# PROGENITOR CELLS IN HUMAN MYELOID LEUKEMIA

In vitro studies with PHA supplemented colony cultures

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PROEFSCHRIFT

Ter verkrijging van de graad van doctor in de
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Prof.Dr.J.Sperna Weiland
en volgens besluit van het college van decanen.
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door

Klaas Swart

geboren te Ferwerd

Promotor : Prof.Dr.J.Abels

Co-referenten : Prof.Dr.D.W.van Bekkum
Prof.Dr.D.Bootsma

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Aan Margreet,
Oan Heit en Mem.

# Voorwoord

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# Contents

# List of abbreviations

| | |
|---|---|
| ACD - CSA | adherent cell dependent colony stimulating activity |
| ANLL | acute non-lymphocytic leukemia |
| BC | blast crisis (of CML) |
| BSA | bovine serum albumin |
| CFC | colony-forming cell(s) |
| CIA | colony inhibiting activity |
| CML | chronic myeloid leukemia |
| CML - BC | blast crisis of chronic myeloid leukemia |
| CSA | colony stimulating activity |
| E-RFC | E-rosette forming cell(s) |
| GM - CFC | granulocyte-monocyte colony-forming cell(s) |
| PGE | prostaglandin-E |
| $Ph^1$ | Philadelphia chromosome, i.e. t(9,22)(q34:q11) |
| PHA | phytohemagglutinin |
| PHA - LCM | PHA-leukocyte conditioned medium, i.e. medium harvested from PHA stimulated leukocyte cultures |
| PHA-l.f. (assay) | PHA-leukocyte feeder colony assay |
| TL - CFC | T lymphocyte colony-forming cell(s) |

CHAPTER 1

# INTRODUCTION

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# Introduction

## 1.1. Rationale of the study.

The finite lifespan of differentiated cells in almost all tissues of multicellular organisms requires a supply of newly formed equivalents to ensure continuity of tissue function. The lifespan of mature blood cells is relatively brief and, consequently, the renewal rate is high. The cells necessary for replacement are generated by a process of exponential proliferation and subsequent differentiation and maturation. This process has its origin in the pluripotent hematopoietic stem cell. Thus, only a small number of stem cells is required to fulfill the demand for mature cells. Due to the capacity of stem cells to replicate, the stem cell supply is generally not exhausted. Several intermediate cell stages can be distinguished between stem cells and mature blood cells. The early stages of stem and precursor cells are of interest for understanding the control of proliferation and differentiation of hematopoietic cells.

The myeloid leukemias are neoplastic diseases which are supposed to originate from the hematopoietic stem cells and are characterized by an overproduction of immature blood cells. Two main categories of myeloid leukemia are distinguished by morphological criteria and clinical features: chronic myeloid leukemia (CML) and acute non-lymphocytic leukemia (ANLL). CML is a myeloproliferative disorder with leukocytosis and apparently normal cellular differentiation. The early, relatively benign, phase of CML has a mean duration of three years. Then, progressively increasing abnormalities with respect to cellular proliferation and differentiation appear (accelerated phase), finally resulting in a phase known as blast crisis (CML-BC). The blast crisis of CML is characterized by an increasing number of blast cells in bone marrow and peripheral blood, as in acute leukemia. In ANLL there is a marked increase of immature cells (mainly blasts) of the myeloid series. The leukemic cell population replaces the normal hematopoietic cells and the disease is, just as CML-BC, fatal within a few months if untreated.

Morphological studies of blood and bone marrow cells are the basis of the diagnosis and evaluation of treatment in myeloid

leukemia. Based on the morphological appearance of the dominating cell type, six subgroups of ANLL can be distinguished (French-American-British (FAB) classification, Bennettet al., 1976). CML-BC comprises two major subgroups: predominance of either myeloblasts or lymphoblasts (Boggs, 1974; Bennettet al., 1976). The subgroups are not only distinct with respect to cellular characteristics but may also differ in clinical features. Microscopic examinations of leukemic blood and bone marrow cells evaluate morphological aspects of leukemic cells but do not provide information on the proliferation and differentiation capacities of leukemic cells as an active process.

Evidence is increasingly available to postulate that the myeloid leukemias are of clonal origin. Iso-enzyme marker studies (Fialkow et al., 1967; Fialkow, 1976) and cytogenetic investigations (Fitzgerald et al., 1971; Krogh-Jensen and Killman, 1971; Gahrton et al., 1974; Blackstock and Gahrson, 1974) have shown that the primary lesion stems from a single pluripotent hematopoietic stem cell. Once a stem cell has been transformed, proliferation, accumulation and replacement of normal cells, may lead to a predominance of leukemic cells in the hematopoietic tissues. To understand the pathophysiological events in leukemia, it is important to unravel the properties of proliferation of clonogenic leukemic cells. To this end, in vitro methods are essential, in particular because colony culture techniques permit comparative investigations on normal hematopoietic as well as leukemic progenitor cells.

## 1.2. In vitro colony growth by normal hematopoietic cells.

The indroduction of the spleen colony method as an assay for multipotent hematopoietic stem cells by Till and McCulloch (1961), had offered an approach to study the proliferation and differentiation of hematopoietic cells. The spleen colony assay, however, is an in vivo technique and its use is limited only to animal experiments. A few years later, Pluznik and Sachs (1965, 1966) and Bradley and Metcalf (1966) discovered independently the ability of early murine hematopoietic cells to form colonies of mature granulocytes and macrophages in semisolid culture media, by which technique some of the limitations of the in vivo colony method could be overcome. In the original culture systems mouse bone marrow cells were suspended in agar medium and plated in petri dishes on top of a feeder layer containing embryonic fibroblasts or neonatal kidney cells,which provided colony stimulating activity (CSA). Following incubation,

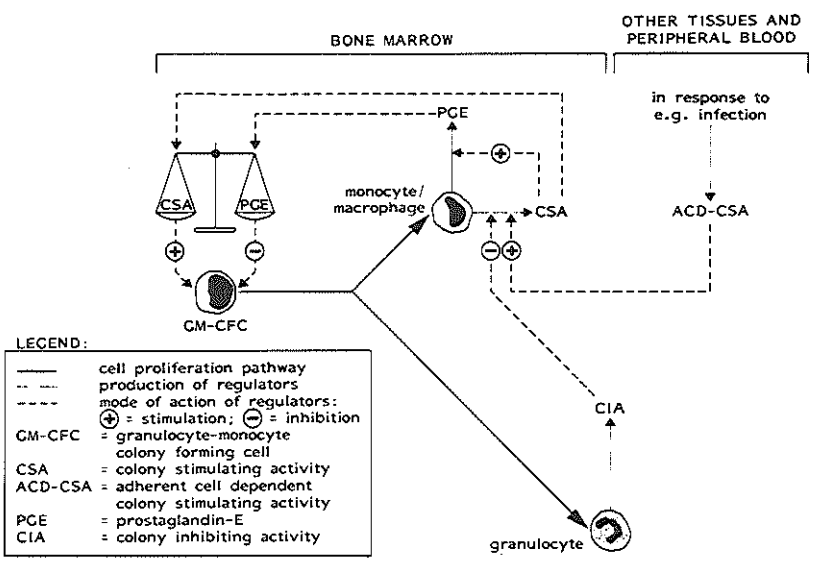
marrow cells proliferate and produce discrete clones of cells which can be recognized as colonies and counted by making use of a microscope. In vitro colony growth from human hematopoietic cells was first reported by Pike and Robinson (1970). Their culture system was analogous to the mouse marrow culture technique, however, human peripheral blood leukocytes were used in the basal agar layer as a source of CSA. Since the original reports, many culture variants, with different sources of CSA, have been described. The in vitro culture methods offered possibilities for more quantitative analysis of proliferation and differentiation of bone marrow cells. In human studies the double layer agar culture technique is presently widely used, which permits the growth of about 10 - 60 colonies out of 100.000 normal bone marrow cells. Colonies are composed of granulocytes, monocytes and macrophages. Occasionally, colonies of eosinophils are present. Single colony analyses revealed that pure granulocytic, pure monocytic-macrophage and mixed colonies occur. It was established that in vitro colonies originate from a single cell (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966; Robinson et al., 1967; Paran and Sachs, 1969; Senn and McCulloch, 1970; Moore et al., 1972; Metcalf, 1977) and single cell transfer studies brought evidence that the colony precursor is a stem cell committed to granulocytic and monocytic cell differentiation. Differentiation into the granulocytic or monocytic lineages can be influenced by modifying the culture conditions, e.g., the source of CSA and amount of CSA added (Metcalf, 1980). With the technique of G-6-PD isoenzyme determinations in separate colonies, Singer et al. (1979) confirmed the clonal nature of each in vitro colony. Their results, however, also indicated that the clonal origin of colonies was only certain when crowding of colonies was prevented by plating low cell numbers.

Arbitrarily, in vitro granulocyte-monocyte colony forming cells (GM-CFC) are usually defined as cells which are capable to proliferate into colonies of at least 40 - 50 cells, thus requiring at least six cell division cycles. Smaller clones of cells are commonly designated clusters, originating from more differentiated,so-called cluster forming cells.

In vitro proliferation of GM-CFC depends on the presence of CSA. Among the cells in the peripheral blood the monocytes have been found to be the main source of CSA (Golde and Cline, 1972; Chervenick and LoBuglio, 1972; Moore and Williams, 1972). Thus, monocytes and their progeny, the tissue macrophages, may have an important function in the stimulation of granulocyto- and monocyto-

poiesis. On the other hand, mature granulocytes (Haskill et al., 1972; Baker et al., 1975; Bruch et al., 1978) and products released by these cells (Broxmeyer et al., 1977; Mendelsohn et al., 1978) have been shown to inhibit granulocyte-monocyte colony growth. The studies of Broxmeyer et al. (1977) indicate that humoral factors from granulocytes, which were called "colony inhibiting activity" or CIA, repress the production and/or release of CSA from monocytes. GM-CFC proliferation has also been found to be inhibited by E-type prostaglandins (PGE) (Kurland et al., 1978a; Taetle and Koesler, 1980; Pelus et al., 1980; Aglietta et al., 1980). Prostaglandins are present in many mammalian tissues and their biological effects are numerous. In the hematopoietic system, monocytes and macrophages can produce PGE and this production is stimulated by CSA (Kurland et al., 1978b). CSA and PGE act directly on GM-CFC and may be competitive. It has been proposed that the balance between PGE and CSA is part of the regulatory control of granulocyto- and monocytopoiesis (Kurland et al., 1978b; Broxmeyer and Moore, 1978). Thus, the monocyte-macrophage cell lineage appears to be an important link in the positive and negative feedback regulation of in vitro GM-CFC proliferation (Figure 1).

Figure 1. A scheme for the control of granulo- and monocytopoiesis by monocytes/macrophages



More recent experimental data favour a modification of the above model (Francis, 1980; Francis et al., 1980): the positive feedback pathway comprises two steps in sequence (Figure 1). When mature granulocytes and monocytes are in demand, e.g., during an infection, a humoral factor which acts indirectly (designated the adherent cell dependent colony stimulating activity, ACD-CSA), is released into the circulation (step 1). This circulating factor stimulates the production of CSA by monocytes and macrophages which reside locally in the bone marrow (step 2). Evidence for the involvement of two distinct factors was given by the fact that the indirect acting stimulating activity was separable from direct acting, bone marrow endogenous CSA in gel filtration (Francis et al., 1980).

It is likely that the factors described above act in vivo as well as regulators of hematopoiesis. Partly due to technical limitations, investigations on the in vivo aspects have been scarce. It is e.g., difficult to draw definite conclusions from in vivo experiments in which impure preparations of CSA, which contain factors with potentially multiple effects, have been used. Furthermore, the in vivo half-life of CSA is short. On the other hand, a cyclic pattern of serum CSA in familial cyclic neutropenia was observed with peaks of CSA levels coinciding with peaks of monocytes and nadirs of neutrophilic granulocytes (Moore et al., 1974a), which may suggest an in vivo role of CSA. The correlation between the level of bone marrow endogenous CSA and the trend in granulocyte production has indicated the importance of endogenous CSA concentrations in human marrow (Francis et al., 1981a). Studies in mice also have suggested a role of marrow endogenous CSA in the regeneration of granulocyte progenitor cells in vivo (Chan and Metcalf, 1973). With respect to the negative feedback pathway in hematopoiesis the in vivo effects of cell-free extracts from human polymorphonuclear neutrophilic granulocytes (CIA) were investigated by Broxmeyer (1978). The injection of CIA in cyclophosphamide treated mice decreased CSA production and inhibited rebound granulopoiesis, which may indicate a regulatory role of CIA in vivo. The in vivo situation, however, is potentially very complex with the involvement of numerous humoral factors and, in addition, cellular interactions.

The features of normal granulocyto- and monocytopoiesis as outlined above represent the physiological base line from which studies in myeloid leukemias are undertaken with the objective to disclose pathophysiological aspects of these diseases.

## 1.3. Abnormal features of colony forming cells and colony formation in myeloid leukemia.

In the past decade many investigations have established characteristics of in vitro growth of leukemic cells. With the double layer agar culture technique marked differences became apparant between normal and leukemic colony growth. However, as will be discussed below, this culture method has major limitations for the analysis of progenitor cells in the acute phase of CML (BC) and in ANLL.

### 1.3.1. Chronic myeloid leukemia.

Blood and bone marrow from patients with chronic myeloid leukemia, in culture, give rise to granulocyte-monocyte colonies of macroscopically normal appearance (Paran et al., 1970; Moore et al., 1973; Goldman et al., 1974; Moberg et al., 1974). Cytogenetic studies revealed that the Philadelphia ($Ph^1$) chromosome, if present in patients cells, was demonstrable too in colony cells (Chervenick et al., 1971; Shadduck and Nankin, 1971; Moore and Metcalf, 1973; Aye et al., 1974a), indicating that CML colonies originate from leukemic progenitor cells. Colony formation of CML cells in agar cultures also depends on stimulation by CSA (Moore et al., 1973; Goldman et al., 1974; Metcalf et al., 1974). GM-CFC in CML have been found to be equally or only slightly less sensitive than normal GM-CFC to CSA (Metcalf et al., 1974; Francis et al., 1979). In untreated patients the occurrence of colony forming cells is in general considerably increased. On the average, the number of GM-CFC in the bone marrow is 5 to 10 times, and in the blood even 50 to 100 times normal values (Moore et al., 1973; Goldman et al., 1974; Moberg et al., 1974). GM-CFC in bone marrow and blood of CML patients are cells of low buoyant density. The peaks of the distribution profile of GM-CFC was shown to be at a density of approximately 1.060 g/ml (Moore et al., 1973; Swart et al., 1981), in contrast with the peak of the density distribution of normal GM-CFC at 1.065 to 1.068 g/ml (Moore et al., 1973; Swart and Löwenberg, 1980). The morphology of GM-CFC in normal bone marrow resembles small lymphocytes (Moore et al., 1973), while CML colony forming cells have the appearance of myeloblasts in cytological preparations of stem cell concentrates (Moore et al., 1973; Swart et al., 1982a).

