphorylation at a site deleted during oncogenic activation. Nature 344:517, 1990.
41. Holt JT, Redner RL and Nienhuis AW. An oligomer complementary to c-myc mRNA inhibits proliferation of HL-60 promyelocytic cells and induces differentiation. Mol Cell Biol 8:963, 1988.
42. Santini V, Colombat Ph, Delwel R, Gurp R van, Touw I and Löwenberg B. Induction of granulocytic maturation in acute myeloid leukemia by G-CSF and retinoic acid. Leuk Res, in press.
43. Colombat Ph, Santini V, Delwel R, Krefft J, Bremond JL and Löwenberg B. Primary human acute myeloblastic leukemia (AML): An analysis of the abilities for granulocytic maturation in vitro following stimulation with retinoic acid and G-CSF. Submitted.

44. Gough NM and Nicola NA. Granulocyte-macrophage colony-stimulating factor. In: Colony stimulating factors. Molecular and cellular biology. Eds: Dexter TM, Garland JM and Testa NG. 49:111, 1990.
45. Nicola NA. Granulocyte colony-stimulating factor. In: Colony-stimulating factors. Molecular and cellular biology. Eds: Dexter TM, Garland JM and Testa NG. 49:77, 1990.
46. Kawasaki ES and Ladner MB. Molecular biology of macrophage colony-stimulating factor. In: Colony-stimulating factors. Molecular and cellular biology. Eds: Dexter TM, Garland JM and Testa NG. 49:155, 1990.
47. Elbaz O, Budel LM, Hoogerbrugge H, Touw IP, Delwel R, Mahmoud LA and Löwenberg B. Tumor necrosis factor (TNF) downregulates G-CSF receptor expression on human acute myeloid leukemia (AML) cells and granulocytes. Submitted.
48. Elbaz O, Budel LM, Hoogerbrugge H, Touw IP, Delwel R, Mahmoud LA and Löwenberg B. Tumor necrosis factor (TNF) regulates the expression of GM-CSF and IL-3 receptors on human acute myeloid leukemia (AML) cells. Submitted.
49. Shieh JH, Peterson RHF, Warren DJ and Moore MAS. Modulation of colony-stimulating factor-1 receptors on macrophages by tumor necrosis factor. J Immunol 143:2534, 1989.
50. Backx B, Broeders L, Bot FJ and Löwenberg B. Positive and negative effects of tumor necrosis factor on colony growth from highly purified normal marrow progenitors. Submitted.
51. Lopez AF, Williamson DJ, Gamble JR, Begley CG, Harlan JM, Klebanoff SJ, Waltersdorph A, Wong G, Clark SC and Vadas MA. Recombinant human granulocyte-macrophage colony-stimulating factor stimulates in vitro human neutrophil and eosiniphil function, surface receptor expression, and survival. J Clin Invest 78:1220, 1986.
52. Walther Z, May LT and Sehgal PB. Transcriptional regulation of the interferon-β_2/B cell differentiation factor BSF-2/hepatocyte-stimulating factor gene in human fibroblasts by other cytokines. J Immunol 140:974, 1988.
53. Elias JA, Reynolds MM, Kotloff RM and Kern JA. Fibroblast interleukin-1β: Synergistic stimulation by recombinant interleukin-1 and tumor necrosis factor and post transcriptional regulation. Proc Natl Acad Sci USA 86:6171, 1989.

54. Nimer SD, Gates MJ, Koeffler HP and Gasson JC. Multiple mechanisms control the expression of granulocyte-macrophage colony-stimulating factor by human fibroblasts. J Immunol 143:2374, 1989.
55. Bot FJ, Schipper P, Broeders L, Delwel R, Kaushansky K and Löwenberg B. Interleukin-1α also induces granulocyte-macrophage colony stimulating factor in immature normal bone marrow cells. Blood 76:307, 1990.
56. Ashmun RA, Look T, Roberts WM, Roussel MF, Seremetis S, Ohtsuka M and Sherr CJ. Monoclonal antibodies to the human CSF-1 receptor (c-fms proto-oncogene product) detect epitopes on normal mononuclear phagocytes and on human myeloid leukemia blast cells. Blood 73:827, 1989.
57. Ridge SA, Worwood M, Oscier D, Jacobs A and Padua RA. FMS mutations in myelodysplastic, leukemic, and normal subjects. Proc Natl Acad Sci USA 87:1377, 1990.
58. Roussel MF, Downing JR, Rettenmier CW and Sherr CJ. A point mutation in the extracellular domain of the human CSF-1 receptor (c-fms proto-oncogene product) activates its transforming potential. Cell 55:979, 1988.
59. Bos JL, Toksoz D, Marshall CJ, Verlaan-de Vries M, Veeneman GH, Eb AJ van der, Boom JH van, Janssen JWG and Steenvoorden ACM. Amino-acid substitutions at codon 13 of the N-ras oncogene in human acute myeloid leukaemia. Nature 315:726, 1985.
60. Bos JL, Verlaan-de Vries M, Eb AJ van der, Janssen JWG, Delwel R, Löwenberg B and Colly LP. Mutations in N-ras predominate in acute myeloid leukemia. Blood 69:1237, 1987.

Summary - Samenvatting

SUMMARY

Blood cell formation takes place primarily in the bone marrow. Multipotential bone marrow stem cells are able to undergo selfrenewal or produce progenitor cells which are predestinated to mature towards certain functional blood cells, e.g. erythrocytes, granulocytes, monocytes, platelets or lymphocytes. The process of blood cell production is regulated by hematopoietic growth factors (HGFs). In the past two decades culture systems have been developed, which enabled the study of the role of HGFs in the proliferation and maturation of bone marrow progenitor cells **in vitro.** When appropriately stimulated the different progenitor cells give rise to colonies containing mature cells. In acute myeloid leukemia (AML), myelomonocytic cells are arrested at an early stage of maturation (blasts) and accumulate in the marrow and in the blood. AML blasts may be cultured **in vitro** as well. AML colony forming cells (AML-CFU) give rise to colonies containing leukemic blast cells.

Normal bone marrow colony forming cells can be identified among other hematopoietic cells using monoclonal antibodies directed towards membrane antigens and with immunologic cell separation procedures. Using the same techniques, AML-CFUs can also be recognized as distinct subpopulations of cells with membrane surface characteristics different from those of nonclonogenic AML cells. One of the objectives of the investigations presented in this thesis was to search for differences in surface marker expression between AML-CFU and normal bone marrow progenitor cells. Such dissimilarities could be of use in the diagnosis of AML, in particular for the detection of minimal numbers of AML progenitor cells in remission marrow (following chemotherapy or bone marrow transplantation).

In the original cultures a variety of crude sources of growth factors were supplemented, e.g. placenta conditioned medium, cell line conditioned medium, leukocyte conditioned medium or feeder leukocytes. However, as these sources of growth factors are impure and the concentrations of the HGFs variable and unkwown a critical analysis of the role of the individual HGFs in the proliferation and maturation of AML progenitor cells was not possible. During the last five years the genes and the complementary DNAs (cDNAs) of several HGFs have been identified and cloned and the activities of these molecules on normal marrow cells determined. In the studies presented here the role of the distinct HGFs in the proliferation and maturation of precursor cells of human AML **in vitro** has been investigated.

In Chapter 1 the general principles of blood cell formation, the heterogeneity and the **in vitro** growth characteristics of AML, the distinct hematopoietic growth factors (HGFs) and their functions, and the current understanding of neoplastic growth of experimental leukemias (in animals) are introduced.

The reactivity of AML cells with a fucose binding lectin, Ulex europaeus agglutinin coupled to fluorescein-isothiocyanate (UEA-FITC) was studied in 13 cases of AML on a flowcytometer (fluorescence activated cell sorter; FACS) (Chapter 2). UEA binding to AML-CFU was determined by cell sorting and subsequent colony culture of UEA-negative, intermediately positive and highly fluorescent cells. AML-CFU from the four FAB-M1 cases, the one FAB-M5 case and one FAB-M2 case were all UEA negative. However, AML-CFU in one of the four FAB-M2 cases and all four FAB-M4 cases, showed high UEA binding. In contrast, normal bone marrow colony forming cells, i.e. granulocyte-erythrocyte-macrophage-megakaryocyte-colony forming cell (CFU-GEMM) and granulocyte-macrophage-CFU (CFU-GM) were generally UEA negative or weakly positive. Thus, in certain cases, following UEA-FITC labeling AML-CFU and normal hematopoietic progenitors can be distinguished.

In an extension of these experiments (Chapter 3), AML cells were labeled with the monoclonal antibodies (MoAbs) CD34 or Vim-2 (CD_w65), sorted by FACS into negative, weakly and strongly positive cells and subsequently cultured in colony assays. The majority of normal bone marrow progenitor cells, i.e. CFU-GEMM, CFU-GM and erythrocyte-burst forming cell (BFU-e) expressed an identical phenotype: CD34 strongly positive and Vim-2 negative or Vim-2 weakly positive ($CD34^{++}/Vim\text{-}2^{-/+}$). On the contrary, the majority of AML-CFU expressed different immunophenotypes in 12 of 20 AML cases. In 8 cases a difference was apparent for one marker ($CD34^{-/+}$ or $Vim\text{-}2^{++}$) and in 4 cases the immunophenotypes differed for both markers ($CD34^{-/+}$ and $Vim\text{-}2^{++}$). To verify the utility of these discrepancies for the detection of AML cells in remission bone marrow, artificial mixtures of normal bone marrow cells and minimal numbers of AML cells (0.1-1%) were prepared. Based on the discrepant phenotypes AML cells were sorted and cultured and AML metaphases could be traced in those mixtures.