Despite increased numbers of colonies as well as clusters in cultures of marrow and blood of untreated CML patients, there is a

relative shortage of cluster forming cells. The ratio of cluster to colony numbers is only about 2 as compared to a normal value of 5 - 10 (Moore et al., 1973). This is consistent with the general phemonenon that the hyperplasia of blood and bone marrow cells in untreated CML is more pronounced in the colony forming cell compartment than in any of the more differentiated categories of cells. In other words, on the average a comparatively low number of mature cells is produced per progenitor cell in CML. Apparently, the ultimate potential of the population of colony forming cells is not expressed in vivo. This corresponds with a low percentage of GM-CFC in the S-phase of the cell cycle in untreated CML, because only 10 % to 15 % of these cells are killed in $^{3}$HTdR incubations as compared to a value of 30 % to 40 % for normal GM-CFC (Moore et al., 1973; Moore et al., 1976; Rickard et al., 1979). The low percentage of S-phase GM-CFC in CML could be due to an increase of the cell cycle time of GM-CFC and/or the fact that part of the GM-CFC are not involved in cell division (Go phase), resulting in a small growth fraction within the leukemic cell population. The abnormalities of cell cycle characteristics of GM-CFC in CML are probably not intrinsic but reflect the suppression of proliferation by the expanded granulopoietic compartment. Following reduction of cell numbers by treatment, CML patients show normal percentages of cycling GM-CFC (Moore et al., 1973; Moore et al., 1976). Short term exposures of bone marrow and blood cells of untreated CML patients to CSA preparations increased the proportion of GM-CFC in S-phase (Moore et al., 1976). These observations suggest that, in untreated CML, the percentages of progenitor cells in the active phase of the cell cycle may be suppressed.

Several factors which modulate the proliferation of CML progenitor cells in culture have been disclosed. It has already been mentioned that the GM-CFC remain responsive to CSA. Sera of patients with CML contain elevated levels of CSA in 45 % of the cases (Metcalf, 1977). This could mean that progenitor cells in CML are subjected to higher levels of stimulators than normal GM-CFC in healthy individuals. The neutrophil associated inhibitory action on granulopoiesis is deficient in CML patients and, also CSA producing cells in CML are less responsive to this colony inhibiting activity (Broxmeyer et al., 1976). Furthermore, direct inhibition of colony forming cells by prostaglandins of the E-series is severely impaired in CML. A concentration of PGE-1 which reduces normal GM colony formation by 50 % does not affect CML-CFC (Taetle and Koesler, 1980; Pelus et al., 1980; Aglietta et al., 1980). It is not

clear whether these abnormalities are an epiphenomenon of the disease and represent secondary changes or whether they play a primary, pathogenic role permitting the outgrowth of the neoplasm. In any case, if these abnormal control mechanisms as shown in vitro, i.e., the elevated levels of CSA, the deficiency in neutrophil derived CIA, and the impaired control of GM-CFC proliferation by PGE, apply similarly to the in vivo situation, they do readily explain the myeloid hyperplasia in CML.

Chemotherapeutic treatment of CML generally reduces the number of in vitro colony forming cells. During clinically "complete" remission bone marrow colony counts become within normal limits in most patients, whereas GM-CFC numbers in the blood frequently remain slightly elevated. Transformation of the disease into acute leukemia (blast crisis) may be preceded by a fall in colony numbers (Paran et al., 1970; Moore et al., 1973; Greenberg et al., 1976). This feature is not specific because low colony counts are also found following a relatively long duration of treatment. Conflicting data have been reported on colony growth during blast crisis. Colony growth patterns similar to those in acute myeloid leukemia, i.e., mainly cluster formation and low numbers of colonies have been reported by Iscove et al. (1971), Moore et al. (1973), Paran et al., (1970) and Moore (1977), whereas others have seen increased numbers of colony forming cells in most cases of CML-BC (Brown and Carbone, 1971; Berthier et al., 1977; Rhodes et al., 1978; Leong et al., 1979). In this respect, it has to be realized that in vitro culture conditions, e.g., sources of CSA and day of colony scoring, have varied considerably from one laboratory to another, which may explain the divergent results.

In summary, using the double layer agar culture method, many aspects of in vitro myelopoiesis in CML have been investigated and specific characteristics of CML cells in culture have been established. Although nearly all cases of CML transform into acute leukemia (CML-BC), relatively little is known of whether clinical evolution is preceded by typical early changes in the progenitor cell compartment which can be assessed in vitro. This certainly is in part due to the absence of colony growth in many cases of CML-BC. The introduction of the PHA supplemented cultures which enable investigation of progenitor cells in chronic phase of CML as well as in BC has given an opportunity to approach this question experimentally.

### 1.3.2. Acute non-lymphocytic leukemia.

Marrow and blood cells of patients with acute non-lymphocytic leukemia, at diagnosis and in relapse, generally lack the ability to generate normal sized colonies in culture (Brown and Carbone, 1971; Greenberg et al., 1971; Moore et al.,1973; Bull et al., 1973; Moore et al., 1974b; Van Bekkum et al., 1976; Knudtzon, 1977). Clonogenic growth in agar cultures is limited to cluster formation in most cases. These clusters exhibit the cytogenetic abnormalities typical of the acute leukemia in question, which is evidence for their leukemic nature (Duttera et al., 1972; Moore and Metcalf, 1973; Aye et al., 1974a). The in vitro proliferation of ANLL cells is absolutely dependent on stimulation by GM-CSA (Paran et al., 1970; Iscove et al., 1971; Moore et al., 1973; Metcalf et al., 1974). Studies by Francis et al. (1979) indicate that ANLL cells have a decreased sensitivity to CSA and may, in this regard, resemble the most immature GM-CFC in normal bone marrow (Francis et al., 1981c).

Acute leukemia cells cultured in the conventional media do not differentiate into morphologically normal granulocytes, monocytes and macrophages and a maturation arrest is observed at the myelocyte or promyelocyte stage in the majority of cases (Moore et al., 1974b). Acute leukemia cluster forming cells have an abnormally light buoyant density (Moore et al., 1973).

Several investigators have analyzed the in vitro growth patterns of leukemic cells in large numbers of patients with ANLL, and classified the patients in different categories. Different growth patterns were found to correlate with response to chemotherapeutic treatment. A summary of these data is given in Table I. In these studies different criteria for classification were used and the overall incidence of remission is influenced by advances in treatment in the past decennium. A consistent finding is that limited leukemic growth in vitro (i.e. low numbers of clones or small cluster growth or no growth of leukemic cells) seems bo be associated with high remission percentages. Patients with large cluster growth or with a high incidence of clone forming cells were found to be poor responders to treatment. The "no growth" group in the study of Moore and coworkers (1974b) does not fit in this general scheme, probably because they gathered material from several regional hospitals with a variety of treatment schedules. In addition, it is to be noted that remission induction was successful in a comparatively low number of patients in this study.

The relatively long duration of the colony cultures (at least 7

*Table I. In vitro growth patterns and responsiveness to therapy in acute non-lymphocytic leukemia.*

| author | number of patients | mean percentage C.R. | | growth patterns | | | |
|---|---|---|---|---|---|---|---|
| Moore et al. 1974b | | | | no growth | colony forming | small clusters | large clusters |
| | 108 | | patients in subclass (%) | 12 | 17 | 47 | 24 |
| | | 35 | C.R. within subclass (%) | 15 | 39 | 53 | 12 |
| Spitzer et al. 1976 | | | | no growth or normal colonies | | small clusters | large clusters |
| | 76 | | patients in subclass (%) | 28 | | 47 | 25 |
| | | 47 | C.R. within subclass (%) | 76 | | 75 | 21 |
| Vincent et el. 1977 | | | | no growth | growth (small or large clusters or colonies) | | |
| | 43 | | patients in subclass (%) | 53 | 47 | | |
| | | 49 | C.R. within subclass (%) | 65 | 30 | | |
| Knudtzon 1977 | | | | total number of colonies plus clusters | | | |
| | | | | <100 | 100 - 1000 | >1000/2 x $10^5$ | |
| | 38 | | patients in subclass (%) | 42 | 24 | 34 | |
| | | 55 | C.R. within subclass (%) | 75 | 56 | 31 | |
| Beran et al. 1980 | | | | no growth or small cluster predominance | | excessive growth | |
| | 26 | | patients in subclass (%) | 58 | | 42 | |
| | | 54 | C.R. within subclass (%) | 87 | | 10 | |

C.R. = complete remission

days) is a major hindrance to apply in vitro data to the treatment strategy. In recent studies by Francis and colleages a correlation was found between in vitro culture pattern and sensitivity of leukemic cells to CSA, i.e., cells which produce small clones have lower thresholds to stimulation by CSA than those which produce larger clones (Francis et al., 1979). In a subsequent study Francis et al. (1981b) showed a correlation between the degree of sensitivity of patient cells to CSA and response to induction chemotherapy. To achieve complete remission more courses or chemotherapy were required by those patients whose cells were least sensitive to CSA. Unfortunately, this study involved a small number of patients. The sensitivity of hematopoietic cells to CSA can be assessed in 2 or 3 days (Francis et al., 1979) and therefore might be available prior to the beginning of chemotherapy in many patients. Clinical CSA sensitivity determinations could therefore be potentially useful but extension and confirmation of the data is certainly required. In theory, determinations of CSA sensitivities of GM-CFC in CML and preleukemic conditions in a follow up study could also be helpful to predict changes in the course of the disease.

There is still scepticism about the predictive value of in vitro culture data with regard to response to treatment and they are currently not yet used to plan patient treatment. This is also because the progressive evolution of treatment schedules has interfered with previously established correlations. The theoretical drawback of using in vitro data relates to the lack of a known causative relationship between cellular proliferation in culture and susceptibility to cytostatic agents. Francis et al. (1981b) have suggested a link between cellular functional phenotype and sensitivity to CSA and, hence, response to treatment. Elaboration along this line may improve knowledge about the basic cellular characteristics in individual leukemias and the heterogeneity of acute leukemias as far as response to treatment is concerned. However, with the aim to increase knowledge about the pathogenesis of acute leukemias it is of interest to identify and characterize specifically the subpopulations of leukemic cells which exhibit the highest proliferative capacities. These cells may be representatives of the stem cell compartment of the neoplasm and methods which allow investigations on such progenitor cells could initiate approaches directed to the primary abnormalities of proliferation and differentiation in leukemia. For this, PHA supplemented colony methods, of which the development will be summarized in the next chapter, have been introduced.

## 1.4. Development of PHA dependent assays for leukemic colony forming cells.

In the leukocyte feeder (double layer agar) technique according to Pike and Robinson (1970) acute myeloid leukemia cells mainly generate clusters and rarely colonies. Therefore, in different studies attempts were undertaken to improve leukemic cell proliferation in vitro. In suspension cultures, Aye et al. (1974b) described that ANLL cells are responsive to the mitogen phytohemagglutinin (PHA). Their results suggest that only a subpopulation of leukemic cells responds to PHA and that the majority of cells does not proliferate (Aye et al., 1975). In 1976, Dicke et al. reported large size colony formation following incubation of acute leukemia cells in liquid culture medium with PHA for 14 hrs and subsequent plating into a semi solid agar culture phase. Cells from untreated and relapsed ANLL patients formed 14 - 440 colonies per $10^5$ cells when cultured for 7 days in the semi solid medium with or without a leukocyte feeder layer. Following PHA preincubation, normal marrow also produced colonies, however, this colony growth was found to be dependent of leukocyte feeder layer stimulation. The leukemic origin of a sample of colonies from ANLL patients was verified by cytogenetic analysis and electron microscopy. The independence of leukocyte feeder stimulation provided a possibility to recognise leukemic stem cells in hematological remissions of ANLL. As yet, however, no data have been reported that this culture system enables the selective assay of leukemic stem cells and thus could be of value for the early detection of relapse or residual leukemia following chemotherapeutic treatment. Investigations by three other groups, using the same culture technique, revealed that cells of half of the patients failed to generate colonies (Wu et al., 1977; Hiraoka et al., 1980; Marie et al., 1982). Hiraoka et al. found that leukemic colony formation in only 9 of 32 acute leukemia patients was really dependent on PHA preincubation. The data summarized above indicate the insufficiency of the culture system to trace minimum numbers of leukemic stem cells. Studies on leukemia cell growth in vitro were extended at the same time mainly at the Ontario Cancer Institute in Toronto (McCulloch and coworkers) and at the Institute of Hematology in Rotterdam (Löwenberg and coworkers). Experiments on leukemia cell proliferation in suspension cultures had indicated that only a minor population of leukemic blast cells exhibited proliferative capacity (Aye et al., 1974b). Proliferation of this population was dependent on a second blast cell subset and was enhanced by the addition of

PHA (Aye et al., 1975). Leukemia cell growth could also be stimulated by culture medium, conditioned by PHA stimulated normal or leukemic leukocytes (PHA-LCM) (Till et al., 1974). Based on these properties of leukemia cell growth in suspension cultures a colony assay was developed. In this technique leukemic cells were suspended in methylcellulose and supplemented with serum and PHA-LCM to stimulate the formation of compact colonies containing 20 - 200 cells (Buick et al., 1977). The leukemic origin of the colonies was documented by morphology, the lack of T and B lymphocyte markers and, in a few instances, by cytogenetic analysis (McCulloch et al., 1978a). Colony growth was obtained from 10 of 11 patients (Buick et al., 1977) and in a group of 21 patients colony numbers correlated with blast cell counts (McCulloch et al., 1978b). This colony assay has the advantage of being a one phase technique.
Another one phase semi solid agar culture technique was introduced by Park et al. (1977). Leukemic colony formation was stimulated by PHA-LCM which was added daily to the cultures; excess medium was allowed to drain continually through holes in the bottom of the culture plates. Colony growth appeared in 8 of 10 acute leukemia patients and was shown to be enhanced by daily feeding with fresh medium. Evidence for the leukemic origin of the colonies was obtained by morphology and chromosome studies on the colonies of one patient. A major disadvantage of this culture method is the susceptibility to infection of the cultures due to the handling required for the daily feeding. In 1978, Löwenberg and Hagemeijer introduced a culture technique for the growth of leukemic colonies, in which patient cells were plated in a thin layer of liquid medium (supplemented with PHA) on top of an agar feeder layer, containing normal peripheral blood leukocytes. In the initial study cytogenetic analyses of colony cells revealed that with karyotypically marked acute leukemia cells, only a minority of the cells in the colonies had the leukemic karyotype. In addition, rather high percentages of E-rosette forming cells were present. Subsequently, it was shown that the technique was also efficient in promoting T lymphocyte colony growth (Löwenberg and De Zeeuw, 1979). The T lymphocyte colony forming cells (TL-CFC) were shown to form E-rosettes and E-rosette depletion was employed to selectively remove the TL-CFC. Thus, the selectivity for leukemia colony formation could be improved considerably (Löwenberg and Hagemeijer, 1978).