In Chapter 4 it is investigated in 7 cases whether the three human recombinant HGFs, interleukin-3 (IL-3), granulocyte macrophage-colony stimulating factor (GM-CSF) and granulocyte-CSF (G-CSF) could substitute the feeder leukocytes of the PHA leukocyte feeder (PHA l.f.) assay and stimulate proliferation of AML-CFU. IL-3 (or multi-CSF) stimulated AML-CFU in 4 of the 7 cases and in the same cases GM-CSF was an efficient growth

factor as well. In 2 other cases IL-3 stimulated AML cell growth only weakly. In those cases G-CSF and in 1 case GM-CSF was an efficient inducer of proliferation. In the single remaining case none of the factors were able to induce growth **in vitro**, whereas the cells of this particular patient were effectively stimulated in the PHA l.f. assay. In 5 cases the PHA l.f. was more active in supporting AML proliferation than the combination of three factors. This suggests that feeder leukocytes produce at least one other cytokine that stimulates proliferation of AML cells. IL-3 nor GM-CSF were able to induce maturation of AML blast cells **in vitro**. However, in 1 of the 3 G-CSF responding cases AML cells matured towards (meta)myelocytes and granulocytes when stimulated with G-CSF. The results indicate that the responsiveness of AML precursors to IL-3, GM-CSF and G-CSF in different cases is variable. This may indicate that AML precursor cells represent normal marrow progenitor cells at different stages of maturation with distinct HGF requirements.

In Chapter 5 the effects of five hematopoietic growth factors, IL-3, GM-CSF, G-CSF, macrophage-CSF (M-CSF) and erythropoietin (Epo) on AML proliferation were investigated in a serum-free culture system. The effects of single and combined factors were studied in 25 cases of AML in suspension-(^{3}H-thymidine incorporation) and colony culture. DNA synthesis experiments revealed that IL-3, GM-CSF and G-CSF stimulated AML growth frequently, i.e. in 19, 15 and 13 cases respectively. M-CSF induced DNA synthesis in 4 cases and Epo only activated thymidine uptake of the cells from an erythroleukemia. Remarkably, the stimulation of DNA synthesis by IL-3 and GM-CSF frequently coincided. Furthermore, when GM-CSF was added to cultures containing IL-3 it did not further increase DNA synthesis that was evoked by IL-3 alone. Most likely, IL-3 and GM-CSF stimulate identical cell populations, sothat stimulation by one factor gives optimal proliferation. On the contrary, in certain cases G-CSF enhanced the IL-3 or GM-CSF stimulated thymidine uptake. It is possible that in those cases G-CSF stimulates a different population of AML cells than does IL-3 or GM-CSF. The DNA synthesis assay appeared more sensitive for determining growth factor responses of AML cells than were colony cultures. Based on these results, we assume that cell-cell contact, which is facilitated in suspension culture, is important for optimal proliferation of AML cells.

The ability of the five HGFs IL-3, GM-CSF, G-CSF, M-CSF and Epo to induce maturation of AML cells **in vitro** was investigated in the study presented in Chapter 6. AML cells from the same 25 cases were cultered serum free in suspension for seven days without factors or with single or

combined HGFs. Some degree of monocytic maturation was observed in about 50% of the cases in the absence of factors. The number of monocyte/macrophages further increased following stimulation with IL-3 (3 cases), GM-CSF (2 cases), G-CSF (1 case) and M-CSF (6 cases). The combination of factors increased the number of mature monocytic cells in 9 cases even further. Neutrophils appeared in culture in only two cases in the absence of HGFs. An additional increase was seen in 2 cases only, i.e. following exposure to IL-3 (2 cases), GM-CSF (1 of those 2 cases) and G-CSF (the other case). Although AML cells may proliferate in response to IL-3, GM-CSF or G-CSF, the ability of the cells to mature towards neutrophils is disturbed and can generally not be restored by these HGFs.

In Chapter 7 the role of tumor necrosis factor (TNF) α and β on AML growth was investigated. The effects of these cytokines were studied in 11 cases when the cells were stimulated by IL-3, GM-CSF, G-CSF or M-CSF in a serum free culture system. TNF inhibited the G-CSF stimulated DNA synthesis and colony or cluster formation. In contrast, TNF elevated ^{3}H-TdR incorporation and colony or cluster numbers induced by IL-3 or GM-CSF. Since TNF is a stimulator of HGF production in other cell types (e.g. endothelial cells or fibroblasts) it was investigated whether the costimulatory effect of TNF was indirect. Specific radioimmunoassays for individual HGFs did not demonstrate detectable amounts of GM-CSF, G-CSF or M-CSF in culture supernatants of TNF stimulated AML cells. Blocking of AML growth with anti-HGF neutralizing antibodies neither indicated an autocrine mechanism of TNF/IL-3 or TNF/GM-CSF costimulated AML cells.

Tumor necrosis factor (TNF) and interleukin-1 (IL-1) are the two most important regulators of HGF production in different hematopoietic and non-hematopoietic cells. In Chapter 8, the role of IL-1 in AML cell proliferation was investigated. In 13 of 28 AML cases IL-1 stimulated DNA synthesis. In a selected number of cases it was shown that IL-1 also stimulated AML colony or cluster formation. Experiments performed to investigate by which mechanism IL-1 activated the growth of AML cells, demonstrated that IL-1 stimulated AML growth indirectly, i.e. through the induction of GM-CSF release. This conclusion is based on the findings that: 1) IL-1 induced AML growth could be inhibited by anti GM-CSF neutralizing antibodies, 2) conditioned media (CM) from IL-1 stimulated AML cells contained factors that could stimulate normal bone marrow precursors, whereas CM from nonstimulated AML blasts could not, and 3) GM-CSF was demonstrated in IL-1/AML-CM with a specific radioimmunosassay. Detectable levels of GM-CSF were not apparent in control AML-CM. Thus IL-1 can

activate an autocrine growth mechanism in human AML involving GM-CSF as the HGF.

From the experiments of Chapter 9 it appears that the role of IL-1 is even more complex. When optimal concentrations of IL-3 or GM-CSF were added to AML cultures, IL-1 further elevated colony and cluster numbers in 8 of 12 cases studied. This costimulating effect of IL-1 was mediated by endogenous TNF-α released following IL-1 stimulation. Anti TNF-α abolished the increase of colony/cluster numbers in IL-1/IL-3 or IL-1/GM-CSF stimulated AML cultures. Furthermore, with a TNFα specific radioimmunoassay it was demonstrated that IL-1 stimulated AML blasts released TNFα into the culture medium. We had already shown (Chapter 7) that TNF synergized with IL-3 or GM-CSF. Thus the costimulating effects of IL-1 on AML-CFU were not the result of direct activation of growth, but were caused by TNFα released by IL-1 activated AML blasts.

Previous studies had already suggested that AML cells produce their own HGFs constitutively, i.e. nonregulated. From the experiments of Chapters 8 and 9 it has become evident that HGF production by AML cells can be induced by IL-1. In Chapter 10 sponaneous growth was compared with IL-1 regulated growth of AML blasts. This was done to examine whether spontaneous growth was also dependent on endogenously released HGFs. Anti-HGF blocking studies and Northern blot analysis revealed that IL-1 stimulated AML cells may produce GM-CSF, G-CSF, M-CSF, IL-6 or IL-1β (n=6 cases), and that these factors subsequently may activate proliferation of those cells. The cocktail of neutralizing antibodies completely abolished the IL-1 induced proliferation of AML blasts of those 6 patients. On the other hand, in 9 of 11 spontaneous growers this nonregulated proliferation could not be inhibited completely with the cocktail of anti(a)GM-CSF, aG-CSF, aM-CSF, aIL-1, aIL-3, aIL-6 and aTNF antibodies, although some suppressive effect was evident in most cases. In 2 of those latter 9 cases neutralizing antibodies did not affect spontaneous proliferation at all. Northern blot analysis in four of those spontaneous growers did not reveal detectable levels of HGF mRNAs. Conditioned media prepared from AML blasts of the latter cases did not show HGF activity when added to factor dependent cells **in vitro**. These data are consistent with the hypothesis that in these particular AML cases the cells proliferate spontaneously independent of endogenous HGFs.

Several aspects of growth activation of AML cells **in vitro** have been discussed in Chapter 11. 1) It is suggested that the variable HGF responses may be of use in the diagnosis of AML, i.e. for the classification of AML. 2) The observation that IL-3 and GM-CSF stimulation of AML cells frequently

coincide is discussed in view of recent findings that IL-3 and GM-CSF share a common receptor complex. 3) G-CSF stimulation of AML cells is discussed with respect to the ability of G-CSF to activate proliferation of AML cells without inducing maturation towards neutrophils. 4) Mechanisms are suggested that may explain the stimulatory and inhibitory effects of TNF on HGF induced proliferation of AML cells. The stimulatory effects of TNF on IL-3 and GM-CSF induced proliferation may be explained by the recent observations that the number of high affinity IL-3 and GM-CSF receptors on the membranes of AML cells are increased following TNF stimulation. In contrast, the inhibitory effects of TNF on G-CSF induced growth may be attributed to the elimination of G-CSF receptors from the AML cell surface following exposure to TNF. 5) As regards autocrine growth, spontaneous or induced by IL-1, the question is raised whether normal marrow precursors may produce their own HGFs as well or whether these features are leukemia specific. 6) The factor independent growth of AML cells, observed in certain cases, is considered in the light of mechanisms of abnormal growth that have been described for experimental leukemias in animals.