The techniques, referred to above, differ from each other in many respects, e.g., preincubation in liquid medium, solidifying the culture medium with agar or methylcellulose, daily feeding with

fresh medium, the use of conditioned medium or leukocyte feeders. They have in common that leukemic colony growth is stimulated by PHA or PHA-leukocyte conditioned medium. It was suggested in the early studies of Aye et al. (1974b) that PHA has an indirect effect in leukemia cell stimulation. This possibility was further supported by the findings that PHA-LCM, containing only low concentrations of PHA, appeared to be a potent stimulator of leukemia colony growth (Buick et al., 1977; Park et al., 1977). In the two-phase culture method of Dicke and coworkers, a direct stimulatory effect of PHA on leukemic colony forming cells has been reported (Dicke et al., 1976; Spitzer et al., 1978). It is to be noted, however, that only the initial phase of this culture method (14 hrs incubation in liquid medium) is supplemented with PHA.

Whether or not these PHA and PHA-LCM supplemented cultures trigger the proliferation of a precursor cell of the leukemic blast cells (e.g., a leukemia stem cell) has to be established. Some evidence for the relationship between colony forming cells and leukemia stem cells has been provided by the observations of McCulloch and coworkers, who found a significant rank correlation between the number of blast colonies and the concentration of blast cells in the peripheral blood of ANLL patients (Buick et al., 1977; McCulloch et al., 1978b). Of even more importance is their finding that the majority (mean value of 53 %) of leukemic colony forming cells is killed in $^{3}$HTdR "suicide" incubations (Minden et al., 1978). These data are different from cell cycle status measurements of acute leukemia cluster forming cells assayed in the double agar layer technique by Moore et al. (1973). These investigators observed only 17 % reduction in cluster numbers following incubation with $^{3}$HTdR. On the contrary, autoradiography measurements on all leukemic blasts revealed a very low percentage of cycling cells, generally zero to ten percent (Killmann et al., 1963; Clarkson et al., 1970; Gavosto et al., 1967). Thus, a comparatively high percentage of the blast cell progenitors (leukemic colony forming cells) is in the S-phase of the cell cycle and, presumably, they are proliferating rapidly and many have a role in maintaining the entire blast cell population (Minden et al., 1978). Experiments by the same group (Buick et al., 1979; Chang et al., 1980) revealed that blast colony forming cells were capable of self-replication which is in agreement with stem cell like properties of these cells.

If the new PHA and PHA-LCM supplemented leukemia colony methods provide an assay for progenitor cells which are the ancestors of the recognizable leukemic cell population, they would enable to study proliferation and differentiation features of the leukemia stem cell compartment.

CHAPTER 2

# INTRODUCTION TO THE EXPERIMENTAL WORK AND OBJECTIVES OF THE STUDIES

# CHAPTER 2

# Introduction to the experimental work and objectives of the studies

The first experiences with the PHA-leukocyte feeder culture technique revealed its applicability for the growth of leukemia cell colonies in vitro (Löwenberg and Hagemeijer, 1978). Meanwhile, it became clear that in this culture system also T lymphocyte colonies are growing (Löwenberg and De Zeeuw, 1979). Consequently, the initial studies on leukemia cell proliferation in the PHA-l.f. assay were hampered by the simultaneous occurrence of two types of colonies, one leukemic and the other T lymphocytic. Methods were therefore developed to improve the specificity of the PHA-l.f. assay for leukemia colony growth. A characterization of T lymphocyte colony forming cells (TL-CFC) in normal bone marrow and blood was made to provide a baseline for a procedure to separate TL-CFC from other bone marrow and blood cells of leukemia patients. These experiments are described in CHAPTER 3. A concurrent goal of these studies was to assess the density properties of normal marrow GM-CFC. Previously, buoyant density determinations have been used to characterize normal bone marrow colony forming cells (Haskill et al., 1970; Moore et al., 1972; Moore and Williams, 1972; Moore et al., 1973), by which methods GM-CFC has been identified as a characteristic population of relatively light cells. Our determinations of the density properties of normal marrow GM-CFC would serve to compare their characteristics with those of leukemic colony forming cells in the PHA-l.f. assay.

In CHAPTER 4, the application of the PHA-l.f. colony method for the culture of bone marrow and blood cells from leukemic patients is described. The experiments were undertaken a) to examine the usefulness of this new technique for the analysis of colony forming cells in human myeloid leukemia and b) to test the value of a cell separation procedure for the elimination of E-rosette forming cells (E-RFC) in order to obtain selective growth of leukemic colonies. Leukemia associated chromosomal aberrations are present in nearly all patients with CML and in the majority of the patients with ANLL. Cytogenetic studies of cultured colony cells were done to trace the leukemic origin of colonies in the PHA-l.f. technique. Besides this specific, though laborious method, the nature of colonies was evaluated by E-rosetting to discriminate between

leukemic (i.e. E-negative) and normal (T lymphocytic, i.e. E-positive) colony growth. The procedure of E-RFC depletion was not always successful for eliminating TL-CFC in order to obtain pure leukemic growth. Therefore, density gradient separation was tried for the purification of leukemic colony forming cells of blood and bone marrow of CML patients. These experiments, described in CHAPTER 5, were also aimed at establishing the density properties of CML colony forming cells in the PHA-l.f. culture method and in the standard leukocyte feeder cultures. These experiments, however, were also not conclusive because the separation of leukemic and T lymphocytic colony forming cells was not complete. CHAPTER 6, therefore, describes an extension of these studies. A series of experiments were undertaken to improve the separation method. It was investigated whether discontinuous density gradient centrifugation with concurrent depletion of E-rosette forming cells (E-RFC) could fulfil our criteria of purification of leukemic colony forming cells. Another purpose of these investigations was to characterize in more detail the properties of leukemic cells forming blast cell colonies in the PHA-l.f. cultures in direct comparison with those producing colonies of maturing cells in the standard leukocyte feeder method. This comparison was done in the chronic phase of CML and also in CML-BC. It was assumed that more significant dissimilarities between both types of colony forming cells might become evident in CML-BC because of the emergence, in this phase of the disease, of blastic, acute leukemia-like clones with increasing abnormalities in proliferation and differentiation. The investigations in CML provided support for the hypothesis that the PHA-l.f. and standard leukocyte feeder cultures identify different leukemic progenitor cells. Thus, the addition of the PHA-l.f. colony method to the conventional leukocyte feeder culture technique in CML progenitor cell investigations offers possibilities to discriminate between subsets of progenitor cells during evolution of the disease. Following these studies in CML the PHA-l.f. colony method was evaluated as an assay for progenitor cells in ANLL (CHAPTER 7). The buoyant density properties of ANLL colony forming cells (PHA-l.f. culture method) were determined in comparison with a) the leukemic progenitor cells capable of forming colonies in the standard leukocyte feeder cultures and b) the normal bone marrow GM-CFC. The latter comparison could provide the knowledge necessary for separating normal and neoplastic progenitor cells, e.g. for diagnostic purposes and application in autologous bone marrow transplantation.

In analogy with normal hematopoiesis, in ANLL a hierarchical

structure of stem cells to end cells in the leukemic cell population, although with limited morphological variation, can be postulated. Colony culture methods are then of use to verify this hypothesis. In subsequent investigations directed at the proliferation and differentiation abilities of the leukemia cell population, we have focussed on the proliferative (colony forming cell) compartment. The comparison of progenitor cells with abilities to produce colonies in different culture methods was directed to the question whether acute leukemias comprised distinct stem cell subsets with different behaviour in culture. To gain more insight in the behaviour of sub-populations in acute leukemia in culture, we analysed the in vitro growth and the roles in the course of the disease of karyotypically distinctly marked subclones which were identified at diagnosis in the bone marrow and blood cells of a patient with acute monoblastic leukemia (CHAPTER 8). It was also assumed that this approach could be of potential value to specify prospectively the clinical relevance of distinct subsets with defined proliferative capacities within the leukemic cell population.

CHAPTER 9 deals with an analysis of the feeder cell requirements for leukemia cell colony growth in PHA supplemented cultures. This study was carried out to elucidate the stimulative role of the cellular components incorporated into the feeder layer of the PHA-l.f. colony method in order to specify differences between growth requirements of leukemic and normal progenitor cells. Information on the stimulating activities specific for leukemia colony growth could be helpful to unravel abnormalities of proliferation and differentiation in leukemia.

Thus, the studies described in this thesis had the common objective to characterize progenitor cells in human myeloid leukemia, to establish their in vitro growth requirements and to distinguish separate clones of progenitor cells within the leukemic cell population in general.

CHAPTER 3

# A CHARACTERIZATION OF T LYMPHOCYTE COLONY-FORMING CELLS (TL-CFC) IN HUMAN BONE MARROW

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# A characterization of T lymphocyte colony-forming cells (TL-CFC) in human bone marrow

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## SUMMARY

T lymphocyte colony-forming cells (TL-CFC) present in the peripheral blood of healthy individuals have been studied by several investigators but an analysis of the properties of marrow TL-CFC is still lacking. The experiments reported here represent a first attempt to define some characteristics of marrow TL-CFC, in direct comparison with blood TL-CFC, using density gradients, rosette tests and stimulation of DNA synthesis. It was found that marrow TL-CFC and blood TL-CFC have different density properties. Both populations were characterized by distinct profiles with peaks at 1·071 g/ml and 1·065 g/ml respectively. In marrow as well as blood striking similarities between the density distributions of TL-CFC and E rosette-forming cells (E-RFC) were found. From E rosette Ficoll separation experiments it became clear that TL-CFC in bone marrow, as well as in blood, represent a subgroup of the E-RFC population. A marked dissociation was observed between the quantitative values of thymidine incorporation and colony responses following stimulation with PHA. The most prominent finding was that light-dense bone marrow subfractions, which were virtually negative in PHA mitogen (DNA-synthesis) tests, still gave rise to relatively large numbers of T lymphocyte colonies after stimulation with PHA. On the contrary, in blood, T lymphocyte colonies could be grown exclusively from density fractions which were positive in PHA mitogen stimulation tests. Apparently, characteristic differences exist between marrow and blood TL-CFC.


## INTRODUCTION

In recent years methods for growing T lymphocytic colonies have been developed. Colonies arise from single lymphocyte precursors following stimulation with phytohaemagglutinin (PHA). These techniques permit the investigation of a subset of T lymphocytes, which are operationally defined by their ability for clonogenic proliferation. The usefulness of these techniques in the study of human disease (lymphoproliferative malignancies, immunodeficiencies) is currently the object of investigation (Dao *et al.*, 1978; Foa & Catovsky, 1979).

The properties of T lymphocyte colony-forming cells (TL-CFC) and their relationships with other lymphocyte subpopulations have been studied only to a limited extent. It has been reported that cells which form T lymphocytic colonies have a sedimentation velocity rate of 4 mm/hr (Claësson *et al.*, 1977) and are in a quiescent cell cycle state (Claësson *et al.*, 1977; Minden, Till & McCulloch, 1978). Studies on cell fractions depleted and enriched in E rosette-forming cells (E-RFC) have indicated that the colony-forming cells belong to the E-RFC population (Claësson *et al.*, 1977). Most reports on this subject (Claësson *et al.*, 1977; Fibach, Gerassi & Sachs, 1976; Minden *et al.*, 1978; Riou *et al.*, 1976; Rozenszajn, Shoham & Kalechman, 1975; Wilson & Dalton,

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1976), however, have dealt with culture systems which support the growth of colonies from blood cells and so far the properties of marrow TL-CFC have not been defined.

We have previously used a culture method which stimulated colony formation both from blood and from bone marrow cells, with a high cloning efficiency (Löwenberg & De Zeeuw, 1979). In the present report we have extended our investigations with the characterization of TL-CFC in human bone marrow in comparison to blood TL-CFC.

## MATERIALS AND METHODS

*Bone marrow cells and blood leucocytes*

Bone marrow was aspirated from the posterior iliac spine or sternum of patients who underwent a diagnostic marrow puncture. Only marrow samples from patients who, retrospectively, had no haematological disease were included in the analysis. Aspirates were collected in glass bottles containing Hanks' balanced salt solution (HBSS) and preservative-free heparin. Nucleated cells were obtained after unit gravity sedimentation (20 min) of the erythrocytes in 0·1% methylcellulose. Blood was obtained from normal individuals by venipuncture and was handled in the same manner as bone marrow.

*Myeloid colony assay*

Myeloid colonies were grown in agar-gel culture according to Pike & Robinson (1970). Methylcellulose-separated bone marrow cells ($2 \times 10^5$) were cultured in a 0·2-ml agar layer (0·25% agar) on top of a feeder layer (0·5% agar) with $1 \times 10^6$ peripheral blood leucocytes in plastic tissue culture petri dishes (Flow; diameter 35 mm). The culture medium consisted of Dulbecco's modified Eagle's minimum essential medium, horse serum (6·7%), foetal calf serum (6·7%), trypticase soy broth (6·7%) and was supplemented with a mixture (10%) composed of dialysed BSA (10%), egg lecithin ($3{\cdot}75 \times 10^{-3}$ M), $Na_2SeO_3$ ($1{\cdot}25 \times 10^{-5}$M), human transferrin ($9{\cdot}62 \times 10^{-4}$ M) in a $FeCl_3$-solution ($1{\cdot}92 \times 10^{-3}$ M) and $\beta$-mercaptoethanol ($10^{-1}$ M) in ratios of respectively 75:8:8:8:1 (Guilbert & Iscove, 1976, modified). The plates were incubated in a fully humidified atmosphere of 7·5% $CO_2$ in air at 37°C for 14 days. Colonies of more than 50 cells were scored using an inverted microscope.

*PHA leucocyte feeder colony assay (T lymphocyte colony assay)*

Bone marrow and blood cells were plated ($1 \times 10^5$) in the PHA leucocyte feeder assay as described by Löwenberg & De Zeeuw (1979) with minor modifications. The liquid overlayer measured 0·4 ml and was supplemented with 0·01 ml PHA (Wellcome). The culture medium was the same as for the myeloid colony assay. After 7 days of incubation (37°C, 7·5% $CO_2$ in air) colonies of more than 50 cells were scored.

*E rosette test*

In E rosette tests the cells (approximately $2 \times 10^6$) were mixed with an equal volume (0·25 ml) of a 0·5% (v/v) untreated sheep red blood cell (SRBC) suspension in foetal calf serum, absorbed with SRBC. The cell mixture was incubated for 10 min at 37°C, centrifuged (130 *g*, 5 min) and incubated overnight at 4°C. The pellet was then gently resuspended and one drop was put on a microscope slide for scoring. The slides were stained with brilliant cresyl blue and at least 200 cells per sample were counted.

*PHA microstimulation test*

The PHA mitogen reactivity of cell suspensions was determined according to DuBois *et al.* (1973). In brief: $1 \times 10^5$ nucleated cells were cultured in microtitre plates in 150 $\mu$l medium (RPMI 1640 + 10% human AB serum) supplemented with 2 $\mu$l PHA (Wellcome). Control cultures were run without PHA. Plates were incubated at 37·5°C in a humidified atmosphere (7·5% $CO_2$ in air) for 5 days (blood) or 7 days (bone marrow). All cultures were done in triplicate.

Twenty-four hours before harvesting 20 $\mu$l $^3$H-TdR (0·08 $\mu$Ci, specific activity 2 Ci/mmol) was added to the cultures. The cultures were harvested on glass fibre filters with an automatic cell culture

harvester. The filters were dried and put into counting vials. After addition of 3 ml scintillation fluid (15 g PPO + 25 mg POPOP per 2·5 l toluol) counting was performed in an Isocap/300 liquid scintillation system (Searle). In each experiment at least four standard vials were counted to determine the counting efficiency. Subsequently, the counts per minute (c.p.m.) were transformed to disintegrations per minute (d.p.m.).

PHA reactivity was expressed as the difference in d.p.m. between the test culture (with PHA) and the control cultures (without PHA).

*Cell separations*

*Mononuclear cells.* Mononuclear cells were obtained from whole blood or bone marrow by centrifugation (30 min, 400 *g*) through Ficoll–Isopaque with a density of 1·077 g/ml (Böyum, 1968).

*E rosette Ficoll separation.* Rosette-forming and non-rosette-forming cells were separated by centrifugation through Ficoll–Isopaque. Rosettes had been prepared by incubating mononuclear cell suspensions ($10 \times 10^6$ nucleated cells/ml) with neuraminidase-treated SRBC. Treatment of SRBC with neuraminidase (*Vibrio cholerae* test neuraminidase; Behring, West Germany) was done according to Weiner, Bianco & Nussenzweig (1973) with minor modifications: a 1% (v/v) suspension of SRBC was incubated with neuraminidase in a final concentration of $8 \times 10^{-4}$ u/ml. Following E rosetting, the cells were placed on the gradient, centrifuged (30 min, 400 *g*) and subsequently the E-RFC-depleted and E-RFC-enriched fractions were collected separately from the interlayer and the sediment.

*Density gradient separations.* Bone marrow and blood cells separated in methylcellulose were fractionated on discontinuous bovine serum albumin (BSA) gradients. BSA (Sigma, fraction V) was prepared as described by Shortman, Williams & Adams (1972): it was dialysed against distilled water, lyophilized and dried over $P_2O_5$. A 35% (w/w) stock solution was prepared in unbuffered balanced salt solution (269 milliosmolar, equivalent to 0·147 M NaCl), and subsequently aliquots were diluted to varying densities. Density determinations were performed in a digital density meter, DMA-40 (Anton Paar, Austria), with an accuracy of 0·0001 g/ml.

A discontinuous gradient was prepared in a test-tube by careful layering of fractions with the following densities (from bottom to top): 1·083, 1·080, 1·077 and so on to 1·059 g/ml. Cells suspended in BSA of density 1·050 g/ml were pipetted on top of the gradient. Then the gradient was centrifuged at 4°C for 30 min (2,000 *g* on the bottom of the tube). Finally, cells from each interface were harvested, washed and counted in a haemocytometer.

Ten cell fractions were obtained of the following densities: 1 < 1·059 g/ml; 2–9 stepwise increasing with 0·003 g/ml; and 10 > 1·083 g/ml. In a few experiments the density layer of 1·059 g/ml was not included. Density distributions of cell populations are expressed as recovered values relative to the peak value of 100%.

## RESULTS

*Discontinuous albumin density gradient fractionation*

Discontinuous albumin density gradients were applied to determine the density properties of TL-CFC in bone marrow (Fig. 1) and blood (Fig. 2). TL-CFC were recovered in areas of light and medium density and characterized by a distinct density range. The actual density peaks of marrow and blood TL-CFC were different. Marrow TL-CFC showed a peak at 1·071 g/ml and blood TL-CFC at 1·065 g/ml. It is to be noted that the density distributions of marrow and blood TL-CFC closely paralleled those of E-RFC.

In bone marrow cells the density distribution of TL-CFC was clearly different from myeloid colony-forming cells, which were present in relatively light fractions with a density peak at 1·065 g/ml.

*Relationship of TL-CFC and E-RFC*

Bone marrow TL-CFC, further analysed by E rosette Ficoll separation, sedimented in the pellet (E-RFC-enriched) and only small numbers of TL-CFC were recovered from the E-RFC-depleted interphase (Table 1). Similar results were obtained with blood TL-CFC.

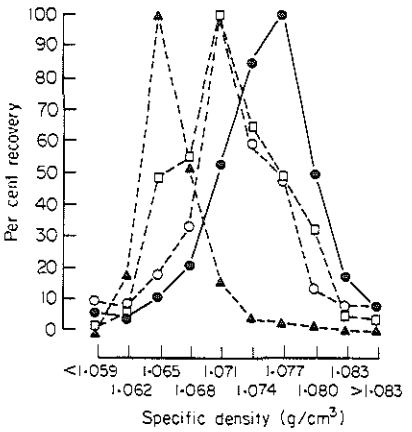


Fig. 1

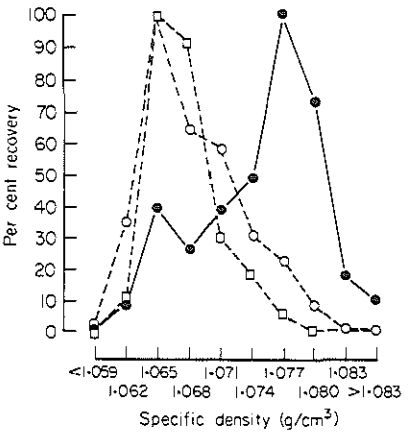


Fig. 2

**Fig. 1.** Discontinuous density gradient separation of normal bone marrow cells. Distribution of nucleated cells (●——●), E-RFC (o - - - o), TL-CFC (□— —□) and myeloid colony-forming cells (▲— —▲). Graphs are normalized to the peak value (100%).

**Fig. 2.** Discontinuous density gradient separation of normal blood cells. Distribution of nucleated cells (●——●), E-RFC (o - - - o) and TL-CFC (□— —□). Graphs are normalized to the peak value (100%).

**Table 1.** E rosette Ficoll separation of bone marrow and blood

| | | Per cent E-RFC | Per cent recovery TL-CFC |
|---|---|---|---|
| Bone marrow | E-RFC-depleted | 5·5 ± 1·3 | 3·5 ± 1·4 |
| | E-RFC-enriched | 89 ± 2·6 | 71 ± 11 |
| Blood | E-RFC-depleted | 3·3 ± 2·3 | 0·1 ± 0·1 |
| | E-RFC-enriched | 74 ± 12·1 | 94 ± 8 |

E-RFC = E rosette-forming cells. TL-CFC = T lymphocyte colony-forming cells.

Data represent means ± s.e.m. (bone marrow $n=3$, blood $n=4$).

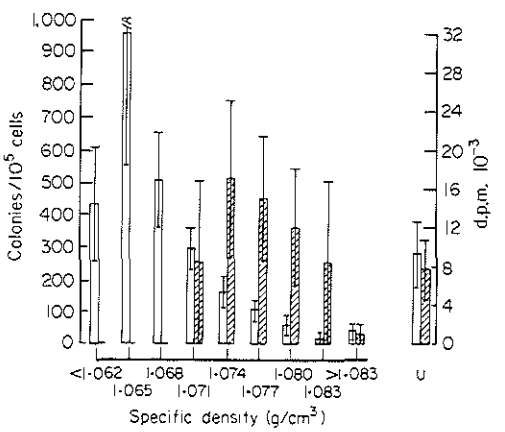


**Fig. 3.** T lymphocyte colony-forming ability and PHA stimulation reactivity (increased DNA synthesis) of density gradient fractions of bone marrow. (□) T lymphocyte colony formation ± s.e.m. ($n=3$); (▨) $^3$H-TdR incorporation (▵ d.p.m.) ± 1 s.e.m. ($n=3$); U = unseparated bone marrow.

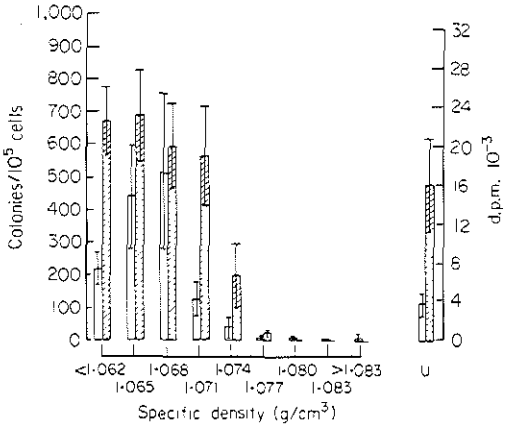


**Fig. 4.** T lymphocyte colony-forming ability and PHA stimulation reactivity (increased DNA synthesis) of density gradient fractions of blood. (□) T lymphocyte colony formation ± s.e.m. ($n=3$); (▨) $^3$H-TdR incorporation (▵ d.p.m.) ± s.e.m. ($n=3$); U = unseparated blood.

*PHA-induced colony formation and PHA-stimulated DNA synthesis*

The T lymphocyte colony-forming ability was compared with the overall mitogenic responsiveness in a stimulation test by assaying marrow and blood density fractions for both TL-CFC and PHA mitogen reactivity. Remarkably, in bone marrow TL-CFC were present not only in fractions which were PHA-positive, as measured by increased DNA synthesis, but also in large numbers in virtually PHA-negative fractions ( < 1·068 g/ml) (Fig. 3). In blood, however, colonies were only formed from fractions which had an increased DNA synthesis rate following PHA stimulation (Fig. 4). Not only PHA negativity in thymidine incorporation tests and PHA positivity in colony assays was seen in the same fractions, but there was also a marked discrepancy between both parameters in other density fractions. The quantitative ratios of TL-CFC/d.p.m. were not a constant value but varied over a broad range. This variable interrelationship between the values of the thymidine incorporation and the colony assays suggests that they measure the result of different processes of cell division following exposure to PHA.

## DISCUSSION

We have done a comparative analysis of some properties of T lymphocyte colony-forming cells (TL-CFC) in human bone marrow and blood, using an *in vitro* culture technique, previously shown to support the growth of T lymphocyte colonies (Löwenberg & De Zeeuw, 1979). The results indicate that marrow and blood TL-CFC are representative of cell fractions which are different in physical characteristics. Most marrow TL-CFC had a comparatively high density, with a peak at $\rho = 1{\cdot}071$ g/ml, whereas blood TL-CFC had a lower peak density at $\rho = 1{\cdot}065$ g/ml. In marrow and in blood there was a striking congruency between the density profiles of TL-CFC and E-RFC. Accordingly, a similar density difference was found between E-RFC in marrow and blood. E rosette Ficoll separation data indicate that TL-CFC—the majority at least—are part of the E-RFC population. These observations taken together demonstrate a close relationship between TL-CFC and E-RFC, not only in the blood (Claësson *et al.*, 1977) but also in the bone marrow.

In the experiments reported here the T lymphocyte colony-forming ability and the overall mitogenic responsiveness in a thymidine incorporation test were also compared. Both *in vitro* phenomena, colony formation and increased DNA synthesis are PHA-dependent. In blood, TL-CFC were found to reside in density fractions which were PHA-responsive in the thymidine incorporation test, but T lymphocyte colonies did not grow from non-responsive subpopulations. This is consistent with the fact that blood TL-CFC represent a subpopulation of all PHA-reactive cells. In bone marrow, PHA responsiveness in the thymidine assay was detected in relatively high

density fractions in which TL-CFC were also present. It is remarkable, however, that in addition large numbers of TL-CFC could be demonstrated in PHA-negative light density fractions. These results indicate a discrepancy between thymidine incorporation and colony formation following stimulation with PHA. From the available data it cannot be concluded if the PHA-responsive cell population in these fractions is indeed absent. It cannot be ruled out that PHA-responsive cells are in fact present in the negative fractions, but this cannot be measured reliably due to the relatively large admixture of spontaneously dividing, $^3$H-TdR-incorporating, immature haematopoietic cells in marrow fractions of light density.