Bloedcelvorming vindt voornamelijk in het beenmerg plaats. Multipotentiële beenmerg stamcellen kunnen zichzelf vermeerderen (zelfreplicatie) of voorlopercellen produceren die voorbestemd zijn om uit te rijpen tot bepaalde functionele bloedcellen, bijvoorbeeld erythrocyten, granulocyten, monocyten, bloedplaatjes of lymphocyten. Het proces van bloedcelvorming wordt gereguleerd door hematopoietische groeifactoren (HGFs). In de afgelopen twee decaden zijn kweeksystemen ontwikkeld, die het mogelijk hebben gemaakt om de rol van HGFs in de proliferatie en maturatie van beenmerg voorlopercellen **in vitro** te bestuderen. Wanneer de verschillende voorlopercellen op de juiste wijzen worden gestimuleerd kunnen ze kolonies van uitgerijpte cellen vormen. In acute myeloïde leukemie (AML) zijn myelo/monocytaire cellen gearresteerd in een vroeg stadium van de maturatie (blasten) en hopen zich op in het beenmerg en in het bloed. AML blasten kunnen ook **in vitro** gekweekt worden. AML kolonie-vormende cellen (AML-CFU) produceren kolonies met leukemische blasten.

Normaal beenmerg kolonie-vormende cellen kunnen worden geïdentificeerd tussen de andere hematopoïetische cellen met behulp van monoclonale antilichamen die gericht zijn tegen membraan antigenen en met immunologische celscheidingstechnieken. Met behulp van dezelfde technieken kunnen AML-CFUs ook worden herkend als aparte subpopulaties van cellen met membraan oppervlakte kenmerken die verschillen met die van niet-klonogene AML cellen. Eén van de doelen van de onderzoekingen gepresenteerd in deze thesis was te zoeken naar verschillen tussen merkerexpressie op het celoppervlak van AML-CFUs en normale beenmerg voorlopercellen. Zulke verschillen zouden gebruikt kunnen worden in de diagnose van AML, in het bijzonder voor de detectie van minimale aantallen AML voorlopercellen in remissie beenmerg (na chemotherapie of beenmergtransplantatie).

In de oorspronkelijke kweken werden verschillende groeifactoren uit ruwe bronnen toegevoegd, bijvoorbeeld placenta geconditioneerd medium, cellijn geconditioneerd medium, leukocyten geconditioneerd medium of leukocyten "feeders". Echter, omdat deze groeifactor bronnen onzuiver waren en concentraties van de HGFs variabel en onbekend, was het niet mogelijk om de rol van de individuele HGFs in de proliferatie en maturatie van AML cellen kritisch te analyseren. Gedurende de afgelopen vijf jaar zijn de genen en de complementaire DNAs (cDNAs) van verscheidene HGFs geïdentificeerd en gekloneerd en de aktiviteiten van deze moleculen op normaal beenmerg voorloper cellen bepaald. In de hier gepresenteerde

studies werd de rol van de verschillende HGFs in de proliferatie en maturatie van voorlopercellen van menselijke AML **in vitro** bestudeerd.

In Hoofdstuk 1 worden de algemene principes van de bloed cel vorming, de heterogeniteit en de **in vitro** groei karakteristieken van AML, de verschillende hematopoiëtische groeifactoren (HGFs) en hun functies, en het huidige inzicht in neoplastische groei van experimentele leukemieën (in dieren) geïntroduceerd.

De reactiviteit van AML cellen met een fucose bindend lectine, Ulex europaeus agglutinine gekoppeld aan fluoresceine-isothiocyanaat (UEA-FITC) werd bestudeerd in 13 gevallen met AML op een flowcytometer (fluorescentie geactiveerde cel sorteerder; FACS) (Hoofdstuk 2). UEA binding aan AML-CFUs werd bepaald door middel van celsortering gevolgd door een koloniekweek van UEA-negatieve, intermediair positieve en sterk fluorescerende cellen. AML-CFUs van de vier FAB-M1 gevallen, het ene FAB-M5 en één FAB-M2 geval, waren allemaal UEA-negatief. Echter, AML-CFUs in één van de vier FAB-M2 en alle vier FAB-M4 gevallen, toonden een sterke UEA binding. Daarentegen waren normale beenmerg voorlopercellen (de granulocyt/erythrocyt/macrofaag/ megakaryocyt kolonie-vormende [CFU-GEMM] en granulocyt/macrofaag-CFU [CFU-GM]) doorgaans UEA-negatief of zwak positief. Dus, in sommige gevallen kunnen AML-CFUs en normale hematopoietische voorlopercellen van elkaar worden onderscheiden met behulp van UEA-FITC labeling.

In een uitbreiding van deze experimenten (Hoofdstuk 3), werden AML cellen gemerkt met monoclonale antilichamen (MoAbs), CD34 of Vim-2 (CD_w65), gesorteerd met behulp van een FACS in negatieve, zwak en sterk positieve cellen en vervolgens gekweekt in koloniekweken. De meerderheid van normale beenmerg voorlopercellen, de CFU-GEMM, de CFU-GM, en de erythrocyt-"burst" vormende cel (BFU-e) hadden een identiek phenotype: CD34 sterk-positief en Vim-2 negatief of zwak-positief ($CD34^{++}/Vim\text{-}2^{-/+}$). Echter, in 12 van de 20 AML gevallen, had de meerderheid van de AML-CFUs een ander immunophenotype. In 8 gevallen was er een verschil in de expressie van één merker ($CD34^{-/+}$ of $Vim\text{-}2^{++}$) en in 4 patiënten verschilde het immunophenotype voor beide merkers ($CD34^{+}$ en $Vim\text{-}2^{++}$). Om de bruikbaarheid van deze verschillen tussen AML-CFUs en normale beenmerg voorlopers voor de detectie van AML cellen in remissie beenmergen te verifiëren, werden artificiele mengsels van normale beenmergcellen en minimale aantallen AML cellen (0,1%-1%) bereid. Gebaseerd op discrepante phenotypen werden AML cellen gesorteerd en gekweekt en konden AML metaphasen opgespoord worden in deze mengsels.