There was no constant relationship between the values of colony and thymidine incorporation responses to PHA. It is remarkable from the data presented in Figs 3 and 4 that in the relatively high density subpopulation of overall PHA-responsive cells the concentration of TL-CFC is low. On the other hand, higher ratios of TL-CFC per d.p.m. PHA reactivity were obtained in fractions of light density. It is likely that the dissociation between TL-CFC and overall PHA-reactive cells is an expression of the difference in the number of divisions following stimulation with PHA. TL-CFC produced scorable colonies only after exponential growth of at least six divisions. PHA-reactive cells, while undergoing fewer cell divisions are, however, included in the radioactivity measurements of a mitogen assay, whereas they may not have colony-forming capacity.

As stated above, marrow and blood TL-CFC have different cellular densities. From our data it is not possible to deduce a kinetic relation between the marrow pool and the circulating pool of T lymphocyte precursors. One explanation of the density differences in the cell populations is that low-density TL-CFC in the marrow enter the blood preferentially. As an alternative it may be possible that high-density TL-CFC selectively migrate to the bone marrow.

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CHAPTER 4

# PHA-INDUCED COLONY FORMATION IN ACUTE NON-LYMPHOCYTIC AND CHRONIC MYELOID LEUKEMIA

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# PHA-INDUCED COLONY FORMATION IN ACUTE NON-LYMPHOCYTIC AND CHRONIC MYELOID LEUKEMIA

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**Abstract**—A method which has previously been introduced for growing colonies *in vitro* from T-lymphocytic cells in normal subjects, has been applied to studying the growth of colonies from the bone marrow and blood of patients with acute non-lymphocytic leukemia and chronic myeloid leukemia. The technique consists of an underlayer of agar containing irradiated leukocytes and a liquid overlayer containing PHA. In leukemic patients, the assay permitted the formation of T-lymphocytic as well as leukemic colonies. Removal of the E-rosette forming cells before culture resulted in the appearance of mainly leukemic colonies. Cytogenetic analysis demonstrated the presence of the acquired karyotypic changes characteristic of the leukemic cells of these patients in the colony cells. Study of eight cases of acute non-lymphocytic leukemia, four cases of blast crisis of CML, and seven cases of CML chronic phase revealed the assay to be efficient in growing large numbers of leukemic blast colonies, as compared to the Robinson culture assay where large colony formation is only found in chronic leukemia [10]. Removal of the progenitors of lymphocytic colonies was sometimes incomplete in acute leukemia but not in CML.

## INTRODUCTION

THE AGAR colony technique according to Pike & Robinson [10] enables the growth of large size colonies of maturing myeloid cells from a normal immature progenitor cell present in bone marrow, but has proved unable to stimulate colony growth from leukemic stem cells (e.g. [5]). Under the standard conditions of this culture system, human acute-leukemia cells give rise to only abortive colonies or clusters which is a drawback for the *in vitro* analysis of proliferating clonogenic leukemic cells.

Recently, leukemic clonogenic cells were shown to respond to phytohemagglutinin (PHA) in culture by the formation of colonies [1, 3, 8]. Dicke *et al.* found evidence that the PHA-colony method may be selective for leukemic colony formation [3]. However, this selectivity is difficult to reconcile with earlier studies which disclosed PHA to be an eminent stimulator for T-lymphocyte colonies *in vitro* as well [2, 4, 7, 13, 15]. We have reported that, in the PHA assay growth of colonies from lymphocytic and leukemic cells were sustained equally well, and thus we could not confirm the selectivity of PHA supplemented cultures for leukemic colony formation [8].

These contradictory results have caused uncertainty about the nature of the different types of colony forming cells in PHA assays and have raised doubts about the usefulness of these colony techniques in studies of patients with leukemias. This report describes the application of a PHA-colony assay for the culture of bone marrow and blood cells from leukemic patients with improved selectivity for leukemic colony formation.

Cytogenetic studies revealed that more than half of the leukemic patients possess acquired clonal chromosomal aberrations in bone marrow cells (leukemic cells) and a normal karyotype in PHA-stimulated blood cells (T-lymphocytes) [14]. Similarly, the $Ph^1$

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*Abbreviations: ANLL*, Acute non-lymphocytic leukemia; *CML* Chronic myeloid leukemia; *E-RFC*, E rosette forming cells; *PHA*, Phytohemagglutinin; *$Ph^1$*, Philadelphia chromosome. Unless otherwise stated, it stands here for the classical translocation t(9;22) (q34:q11).

chromosome anomaly is regularly found in the bone marrow metaphases of patients with chronic myeloid leukemic (CML) but not in their lymphocyte culture [14]. By selecting such patients it was possible to confirm by cytogenetic analysis the leukemic origin of colonies in the PHA-leukocyte feeder assay.

## MATERIALS AND METHODS

Bone marrow was aspirated from the posterior iliac spine or the sternum of patients with untreated acute non-lymphocytic leukemia (ANLL), chronic phase of chronic myeloid leukemia (CML), and CML in blast crisis (BC). Only Philadelphia chromosome ($Ph^1$) positive CML patients were studied. Blood cells were drawn by venipuncture. Our techniques for cell collection, cell handling, myeloid colony formation in culture, cytological preparations and E-rosette tests have been described previously [7].

The PHA-leukocyte feeder colony assay which has been described in detail before [7] will be briefly summarized. The underlayer is a mixture of Eagle's Minimum Essential Medium (as modified by Dulbecco), 10% heat inactivated horse serum, 10% fetal calf serum, 10% trypticase soy broth and a final concentration of 0.5% agar. One milliliter of this mixture was pipetted into sterile plastic petridishes (diameter 35 mm) with $2 \times 10^6$ pre-irradiated (2000 rad X-rays) leukocytes, and allowed to gel. The plates were incubated for 1 day at 37°C in humidified air with 7.5% $CO_2$ before plating the overlayers which contained $0.5 \times 10^5$ marrow cells or $1 \times 10^5$ blood cells. The liquid overlayer had no agar and consisted of the same medium (0.2 ml) with addition of 0.01 ml of PHA (Wellcome). The dishes were then returned to the incubator and after 7 days colonies of more than 50 cells were counted using an inverted microscope. Each experimental value was calculated from the results of three parallel cultures.

In some experiments, prior to culture, rosetting T-cells were separated from non-rosetting cells on an E-rosette Ficoll gradient. For this mononuclear cells were first separated by centrifugation (30 min, 450 $g$) through Ficoll–Isopaque (1.077 g/cm$^3$). Subsequently, these cells were incubated with sheep red-blood cells and spun down through Ficoll–Isopaque. The interphase cells were T-cell depleted and the pellet contained the enriched T-cell fraction.

E-rosette tests were done on colony cells. The colonies were harvested with a Pasteur pipette, pooled, suspended, and incubated with sheep red blood cells as previously described [7]. The percentage of E-rosette forming cells (E-RFC) in colonies resulted from scoring at least 200 isolated cells. Rosette formation by whole colonies or broken colonies was also examined and found similar to the percentages obtained for single cells per culture dish.

Cytogenetic analysis was always done on cells from each bone-marrow sample prior to culture, and on cells from colonies in the PHA-culture at day 8. If no marrow was available, unstimulated blood cells were analysed after culture for 24 h and 48 h. Colchicin (0.25 $\mu$g/ml) was added to the plates before the termination of the cultures. One and a half hours later the colonies were collected from the plates with a Pasteur pipette, transferred to a test tube and suspended in a small amount of isotonic phosphate-buffered salt solution.

Chromosome preparations were made following the standard procedure of swelling the cells with KCl 0.075 M, fixing three times with methanol: acetic acid (3:1), spreading on slides and drying in air. Banding techniques were used to identify the chromosomes: QFA (Q-banding by fluorescence using atebrine), RFA (reverse-banding by fluorescence using acridine orange) and Trypsin-Giemsa according to the Paris Conference (1971) supplement 1975 [11]. Routinely, 32 metaphases from bone marrow and blood were karyotyped and, if feasible, 16 metaphases from colonies of each set of cultures were analyzed. The abbreviation $Ph^1$ used in the text and tables stands for Philadelphia chromosome, which in these studies refers to the classical translocation t(9;22) (q34; q11).

## RESULTS

### *Colony formation in acute non-lymphocytic leukemia (ANLL) and CML blast crisis (BC)*

Bone marrow and blood cells of nine patients with ANLL were cultured in the PHA-leukocyte feeder assay (Table 1). It was observed that the number of colonies in this assay was similar to the results obtained with control cultures, while the number of myeloid colonies in the Robinson system was severely depressed.

In order to investigate more thoroughly the nature of the colonies produced in the PHA-assay in acute leukemia, blood or bone marrow, cells of eight patients with ANLL and four patients with BC were systematically studied (Tables 2 and 3). Of patients with ANLL, four [Nos. 5–8] had acquired chromosomal abnormalities in their leukemic cells at time of diagnosis. The BC patients were $Ph^1$ (+) with additional clonal aberrations which developed during blastic transformation. Culture of total bone marrow or blood cells of these patients gave rise to colony formation in the PHA-assay in all 12 cases (Table 2). E-rosette tests showed that the larger part of the colony cells were E-rosette positive indicating the growth of T-lymphocyte colonies from ANLL and BC marrow. The percentage of E-rosette positive cells varied from 3 to 85% suggesting in some cases—besides T-lymphocytic colonies–the presence

TABLE I. COLONY-FORMING ABILITY OF MARROW AND BLOOD CELLS IN ANLL

| No. of colonies per $10^5$ cells | | |
|---|---|---|
| Marrow | | blood |
| Robinson system | PHA-leukocyte feeder system | PHA-leukocyte feeder system |
| 2 | 209 | n.d. |
| 0 | 35 | 226 |
| 0 | 26 | n.d. |
| 0 | 28 | 25 |
| 0 | 121 | 59 |
| 0 | 404 | 250 |
| 1 | 34 | 91 |
| 0 | 432 | 253 |
| 1 | 80 | 108 |
| Mean ± S.E.M. | 152 ± 54 | 145 ± 36.3 |
| Normal values (mean ± S.E.M.) | 147 ± 17 | 100 ± 17 |
| (normal range) | 42—356 | 8–224 |

Normal values were derived from culturing marrow (n = 31) and blood (n = 12) from hematologically normal subjects during the same period as the cultures of the patients.
n.d. = not done.
S.E.M. = standard error of the mean.

of E-rosette negative colonies. The cytogenetic analysis of the colony cells from patient Nos. 5–12 revealed that most of the metaphases had a normal karyotype and were of lymphocytic origin, except for patient No. 11, where all the metaphases carried the leukemic cytogenetic changes. Colony growth in the Robinson system was in line with data from the literature: severely depressed in acute leukemia [Nos. 1–8] and variable in BC [Nos. 9–11].

The same cell specimens (except patient No. 11) were cultured after depletion of E-RFC in an E-rosette Ficoll gradient (Table 3) and growth of PHA-dependent colonies was still present. However, in contrast to the results with total bone marrow (Table 2), this time the percentage of E-RFC in the colonies was absent or very low in seven of the ten cases studied. Cytogenetic analysis demonstrated the presence of the abnormal karyotype in all metaphases in patients Nos. 5, 9, and 10, confirming the proliferation of leukemic cells in the assay. In patient No. 6 the number of colonies was too limited to allow a reliable E-rosette test and cytogenetic analysis revealed the presence of a majority of cells with a normal karyotype, presumably of lymphocytic origin. In the cultures of three patients (Nos. 7, 8 and 12) colonies of both lymphocytic and leukemic origin were grown as shown by a relatively high percentage of E-RFC in the colonies and a low number of cells with the leukemic karyotype. At the same time, growth of myeloid colonies in the Robinson assay did not change following removal of E-RFC from the ANLL and BC hematopoietic cell suspension.

Distinction between lymphocytic and leukemic colonies in the PHA-leukocyte feeder assay was not possible by examination with the inverted microscope: both were compact and of equivalent size. On handling the colonies with Pasteur pipette, the cohesion between cells was less in leukemic than in lymphocytic colonies. Some of the leukemic colony cells showed a positive reaction when stained with Sudan Black, indicating myeloid origin, but most were relatively undifferentiated blast cells.

TABLE 2. COLONY GROWTH OF MARROW OR BLOOD FROM PATIENTS WITH ANLL AND BC

| Patients | | | Robinson Assay | PHA-leukocyte feeder assay | | | Cytogenetic marker in bone marrow cells |
|---|---|---|---|---|---|---|---|
| No. | Sex | Age | No. of colonies per $10^5$ cells | No. of colonies per $10^5$ cells | % E-RFC in colonies | No. of cells in colonies with cytogenetic marker/No. of cells analysed | |
| ANLL without chromosome abnormalities | | | | | | | |
| 1 | F | 34 | 4 | 188 | 40 | — | — |
| 2 | F | 37 | 0 | 446 | 85 | — | — |
| 3 | M | 78 | 0 | 207 | 75 | — | — |
| 4 | M | 78 | 0 | 67 | 56 | — | — |
| ANLL with chromosome abnormalities | | | | | | | |
| 5 | F | 74 | 0.6 | 58 | 45 | 2/16 | +2i(xp), +8/+2i(xp), +20 |
| 6 | F | 61* | 0 | 73 | 83 | 0/20 | $Ph^1$ |
| 7 | F | 44 | 0 | 136 | 52 | 0/16 | t(8;21) |
| 8 | M | 61 | 0.8 | 232 | 73 | 1/8 | 16q− |
| Blast crisis of $Ph^1$ positive CML | | | | | | | |
| 9 | M | 51 | 9 | 58 | 3 | 3/6 | $Ph^1/Ph^1$, +8, +22q− |
| 10 | F | 66 | 11 | 34 | 34 | 1/3 | $Ph^1/Ph^1$, +19, +22q−/$Ph^1$ + 8, +12, +19, +22 |
| 11 | M | 27 | 90 | 240 | n.d. | 20/20 | $Ph^1$, 17p+, −17, −18 |
| 12 | M | 25† | 9 | 98 | 42 | 0/17 | 14p+/+9, +10, +12 |

n.d. = not done.

* $Ph^1$-positive untreated acute myeloid leukemia.

† blast crisis of originally $Ph^1$-positive CML with disappearance of $Ph^1$ and acquisition of new chromosome abnormalities (see [6]).

TABLE 3. COLONY GROWTH OF E-RFC DEPLETED MARROW OR BLOOD FROM PATIENTS WITH ANLL AND BC

| Patients | | | Robinson Assay | PHA-leukocyte feeder assay | | |
|---|---|---|---|---|---|---|
| No. | Sex | Age | No. of colonies per $10^5$ cells | No. of colonies per $10^5$ cells | % E-RFC in colonies | No. of cells in colonies with cytogenetic marker/No. of cells analysed |
| ANLL without chromosome abnormalities | | | | | | |
| 1 | F | 34 | 7 | 183 | 0 | — |
| 2 | F | 37 | 0.2 | 159 | 2 | — |
| 3 | M | 78 | 0 | 90 | 11 | — |
| 4 | M | 78 | 0 | 106 | 0 | — |
| ANLL with acquired chromosome abnormalities | | | | | | |
| 5 | F | 76 | n.d. | 102 | 0 | 15/15 |
| 6 | F | 61* | 0 | 15 | n.d. | 1/16 |
| 7 | F | 44 | 0 | 75 | 37 | 8/19 |
| 8 | M | 61 | 2.3 | 98 | 65 | 4/16 |
| BC of $Ph^1$-positive CML | | | | | | |
| 9 | M | 51 | 16 | 398 | 0 | 13/13 |
| 10 | F | 66 | 10 | 144 | 0 | 11/11 |
| 12 | M | 25† | 10 | 92 | 25 | 5/15 |

* $Ph^1$ positive untreated acute myeloid leukemia.

† Blast crisis of originally $Ph^1$-positive CML with disappearance of $Ph^1$ and acquisition of new chromosome abnormalities (see [6]); patient was on high dose busulfan treatment at the time of culture.

n.d. = not done.

*Colony formation in chronic phase of CML*

Bone marrow cells of seven patients with CML were studied following the same procedure as for ANLL marrow (Table 4). Only patient No. 5 had not been treated; all others were on intermittent therapy with busulfan or hydroxyurea. The number of PHA-induced colonies generally exceeded that of 'Robinson' colonies (Table 4). The percentage of E-RFC in PHA induced colonies varied from 22 to 88 % and the $Ph^1$ chromosome was found in a variable number of metaphases suggesting growth of a mixture of lymphocytic and leukemic colonies.

Removal of E-RFC prior to culture resulted in the growth of pure leukemic colonies in the PHA-assay: the majority of the colony cells being E-rosette negative and the $Ph^1$ chromosome evident in almost all the metaphases examined (Table 5). Production of myeloid colonies was unchanged or slightly increased.

TABLE 4. COLONY GROWTH OF MARROW FROM PATIENTS WITH CML

| Patients | | | Robinson Assay | PHA-leukocyte feeder assay | | |
|---|---|---|---|---|---|---|
| No. | Sex | Age | No. of colonies per $10^5$ cells | No. of colonies per $10^5$ cells | % E-RFC in colonies | No. of cells in colonies with $Ph^1$ chromosome/No. of cells analysed |
| 1 | M | 25* | 3.5 | 38 | 24 | 12/25 |
| 2 | F | 27* | 2 | 10 | 57 | 3/18 |
| 3 | F | 47 | 0.5 | 207 | 22 | 1/6 |
| 4 | M | 62* | 1 | 83 | 88 | 1/17 |
| 5 | M | 58 | 38 | 36 | 75 | 8/8 |
| 6 | F | 58 | 40 | 47 | 80 | 1/1 |
| 7 | F | 31 | 3 | 73 | 85 | 4/8 |

* Mean values from duplicate experiments at different times.

TABLE 5. COLONY GROWTH OF E-RFC DEPLETED MARROW FROM PATIENTS WITH CML

| Patients | | | Robinson assay | PHA-leukocyte feeder assay | | |
|---|---|---|---|---|---|---|
| No. | Sex | Age | No. of colonies per $10^5$ cells | No. of colonies per $10^5$ cells | % E-RFC in colonies | No. of cells in colonies with $Ph^1$ chromosome/No. of cells analysed |
| 1 | M | 25* | 1 | 170 | 0 | 30/30 |
| 2 | F | 27* | 1 | 33 | 2 | 27/29 |
| 3 | F | 47 | 0 | 226 | 0 | 8/8 |
| 4 | M | 62* | 3 | 137 | 5 | 34/34 |
| 5 | M | 58 | 87· | 56 | 20 | 16/16 |
| 6 | F | 58 | 76 | 236 | 0 | 16/16 |
| 7 | F | 31 | 22 | 453 | 3 | 16/16 |

* Mean values of duplicate experiments at different times.

## DISCUSSION

Formation of leukemic colonies in the PHA-leukocyte feeder assay is dependent on two stimulators: PHA and leukocytes or some factors of leukocytic origin. In this assay, irradiated leukocytes are present in the agar underlayer and PHA in the liquid overlayer. Dicke *et al.* [3] have used a short-term culture in the presence of PHA before plating on agar, usually without leukocytes in the underlayer, and Minden *et al.* [9] have employed PHA-leukocyte conditioned medium in a 0.8% methylcellulose semi-solid medium. The PHA-leukocyte feeder method apparently yields more large colonies than the other systems, but it is not specific for leukemic clonogenic cells because it is also a very efficient culture system for T-lymphocytic colonies [7]. In the present study we demonstrate that cultures of leukemic marrow produce a mixture of T-lymphocytic and leukemic colonies and that elimination of E-RFC from the cultured cells prior to culture improves the specificity of the PHA-leukocyte feeder system for leukemic colony formation. In CML and BC we obtained virtually pure leukemic growth, as demonstrated by the presence of $Ph^1$-chromosome positive cells in the colonies in the absence of E-rosette forming cells. In ANLL the results were less reproducible: in some patients pure leukemic colonies were produced, but in others the admixture of lymphocytic colonies was too high to be ignored. We do not know why, in these experiments, separation of T-lymphocyte precursor cells was incomplete: when an E-rosette Ficoll gradient was applied to marrow and blood cells of non-leukemic patients, T-lymphocyte colony forming cells were effectively separated and recovered in the pellet fraction, and less than 5% of colonies grew out of the E-RFC depleted fraction (unpublished results).

Cytogenetic analysis is a powerful tool in confirming the leukemic origin of colony cells, but it is only possible in patients with acquired chromosomal abnormalities. In patients without chromosomal aberrations we have used E-RFC negativity of colonies as a probable indicator of leukemic growth. Once a reliably selective culture method for leukemic colony formation has been established, E-RFC negativity would be a practical marker, because cytogenetic analysis is too laborious for routine use.

To this end we present a PHA-assay which supports the growth of rather compact colonies of blast cells, which in contrast to the Robinson system do not show signs of maturation. Whereas the Robinson system delivers abortive leukemic colonies (clusters), the PHA-leukocyte feeder assay produces colonies as the result of a larger number of cell divisions. From studies on CML marrow cells after discontinuous albumin-gradient fractionation, we have obtained evidence that the colony-forming cells in the Robinson system and in the PHA-leukocyte feeder assay have identical density properties, but produce different types of

colonies, i.e. maturing myeloid cells are seen in the Robinson system and mainly poorly differentiated blast cells in the PHA-assay (to be published). Further studies along these lines are in progress.

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CHAPTER 5

# DENSITY PROFILES AND PURIFICATION OF CHRONIC MYELOID LEUKEMIA CELLS FORMING COLONIES IN THE PHA-LEUKOCYTE FEEDER ASSAY

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# Density Profiles and Purification of Chronic Myeloid Leukemia Cells Forming Colonies in the PHA-Leukocyte Feeder Assay

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Recently, PHA supplemented culture techniques have been introduced for growing colonies of myeloid leukemia cells. To prepare purified leukemic colony forming cell (CFC) suspensions for further studies, a discontinuous albumin density gradient separation method was applied to bone marrow and blood from patients with chronic myelocytic leukemia. It was found that the PHA-responding CFC were recovered, just as the leukocyte feeder layer stimulated CFC (Robinson CFC), from the light density fractions (1.056, 1.059 and 1.062 g/ml). Density profiles of the precursor cells forming colonies of $Ph^1$ positive cells in the PHA-leukocyte feeder and Robinson assays appeared similar. T-lymphocyte progenitor cells, which also proliferate into colonies in the PHA-leukocyte feeder assay, were in majority harvested from the more dense fractions of the gradient. E-rosette tests and chromosome analysis were used to distinguish between leukemic and lymphocytic colonies. The density distributions of the PHA responsive leukemic CFC ($Ph^1$ chromosome positive) and T-lymphocyte CFC ($Ph^1$ negative) partially overlapped and a complete separation of leukemic and lymphocytic CFC was not achieved.

*Key words:* leukemic colony-forming cells – density distribution – PHA-leukocyte feeder assay – colonies in vitro – chronic myelocytic leukemia

The Robinson colony technique is widely used to define properties of granulocyte-macrophage colony-forming cells (GM-CFC) in chronic myelocytic leukemia (CML). GM-CFC are quantitatively and qualitatively abnormal in bone marrow as well as in blood of patients with CML (1–4). Colony techniques using PHA as a stimulator have been advanced for growing leukemic colonies (5–7), but their value, especially in relation to standard GM-CFC culture techniques, is still to be established.

T-lymphocyte colony-forming cells (TL-CFC), which are also induced to form colonies in these cultures, contam-

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inate leukemic colony growth (8), which hampers specific analysis of leukemic colony formation. On bovine serum albumin (BSA) density gradients, TL-CFC have been found to have a high buoyant density (9), in contrast to GM-CFC in CML patients, which belong to the lightest subpopulation of marrow and blood cells (4).

The present study was designed to investigate the possibility to grow pure leukemic colonies in PHA-supplemented cultures, by separating the TL-CFC on BSA density gradients. The density properties of leukemic CFC in PHA supplemented cultures were investigated and compared to those of leukocyte feeder layer stimulated CFC (Robinson CFC) in the same material. For this purpose 14 bone marrow and 8 blood cell samples from Philadelphia ($Ph^1$) chromosome positive CML patients were analysed.

## MATERIALS AND METHODS

*Bone marrow and blood cells.* Bone marrow was aspirated from the posterior iliac spine or the sternum of patients with CML in chronic phase and in blast crisis (BC). Blood samples were obtained by venipuncture. Bone marrow and blood were collected in perservative free heparin and separation of nucleated cells was obtained as described (9). Thirteen consecutive CML patients were studied, either at first admission or when a bone marrow aspirate and a blood sample were taken for hematologic evaluation. In the myeloid cells of all patients studied the Philadelphia ($Ph^1$) chromosome was present.

*Density gradient separation.* Nucleated cells from bone marrow or blood were further separated on a discontinuous BSA density gradient. BSA (Sigma, fraction V) was prepared as described by Shortman et al. (10): it was dialysed against distilled water, lyophilized and dried over $P_2O_5$. A 35% (w/w) stock solution was prepared in unbuffered balanced salt solution (269 milliosmolar, isotonic with human serum), and subsequently aliquots were diluted with the same salt solution to varying densities. The pH of the BSA solutions was approximately 5.1. Density determinations were performed in a digital density meter, DMA-40 (Anton Paar, Austria).

A discontinuous gradient was prepared in a test tube (inner diameter 20 mm) by careful layering BSA solutions with the following densities (from bottom to top): 1.083, 1.071, 1.068, 1.065, 1.062, 1.059 and 1.056 g/ml. Cells suspended in BSA of density 1.050 g/ml were layered on top of the gradient. Maximally $1.5 \times 10^9$ cells were applied to one gradient. Gradients were centrifuged at 4°C for 30 min (2000 *g* on the bottom of the tube). Cells from each interface were harvested, washed and counted in a hemocytometer.

When minimum cell numbers were recovered from fraction 1.056 g/ml, the fractions 1.056 and 1.059 g/ml were pooled. Results from these combined cells are given as obtained from fraction 1.059 g/ml.

*Colony assays, techniques of cytogenetic and cytologic analysis.* These have been described (8,9,11). The PHA-leukocyte feeder (PHA-l.f.) assay will be briefly summarized. Bone marrow and blood cells were plated ($0.2–1 \times 10^5$) in a 0.4 ml liquid overlayer, on top of a 0.5% agar underlayer containing $2 \times 10^6$ irradiated (2500 rad X-rays) leukocytes. The overlayer was supplemented with 0.01 ml PHA (HA 15, Wellcome). PHA dose response studies had revealed that without the addition of PHA to the cultures colony growth was absent and a concentration of 0.01 ml PHA/culture dish maximally stimulated the formation of leukemic colonies. The culture medium consisted of Dulbecco's modified Eagle's Minimum Essential Medium, horse serum (6.7%), fetal calf serum (6.7%), trypticase soy broth (6.7%), and was supplemented with a mixture (10%) of BSA, egg lecithin, $Na_2SeO_3$, human transferrin and $\beta$-mercaptoethanol as described (9). The culture dishes were incubated for 7 days in a fully humidified atmosphere of 7.5% $CO_2$ in air and colonies of more than 50 cells were scored using an inverted microscope. Cytogenetic analyses were done usually on 30 metaphases from total bone marrow (direct analysis) and on 8–16 metaphases from pooled colony cells of fractions 1.056, 1.059 and 1.062 g/ml. Colony growth was designated pure leukemic when all metaphases were $Ph^1$ chromosome positive. Colony cells from all density fractions were tested for E-rosette forming ability. Colonies from fraction 1.065 g/ml and from higher dense fractions were E-rosette positive and considered T-lymphocytic.