In Hoofdstuk 4 werd in 7 gevallen onderzocht of de drie menselijke recombinant HGFs, interleukine-3 (IL-3), granulocyt macrofaag kolonie-stimulerende factor (GM-CSF) en granulocyt-CSF (G-CSF), de "feeder" leukocyten konden vervangen in de PHA leukocyten "feeder" (PHA l.f.) test en de proliferatie van AML-CFUs stimuleren. IL-3 (of multi-CSF) stimuleerde AML-CFUs in 4 van de 7 gevallen en in dezelfde gevallen was GM-CSF ook een efficiënte groeifactor. In 2 andere gevallen stimuleerde IL-3 de AML groei zwak. In deze twee gevallen bevorderde G-CSF en in één ander geval stimuleerde GM-CSF de proliferatie. In de ene overgebleven patiënt kon geen van de factoren de groei **in vitro** stimuleren, terwijl de cellen van deze bijzondere patiënt effectief gestimuleerd konden worden in de PHA l.f. test. In 5 gevallen werd de proliferatie van AML cellen effectiever gestimuleerd in de PHA l.f. test dan in kweken met de combinatie van drie factoren. Dit suggereert dat "feeder" leukocyten ten minste één andere cytokine produceren die de groei van AML cellen stimuleert. IL-3 noch GM-CSF waren in staat om maturatie van AML cellen **in vitro** te induceren. Echter, in één van de drie op G-CSF reagerende leukemieën rijpten de cellen uit tot (meta)myelocyten en granulocyten wanneer ze gestimuleerd werden door G-CSF. De resultaten tonen aan dat de respons van AML voorloper cellen op IL-3, GM-CSF en G-CSF in de verschillende gevallen variabel is. Dit zou kunnen betekenen dat AML voorloper cellen normale beenmerg voorlopers van verschillende differentiatie stadia en met verschillende HGF behoeften representeren.

In Hoofdstuk 5 werden de effecten van 5 hematopoïetische groeifactoren, IL-3, GM-CSF, G-CSF, macrofaag-CSF (M-CSF) en erythropoïetine (Epo) op AML proliferatie in een serum-vrij kweeksysteem onderzocht. De effecten van afzonderlijke en gecombineerde factoren werd in 25 gevallen van AML bestudeerd en wel in suspensie-(^{3}H-thymidine incorporatie) en koloniekweek. DNA synthese experimenten toonden aan dat IL-3, GM-CSF en G-CSF de groei van AML frequent stimuleerden, namelijk in respectievelijk 19, 15 en 13 gevallen. M-CSF induceerde de DNA synthese in 4 gevallen, en Epo stimuleerde de thymidine inbouw alleen in de cellen van een erythroleukemie. Het was opvallend dat de stimulatie van de DNA synthese door IL-3 en GM-CSF vaak samenvielen. Wanneer GM-CSF werd toegevoegd aan kweken met IL-3, verhoogde dit niet de DNA synthese die werd bereikt door stimulatie met IL-3 alleen. Waarschijnlijk stimuleren IL-3 en GM-CSF identieke celpopulaties, zodat stimulatie door één factor al optimale proliferatie bewerkstelligt. Daarentegen kan G-CSF in sommige gevallen de door IL-3 of GM-CSF gestimuleerde thymidine inbouw wel verder

doen toenemen. Mogelijk stimuleert G-CSF in deze gevallen een andere populatie van AML cellen dan IL-3 of GM-CSF. De DNA synthese test bleek gevoeliger voor de bepaling van groeifactor gevoeligheid van AML cellen dan de koloniekweektechniek. Gebaseerd op deze resultaten, wordt aangenomen dat cel-cel contacten, welke worden vergemakkelijkt in suspensiekweken, belangrijk zijn voor optimale proliferatie van AML cellen.

Het vermogen van de vijf HGFs, IL-3, GM-CSF, G-CSF, M-CSF en Epo om maturatie van AML cellen **in vitro** te induceren, werd onderzocht in de studie beschreven in Hoofdstuk 6. AML cellen van 25 gevallen werden voor zeven dagen zonder factoren of met enkelvoudige of gecombineerde HGFs serum-vrij in suspensie gekweekt. Enige mate van monocytaire uitrijping werd waargenomen in ongeveer 50% van de gevallen in afwezigheid van factoren. Het aantal monocyt/macrofagen nam verder toe na stimulatie met IL-3 (3 gevallen), GM-CSF (2 gevallen), G-CSF (1 geval) en M-CSF (6 gevallen). De combinatie van factoren verhoogde het aantal rijpe monocytaire cellen in 9 gevallen. Neutrofielen verschenen in twee gevallen in afwezigheid van factoren. Een verdere toename werd maar in twee gevallen waargenomen, namelijk na blootstelling aan IL-3 (2 gevallen), GM-CSF (1 van deze 2 gevallen) en G-CSF (het andere geval). Alhoewel AML cellen kunnen prolifereren in respons op IL-3, GM-CSF of G-CSF, is het vermogen van de cellen om uit te rijpen tot neutrofielen kennelijk gestoord en kan doorgaans niet door deze HGFs worden hersteld.