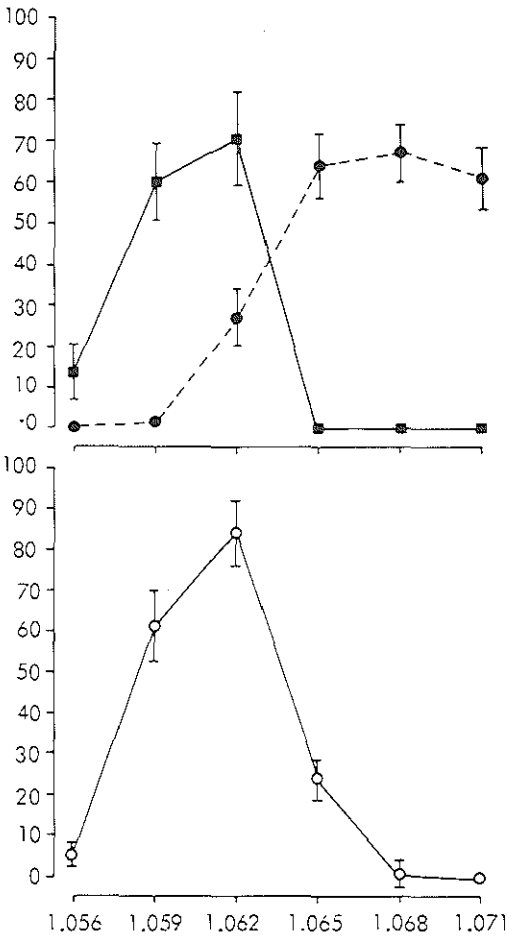


Figure 1. Discontinuous density gradient separation of bone marrow cells from 10 CML patients (including 1 CML-BC patient). Upper part: distribution of leukemic CFC (■——■) and T-lymphocyte CFC (●- - - - -●) in PHA-leukocyte feeder assay. Lower part: distribution of CFC in Robinson assay (○——○). Data represent means ± SEM of 14 experiments (two duplicate and one triplicate experiments at different times of the disease in the same patients). In individual experiments distributions were normalized to the peak value (100%) and subsequently the means of all experiments were calculated. X-axis: specific density g/ml; Y-axis: % recovery.

## RESULTS

Discontinuous albumin density gradient centrifugation was applied to fractionate cell suspensions from 14 bone marrow and 8 blood cell samples from CML patients. Taking all experiments together, mean recoveries of cells from the gradients were: nucleated cells 92% ± 3.7 (SEM), cells forming colonies in PHA-l.f. assay 90% ± 10, Robinson CFC 72% ± 8. Of the 22 experiments, 5 were performed with bone marrow or blood cells from patients with blast crisis (BC). The BC experiments gave results comparable with those in chronic phase. Two additional BC-patients gave very poor growth in Robinson and in PHA-leukocyte feeder (PHA-l.f.) assays and were not included in the following analysis.

The density profiles of the nucleated cells from chronic phase CML patients were about identical to those reported for normal bone marrow and blood (9). Blast cells were concentrated in the light density fractions. Fraction 1.056 g/ml contained 70–100% myeloblasts, fraction 1.059 g/ml 40–90% and fraction 1.062 g/ml 5–50%. In addition, these fractions contained nucleated erythroid cells, promyelocytes, myelocytes and lymphocytes. Cell recoveries of patients with BC were relatively high in the light density fractions and these fractions contained higher percentages of myeloblasts.

In Fig 1 (marrow) & 2 (blood) the mean density distributions of colony-forming cells (CFC) are depicted. In all individual experiments the peaks of the profile were set at 100%. Peaks were either in fraction 1.059 or 1.062 g/ml, which explains that the peak of pooled results was less than 100%. The density distribution profiles of leukemic CFC in PHA-l.f. and Robinson assays were approximately similar in bone marrow and in blood. There was a density overlap of leukemic CFC and T-lymphocyte colony-forming cells (TL-CFC). Neverthe-

less, fraction 1 (1.056 g/ml) was usually free of TL-CFC and gave rise to mere leukemic colonies in the majority of cases. The second fraction (1.059 g/ml), both from blood and bone marrow, gave a high yield of leukemic CFC. In bone marrow these were only in part of the experiments contaminated by low numbers of TL-CFC (Fig 1) but in the cultures from this fraction of blood the TL-CFC contamination was more severe (Fig 2). Fraction 1.062 g/ml and higher fractions contained significant numbers of TL-CFC.

In 4 bone marrow and 5 blood cell separation experiments the three lightest fractions contained enough cells to permit investigations on colony growth from these fractions separately. High numbers of Robinson colonies were grown from the density fractions 1.056, 1.059 and 1.062 g/ml (Table 1), whereas in the more dense fractions colony growth was minimal (data not shown). The PHA-l.f. assay produced leukemic colonies in low dense and T-lymphocytic colonies in high dense fractions. The percentage of E-rosette forming cells (E-RFC) among colony cells in fractions 1.056 and 1.059 g/ml was low and karyotype analysis revealed the $Ph^1$ chromosome in the majority of metaphases (Table 1). The percentages of E-RFC and $Ph^1$ positive cells were variable in the third fraction (1.062 g/ml), indicating the frequent admixture of T-lymphocyte to leukemic colony formation.

A significant number of E-RFC (mean 30%, range 2–67%) had been demonstrated in this fraction before culture. Colony cells from higher dense fractions were overwhelmingly E-rosette positive (not shown).

In 13 experiments, too few cells were harvested from fraction 1.056 g/ml and they were pooled with cells from the following fraction (1.059 g/ml). The results from these experiments were comparable to those reported in Table 1. Taking all experiments together, colony growth was pure leukemic in fraction 1.056 g/ml in most cases. In blood experiments, contaminating T-lymphocyte colonies were frequently present in fraction 1.059 g/ml while in the fol-

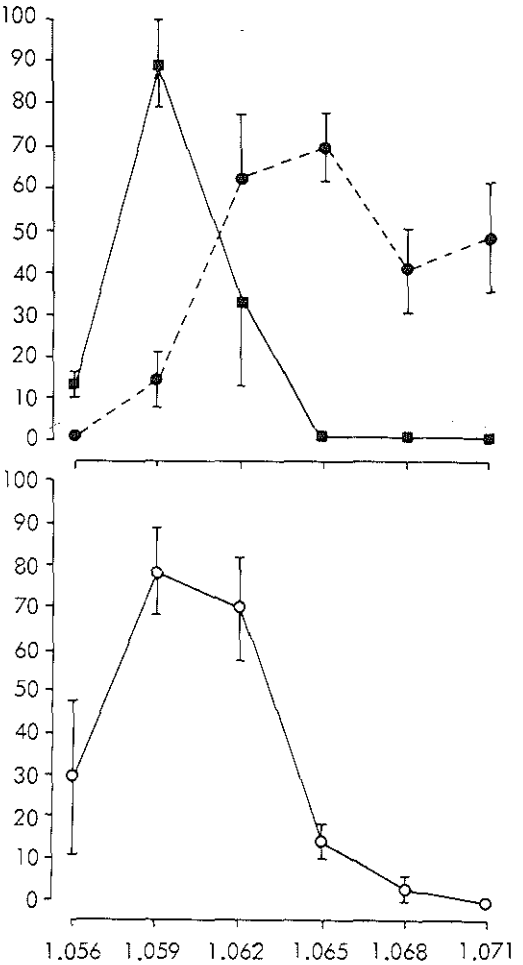


Figure 2. Discontinuous density gradient separation of blood cells from 6 CML patients (including 3 CML-BC patients). Data represent means ± SEM of 8 experiments (two duplicate experiments at different times of the disease in the same patients). For explanation see legend of Figure 1.

TABLE 1

*Colony growth of light density bone marrow and blood cell fractions from patients with CML*

| Patient number/ date | Density fraction | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1.056 g/ml | | | | 1.059 g/ml | | | | 1.062 g/ml | | | |
| | Robinson colonies /$10^5$ | PHA-l.f-assay | | | Robinson colonies /$10^5$ | PHA-l.f.-assay | | | Robinson colonies /$10^5$ | PHA-l.f.-assay | | |
| | | Colonies /$10^5$ | % $Ph^1$ | % E-RFC | | Colonies /$10^5$ | % $Ph^1$ | % E-RFC | | Colonies /$10^5$ | % $Ph^1$ | % E-RFC |
| Marrow | | | | | | | | | | | | |
| 2/ 2-78 | 518 | 380 | 89 (9) | n.t. | 421 | 399 | 100 (8) | n.t. | 72 | 105 | 60 (10) | n.t. |
| 5/ 4-78 | 14 | 5 | 100 (16) | 30 | 274 | 140 | 100 (16) | 10 | 298 | 135 | 75 (16) | 27 |
| 9/ 6-78 | 58 | 752 | 83 (12) | 9 | 910 | 121 | 100 (16) | 3 | 327 | 9 | 50 (8) | 5 |
| 12/ 9-78 (BC) | 207 | 1517 | 100 (14) | 2 | 477 | 1469 | 100 (7) | 2 | 117 | 465 | 0 (8) | 25 |
| Blood | | | | | | | | | | | | |
| 4/ 3-78 | 283 | 12 | 100 (18) | 4 | 196 | 175 | 64 (11) | 43 | 9 | 165 | 0 (2) | 65 |
| 6/ 4-78 | 940 | 1307 | 94 (16) | 10 | 1191 | 1304 | 88 (16) | 5 | 563 | 1524 | n.t. | 15 |
| 9/ 8-78 (BC) | 1297 | 1113 | 100 (16) | 11 | 1453 | 1497 | 63 (8) | 11 | 947 | 689 | 0 (8) | 57 |
| 8/10-78 (BC) | 1270 | 1477 | 100 (8) | 0 | 1067 | 1721 | 88 (8) | 1 | 393 | 1469 | 88 (8) | 7 |
| 2/10-78 (BC) | 109 | 197 | 100 (8) | 20 | 263 | 219 | 100 (8) | 29 | 117 | 736 | n.t. | 88 |

BC = blast crisis.

% $Ph^1$ = Metaphases (%) in colony cells with $Ph^1$ chromosome. In parenthesis: number of metaphases analysed.

% E-RFC = E-rosette forming cells (%) in colony cell suspension.

n.t. = not tested.

lowing fraction (1.062 g/ml) T-lymphocyte colonies grew in all experiments. Chromosome analysis revealed that Ph[1] positive colonies, indicating leukemic growth, were also present in the third (1.062 g/ml) fraction in 13 of the 22 experiments.

## DISCUSSION

Bone marrow or blood cells from 13 consecutive Philadelphia chromosome positive CML patients were separated on discontinuous BSA density gradients and the fractions obtained were assayed for colony growth in the Robinson and the PHA-leukocyte feeder culture techniques.

Results of 22 experiments showed that the density distributions of leukemic CFC in both assays were concordant. Thus, in Ph[1](+) CML, leukemic CFC in Robinson and PHA-l.f.-assays belong to the least dense cells in bone marrow and blood. These findings support the assumption that the same leukemic cell population was stimulated to form colonies in both assays.

The highest concentration of CFC in both assays was found in fraction 1.059 g/ml in the majority of the experiments, and the peak of the density profile was either in fraction 1.059 g/ml or 1.062 g/ml. Colony numbers in the light density fractions of the experiments performed with blood generally exceeded those in the marrow experiments.

As demonstrated in Fig 1 & 2 there were no differences between the mean density profiles of leukemic CFC from marrow and blood in the Robinson assay. With the PHA-l.f. technique, however, the mean density profile for blood CFC appeared less dense than the marrow CFC distribution. The latter observation may be an artifact due to admixture of T-lymphocyte colonies in PHA supplemented cultures (11), since clear-cut conclusions from PHA-l.f. data depend on accurate enumerations of leukemic CFC without interference of TL-CFC.

The shift to lighter fractions of CFC in CML is a well recognized fact (4). We have demonstrated that the same phenomenon applies to leukemic CFC in the PHA supplemented cultures. Whereas in normal bone marrow the peak of the density profile of TL-CFC was found in fraction 1.071 g/ml (9), we expected that gradient fractionation would permit a separation of TL-CFC and leukemic CFC in CML hematopoietic cell suspensions. However, from the experiments reported here it became apparent that subpopulations of TL-CFC and leukemic CFC still shared the same density fractions (Fig 1 & 2) and the separation of lymphocytic and leukemic PHA responsive cell classes was not complete. Chromosome analysis and E-RFC tests revealed that T-lymphocyte colonies grew from fraction 1.056 g/ml only in a few instances, but more frequently from fraction 1.059 g/ml. In the cultures from fraction 1.062 g/ml leukemic colony growth was dominated by T-lymphocyte colonies. Study of the density profiles and other characteristics of leukemic CFC in the PHA-l.f. assay requires better removal of TL-CFC prior to cultures.

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CHAPTER 6

# STUDIES ON CHRONIC MYELOID LEUKEMIA CELL POPULATIONS WITH COLONY FORMING ABILITIES IN PHA-LEUKOCYTE FEEDER AND ROBINSON ASSAYS

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# STUDIES ON CHRONIC MYELOID LEUKEMIA CELL POPULATIONS WITH COLONY-FORMING ABILITIES IN PHA-LEUKOCYTE FEEDER AND ROBINSON ASSAYS*

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**Abstract**—Investigation of leukemic colony-forming cells (CFC) in PHA-supplemented cultures requires removal of T lymphocyte precursors prior to culture. Using a method of discontinuous density gradient centrifugation with concurrent depletion of E-rosette forming cells, T lymphocytes were effectively separated from light density CML bone marrow and blood cell fractions. Consequently, in light density fractions (1.056 and 1.059 g/ml) pure leukemic colony growth was obtained in the PHA-leukocyte feeder (PHA-l.f.) assay. Fraction 1.062 g/ml also yielded pure leukemic colonies in most experiments. Comparison of the density distributions of leukemic PHA-l.f. CFC and Robinson CFC revealed that both CFC populations had congruent density profiles in most patients. In others PHA-l.f. CFC were found to be of somewhat higher density than Robinson CFC. The most striking divergence was apparent in a patient in blast crisis. The findings suggest that different subsets of precursor cells within the CML population proliferate in PHA-l.f. and Robinson colony methods. Both colony techniques are thus potentially useful for discriminating subpopulations of colony-forming cells in chronic myeloid leukemia.

*Key words*: Leukemic progenitor cells, chronic myeloid leukemia, leukemic colonies *in vitro*.

## INTRODUCTION

THE PURITY of leukemic colony growth in a PHA-supplemented culture technique largely depends on the absence of T lymphocyte colony-forming cells (TL-CFC) in the hematopoietic cell samples. The capacity to form E-rosettes [1, 12] and their relatively high buoyant density [12], distinguish TL-CFC from leukemic precursor cells. Removal of E-rosette forming cells (E-RFC) on Ficoll–Isopaque prior to culture did not yield adequate separation of TL-CFC and leukemic colony-forming cells in previous studies [6] and density gradient separation appeared also to be insufficient to obtain cell fractions depleted of TL-CFC [13].