In Hoofdstuk 7 werd de rol van tumor necrosis factor (TNF) α en β op AML groei onderzocht. De effecten van deze cytokinen werden bestudeerd in 11 gevallen waarbij de cellen werden gestimuleerd met IL-3, GM-CSF, G-CSF of M-CSF in een serum-vrij kweeksysteem. TNF remde de G-CSF gestimuleerde DNA synthese en kolonie of kluster vorming. Daarentegen verhoogde TNF de IL-3 of GM-CSF geïnduceerde ^{3}H-TdR incorporatie en kolonie of kluster aantallen. Omdat TNF een stimulator van groeifactor productie is in bepaalde celtypen (bijv. endotheelcellen of fibroblasten) werd onderzocht of het costimulerende effect van TNF indirect was. Specifieke radioimmuun testen voor individuele HGFs toonden geen detecteerbare hoeveelheden GM-CSF, G-CSF of M-CSF aan in kweek supernatanten van AML cellen na stimulatie met TNF. In experimenten waarbij werd onderzocht of the groei van AML met anti-HGF neutralizerende antilichamen kon worden geremd, werd geen aanwijzing gevonden dat onder invloed van TNF groeifactoren uit de AML cellen vrijkwamen die vervolgens de proliferatie stimuleerden. Een autocrien mechanisme in TNF/IL-3 of TNF/GM-CSF gestimuleerde AML cellen is dus onwaarschijnlijk.

Tumor necrosis factor (TNF) en interleukine-1 (IL-1) zijn de twee belangrijkste regulatoren van HGF productie in verschillende hematopoietische en niet hematopoïetische cellen. In Hoofdstuk 8 werd de rol van IL-1 bij de AML proliferatie onderzocht. In 13 van de 28 AML gevallen stimuleerde IL-1 de DNA synthese. In een geselecteerd aantal gevallen werd aangetoond dat IL-1 ook AML kolonie of kluster vorming stimuleerde. Experimenten uitgevoerd om te verklaren door welk mechanisme IL-1 de groei van AML cellen activeerde, toonden aan dat IL-1 de groei van AML cellen indirect stimuleerde, namelijk door de inductie van GM-CSF produktie. Deze conclusie is gebaseerd op de bevindingen dat: 1) IL-1 gestimuleerde groei kon worden geremd door anti GM-CSF neutralizerende antilichamen, 2) geconditioneerde media (CM) van IL-1 gestimuleerde AML cellen factoren bevatten die normale beenmerg voorloper cellen konden stimuleren, terwijl CMs van niet-gestimuleerde AML cellen dat niet konden, en 3) GM-CSF werd aangetoond in IL-1/AML-CM met behulp van een specifieke radioimmuun test. Detecteerbare hoeveelheden GM-CSF waren niet aanwezig in controle AML-CMs. Dus IL-1 kan (autocriene) groei in menselijke AML cellen stimuleren, waarbij GM-CSF de betrokken HGF is.

Uit de experimenten van Hoofdstuk 9 blijkt dat de rol van IL-1 nog complexer is. Wanneer optimale hoeveelheden IL-3 of GM-CSF werden toegevoegd aan AML kweken, verhoogde IL-1 de kolonie of kluster aantallen in 8 van de 12 bestudeerde gevallen. Dit costimulerende effect van IL-1 werd bewerkstelligd door endogeen TNFα, dat werd geproduceerd als gevolg van de IL-1 stimulatie. De verhoging van de kolonie/kluster aantallen in IL-1/IL-3 of IL-1/GM-CSF gestimuleerde AML kweken werd door anti-TNFα teniet gedaan. Verder werd met een TNFα specifieke radioimmuun test aangetoond dat IL-1 gestimuleerde AML blasten TNFα uitscheidden in het kweekmedium. We hadden al reeds aangetoond (Hoofdstuk 7) dat TNF synergistisch werkt met IL-3 of GM-CSF. Dus de costimulerende effecten van IL-1 op AML-CFU waren niet het resultaat van "directe" groeiactivatie, maar werden veroorzaakt door TNFα, uitgescheiden door IL-1 geactiveerde AML blasten.

Eerdere studies hadden al gesuggereerd dat AML cellen hun eigen groeifactoren constitutief, i.e. niet gereguleerd kunnen produceren. Uit de experimenten van de Hoofdstukken 8 en 9 is het duidelijk geworden dat HGF produktie door AML cellen kan worden geïnduceerd door IL-1. In Hoofdstuk 10 werd de spontane groei vergeleken met IL-1 gereguleerde groei van AML blasten. Dit werd gedaan om na te gaan of spontane groei ook het gevolg was van stimulatie door endogeen uitgescheiden HGFs. Anti-HGF blokkering

studies en Northern blot analyse toonden aan dat IL-1 gestimuleerde AML cellen GM-CSF, G-CSF, M-CSF, IL-6 en IL-1β (n=6 gevallen) kunnen produceren en dat deze factoren vervolgens de groei van deze cellen kunnen activeren. De combinatie van neutralizerende antilichamen deed de IL-1 geïnduceerde proliferatie van AML blasten in deze 6 patiënten volledig teniet. Aan de andere kant, in 9 van de 11 spontane groeiers, kon de ongereguleerde proliferatie niet volledig worden geremd met de combinatie van anti(a)GM-CSF, aG-CSF, aM-CSF, aIL-1, aIL-6, aIL-3 en aTNF antilichamen, alhoewel in de meeste gevallen enig onderdrukkend effect werd waargenomen. In 2 van deze 9 gevallen hadden de neutralizerende antilichamen in het geheel geen effect. Northern blot analyse in vier van deze spontane groeiers toonde geen detecteerbare hoeveelheden HGF mRNAs aan. Geconditioneerde media, bereid van AML blasten van deze gevallen, vertoonden geen HGF activiteit wanneer ze werden getest op factor afhankelijke cellen **in vitro**. Bij deze gegevens past de hypothese dat in deze bijzondere AML gevallen de cellen spontaan groeien onafhankelijk van endogene HGFs.

In Hoofdstuk 11 worden verscheidene aspecten van groeiactivatie van AML cellen **in vitro** besproken. 1) Geopperd wordt dat de variabele HGF gevoeligheid van waarde kan zijn in de diagnose van AML, bijvoorbeeld voor de klassifikatie van AML. 2) De observatie dat IL-3 en GM-CSF stimulatie van AML cellen vaak samenvallen wordt bediscussieerd in het licht van recente bevindingen dat IL-3 en GM-CSF een gemeenschappelijk receptor complex delen. 3) G-CSF stimulatie van AML cellen wordt bediscussieerd met betrekking tot het vermogen van G-CSF om proliferatie van AML cellen te activeren zonder uitrijping tot neutrofielen te induceren. 4) Er wordt ingegaan op de mechanismen die de stimulerende en remmende effekten van TNF op HGF geïnduceerde proliferatie van AML cellen kunnen verklaren. De stimulerende effecten van TNF op IL-3 en GM-CSF geïnduceerde proliferatie zou kunnen worden verklaard met de recente observaties dat het aantal IL-3 en GM-CSF receptoren, met hoge affiniteit voor deze factoren, op de membranen van AML cellen wordt verhoogd als gevolg van de TNF stimulatie. In tegenstelling zouden de remmende effecten van TNF op G-CSF geïnduceerde groei kunnen worden toegeschreven aan de eliminatie van G-CSF receptoren van het AML oppervlak na blootstelling aan TNF. 5) Wat betreft autocriene groei, spontaan of geïnduceerd door IL-1, wordt de vraag opgeworpen of normale beenmerg voorlopers hun eigen HGFs op gelijke wijze zouden kunnen produceren of dat deze kenmerken leukemie-specifiek zijn. 6) De factor onafhankelijke groei van AML cellen, waargenomen in sommige gevallen, wordt

besproken in het licht van mechanismen van abnormale groei die zijn beschreven voor experimentele leukemieën in dieren.

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CURRICULUM VITAE

Ruud Delwel was born in Rotterdam on May 1, 1958. In 1977 he completed his secundary education (VWO-B) and started a biology study at the State University in Leiden. During this study he participated in several research projects. He worked for 1 year at the Laboratory of Parasitology in Leiden, under the supervision of Dr. P. Rotmans, on the development of immunological methods for the detection of Schistosomiasis. He was introduced into the cell culture techniques and preparation of monoclonal antibodies by Dr. W. van Ewijk at the Department of Cell Biology and Genetics of the Erasmus University of Rotterdam, where he spent 10 months. At the Silvius Laboratory in Leiden, the author was trained in molecular biology for 9 months by Dr. J.L. Bos and studied gene regulation in adenoviruses. He received his degree in June, 1983. In August 1983, he joint the cell culture laboratory at the Dr. Daniel den Hoed Cancer Center where he investigated membrane phenotypes and growth factor responsiveness of acute myeloid leukemia progenitor cells in vitro, under the supervision of Dr. B. Löwenberg. He was introduced into flow cytometry (FACS) by Dr. J.G.J. Bauman and Dr. J.W.M. Visser at TNO, Rijswijk, received a FACS training at Becton Dickinson, USA, and has been the FACS operator at the Dr. Daniel den Hoed Cancer Center during the past 7 years. The investigations described in this thesis were carried out at this institute.