In the experiments reported here, removal of TL-CFC was attempted utilizing both characteristics of these cells in combination. T lymphocytes in bone marrow and blood samples from CML patients were allowed to form E-rosettes and subsequent discontinuous density gradient separation yielded light density fractions depleted of E-RFC. Cells from these fractions were assayed for colony growth in PHA-leukocyte feeder (PHA-l.f.) and Robinson culture techniques. Colonies in the PHA-l.f. assay were identified as leukemic by the absence of E-rosette forming cells and the presence of the Philadelphia ($Ph^1$) chromosome. Apparent dissociations between the density profiles of leukemic colony-

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*Abbreviations*: *CFC*, colony-forming cell(s); *CML*, chronic myeloid leukemia; *E-RFC*, E-rosette forming cells; $Ph^1$, Philadelphia chromosome, i.e. t(9,22) (q34:q11); *PHA*, phytohemagglutinin; *PHA-l.f.*, PHA-leukocyte feeder colony assay; TL-CFC, T lymphocyte colony-forming cells.

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forming cells in PHA-leukocyte feeder and Robinson assays were found in some patients. This suggests that colony formation in both assays is not generated by the same subset of leukemic cells.

## MATERIALS AND METHODS

*Bone marrow and blood cells*

Bone marrow and blood was obtained from Philadelphia ($Ph^1$) chromosome-positive chronic myelocytic leukemia (CML) patients as described [13]. Nucleated cells were harvested following sedimentation of the erythrocytes in 0.1% methylcellulose [5]. Blood and/or bone marrow samples were obtained from 10 different patients, in six of them on different occasions. Most of the patients were in the chronic phase of the disease: two cases developed blast crisis (2/12-78 and 17/6-79). Patients are identified by a number, consistent with the identification given in a previous study [13]. Since material of the same patients were assayed at different occasions, experiments are given by a date code (month–year).

*Discontinuous density gradient separation after E-rosetting*

Rosettes of T lymphocytes in nucleated cell samples were formed by incubating the cells ($10 \times 10^6$/ml) with an equal volume of a 1% (v/v) neuraminidase-treated sheep red blood cell suspension as described [12]. The sedimented cells were carefully resuspended in bovine serum albumin (BSA) of density 1.050 g/ml and layered on top of a discontinuous BSA gradient. BSA processing and preparation of the density fractions have been reported [12, 13]. Discontinuous gradients were prepared of the following density layers from top to bottom: 1.056, 1.059, 1.062 and 1.065 g/ml. A BSA solution of 1.083 g/ml was used as a bottom cushion. When too few cells could be harvested from fraction 1.056 g/ml the fractions 1.056 and 1.059 g/ml were pooled.

*Colony assays*

Colony cultures were done as described in detail previously [6, 12, 13]. Granulocyte–macrophage colony formation was performed in the double layer agar culture technique with normal leukocytes in the feeder layer as source of CSF. The culture medium consisted of Dulbecco's modified Eagle's medium, horse serum (6.7%), fetal calf serum (6.7%), trypticase soy broth (6.7%) and was supplemented with a mixture (10%) of BSA, egg lecithin, $Na_2SeO_3$, human transferrin and $\beta$-mercaptoethanol as described [12]. From unfractionated bone marrow and blood $1–2 \times 10^5$ cells were plated per dish (35 mm dia). Cells from the density fractions were plated in two concentrations: $0.1 \times 10^5$ and $0.5 \times 10^5$ per dish. Colonies of more than 50 cells were counted between days 10 and 14 using an inverted microscope. Leukemic blast cell colony formation was performed in the PHA-leukocyte feeder (PHA-l.f.) assay [13]. Per culture dish $0.2–1 \times 10^5$ cells were plated in a liquid overlayer (0.4 ml) on top of a 0.5% agar underlayer containing $2 \times 10^6$ irradiated (2500 rad X-rays) normal leukocytes. The overlayer was supplemented with 0.01 ml PHA (HA 15, Wellcome). The culture medium was the same as in the granulocyte–macrophage colony assay. At day 7 colonies of 50 cells and more were counted using an inverted microscope. Cell–dose titration experiments were done with cells from purified fractions (1.056 and 1.059 g/ml) in two instances showing a linear cell–dose response relationship. All cultures were done in triplicate and incubated in a fully humidified atmosphere of 7.5% $CO_2$ in air.

*Cytogenetic analysis*

Cytogenetic analysis was done on fresh bone marrow and/or blood samples by direct harvesting of metaphases and following short-term (24 and 48 h) unstimulated cell culture. Cells from density fractions were investigated after short-term (24 h) culture using the improved technique with methotrexate treatment of the cultures [3].

Cytogenetic analysis of the PHA-l.f. colony cultures was done at day 7 or 8. Methotrexate ($10^{-7}$ M) was added to the Petri dishes of the PHA-l.f. cultures the day before harvesting. Following 17 h additional incubation, colonies were collected, pooled in a sterile plastic tube and spun down; prewarmed fresh medium containing either TdR ($10^{-5}$ M) or BrdU ($10^{-5}$ M) was added to the cell pellet and the tubes were incubated at 37°C for 5–6 h. Colchicine (0.25 $\mu$g/ml) was added for the last 10 min of incubation. Further processing followed standard techniques for chromosome preparations: hypotonic shock, fixation and air spreads. Chromosome identification was done by banding techniques: QFA (Q-banding by fluorescence using atebrine) and RFA (reverse banding by fluorescence using acridine orange) according to the Paris Conference (1971), supplement 1975 [10].

*Rosette tests and cytology on colony cells*

Colonies of the PHA-l.f. cultures were mass harvested and incubated with sheep red blood cells to determine spontaneous E-rosette forming cells [12]. Cytocentrifuge preparations were made for morphologic examination of the cells. The slides were fixed and stained according to standard procedures. Colonies of the Robinson cultures were collected with a finely-drawn pasteur pipette, pooled, washed once and slides were prepared using the cytocentrifuge.

Cytological preparations were made of all PHA-l.f. cultures of the light density fractions and of a sample of the Robinson cultures, including those from blast crisis patients.

## RESULTS

*Nature of colonies*

In 19 experiments CML marrow and blood cell suspensions were incubated with SRBC to allow the T lymphocytes to form E-rosettes and subsequently a discontinuous density gradient separation was performed. Cells from the three lightest fractions (1.056, 1.059 and 1.062 g/ml) were harvested and subjected to a series of tests, i.e. cytological analysis, E-rosette formation, colony growth in PHA-l.f. and Robinson culture techniques. After appropriate time in culture the colonies were counted and the colony cells were harvested and analysed for morphology, E-rosette formation and karyotype.

Results of E-rosette tests prior to culture and the numbers of colonies obtained in both culture techniques are given in Table 1. Clearly, the density fractions 1.056 and 1.059 g/ml were sufficiently depleted of E-RFC by the separation procedure to yield leukemic colonies only in the PHA-l.f. assay. In fraction 1.062 the depletion was sometimes less clean and admixture of T lymphocyte colonies was found in three marrow (15/1-79, 18/2-80, 10/2-80) and two blood (14/4-79, 17/6-79) experiments. In the latter two cases, cytogenetic analyses revealed that all metaphases were of leukemic origin ($Ph^1+$). In two other experiments (15/4-79, 10/1-79) leukemic colonies were not demonstrable in

TABLE 1. NATURE OF COLONIES IN THE PHA-LEUKOCYTE FEEDER ASSAY FOLLOWING DENSITY GRADIENT SEPARATION AND E-RFC DEPLETION

| | Density fraction (g/ml) | | | | | |
|---|---|---|---|---|---|---|
| | 1.056 | | 1.059 | | 1.062 | |
| Patient number/ date | % E-RFC before culture | Colony nature* | % E-RFC before culture | Colony nature | % E-RFC before culture | Colony nature |
| *Marrow* | | | | | | |
| 14/11-78 | p† | p | 0 | leuk | 0 | leuk |
| 12-79 | p | p | 0.5 | leuk | 0.5 | (leuk)‡ |
| 15/1-79 | p | p | 3 | leuk | 12.5 | leuk + T-ly |
| 4-79 | p | p | 3.5 | (leuk) | 18 | (T-ly) |
| 18/11-79 | p | p | 2.5 | leuk | 2 | (leuk) |
| 2-80 | 2 | leuk | 5.5 | (leuk) | 23.5 | (leuk + T-ly) |
| 10/1-79 | 2 | leuk | 0 | leuk | 7 | T-ly |
| 11-79 | p | p | 0 | leuk | 0.5 | (leuk) |
| 2-80 | 2 | leuk | 1.5 | (leuk) | 6 | (leuk + T-ly) |
| 3/1-79 | 0 | no growth | 0.5 | leuk | 2 | (leuk) |
| 11-79 | 2.5 | (leuk) | 1.5 | leuk | 1 | leuk |
| 2-80 | n.t. | leuk | 0 | (leuk) | 0 | (leuk) |
| *Blood* | | | | | | |
| 6/12-78 | 0 | leuk | 2 | leuk | 15 | cluster growth |
| 16/1-79 | 0 | leuk | 0 | leuk | 0.5 | leuk |
| 14/4-79 | n.t. | leuk | 6 | leuk | 8 | leuk + T-ly§ |
| 17/6-79bc‖ | 0 | leuk | 0.5 | leuk | 13 | leuk + T-ly§ |
| 11/10-79 | 0 | leuk | 0 | (leuk) | 0 | (leuk) |
| 2/12-78bc | 0 | leuk | 1 | leuk | 17 | leuk |
| 1-79bc | 0.5 | leuk | 1 | leuk | 1 | leuk |

*Colony nature: leuk = leukemic, defined as containing the $Ph^1$ chromosome in all metaphases examined and with less than 10% E-RFC. T-ly = T lymphocytic, defined by the presence of $Ph^1$-negative metaphases and/or more than 10% E-RFC.

†p = cells pooled with fraction 1.059 g/ml.

‡Data given in brackets indicate experiments where cytogenetic analysis of colony cells was not performed. In those instances, classification was only based on the E-RFC marker: less than 10% E-RFC = leukemic colonies; 10–60% = mixture of leukemic and T lymphocytic colonies; 60–100% = T lymphocytic colonies.

§E-rosette tests indicated the presence of cells with sheep RBC receptors, but all metaphases analysed were $Ph^1$ (+) indicating that the vast majority of proliferating cells at the time of analysis were of leukemic origin.

‖bc = blast crisis.

TABLE 2. LEUKEMIC COLONY GROWTH IN PHA-LEUKOCYTE FEEDER AND ROBINSON ASSAYS FOLLOWING DENSITY GRADIENT SEPARATION AND E-RFC DEPLETION

| | Density fraction | No. of exp. | Colonies/$10^5$ cells Mean values (range) PHA-l.f. assay | Robinson assay |
|---|---|---|---|---|
| CML marrow | 1.056 | 6 | 749 (0-1891) | 777 (1-1790) |
| | 1.059 | 12 | 863 (133-2396) | 1402 (373-3533) |
| | 1.062* | 7 | 447 (115-825) | 545 (13-1593) |
| CML blood | 1.056 | 4 | 2298 (579-4877) | 3109 (680-4733) |
| | 1.059 | 4 | 2831 (354-4996) | 2872 (1633-4047) |
| | 1.062 | 4 | 1080 (0-2773) | 453 (109-1213) |
| BC blood | 1.056 | 3 | 287 (51-515) | 878 (35-1687) |
| | 1.059 | 3 | 263 (91-504) | 407 (29-903) |
| | 1.062 | 3 | 438 (8-1004) | 73 (8-133) |

* = data from selected experiments with pure leukemic colony growth only (see Table 1).

the cultures from fraction 1.062 g/ml and in experiment 6/12-78 cytogenetic analysis of the cluster cells revealed the $Ph^1$ chromosome in 50% of the metaphases.

The concentration of colony-forming cells in the light density fractions varied over a broad range (Table 2). The highest concentrations were found in blood, whereas relatively low numbers were demonstrated in the BC samples. However, no correlation between colony growth and clinical status could be shown, maybe due to the limited number of patients.

Results of four representative experiments are presented in detail in Table 3. The majority of myeloblasts were recovered in the first and second fractions and these fractions also yielded high numbers of leukemic colonies ($Ph^1$-positive, E-RFC-negative). The numbers of colonies formed in the PHA-l.f. and Robinson cultures were comparable in some cell fractions (e.g. experiment 6/12-78, fraction 1.059; experiment 10/1-79, fraction 1.056; experiment 16/1-79, fractions 1.056 and 1.059), but in others considerable differences were evident. A constant quantitative relationship between PHA-l.f.- and Robinson-type CFC did not emerge from analysis of these data and also there was no

TABLE 3. DETAILS OF COLONY GROWTH AND THE DISTRIBUTION OF MYELOBLASTS AND E-ROSETTE FORMING CELLS IN FOUR EXPERIMENTS FOLLOWING DENSITY GRADIENT SEPARATION AND E-RFC DEPLETION

| Patient/ date | Density fraction | Before culture % Myeloblasts | % E-RFC | Robinson assay Colonies/$10^5$ | PHA-leukocyte feeder assay Colonies/$10^5$ | % E-RFC in colony cells | $Ph^1$ chromosone in colony cells* |
|---|---|---|---|---|---|---|---|
| 6/12-78 | 1.056 | 71 | 0 | 4383 | 2557 | 0 | 16/16 |
| blood | 1.059 | 47 | 2 | 3467 | 2616 | 4 | 8/8 |
| | 1.062 | 15 | 15 | 353 | c.o. | n.t. | 4/8 |
| 10/1-79 | 1.056 | 81 | 2 | 1547 | 1891 | 0 | 8/8 |
| marrow | 1.059 | 35 | 0 | 687 | 1645 | 0 | 8/8 |
| | 1.062 | 8 | 7 | 55 | 1301 | 44 | 0/8 |
| 16/1-79 | 1.056 | 100 | 0 | 4733 | 4877 | 0 | 16/16 |
| blood | 1.059 | 69 | 0 | 4047 | 4996 | 0 | 16/16 |
| | 1.062 | 7 | 0.5 | 138 | 1344 | 0 | 8/8 |
| 17/6-79 | 1.056 | 100 | 0 | 1687 | 51 | 1 | 24/24 |
| blood, bc | 1.059 | 98 | 0.5 | 903 | 504 | 0 | 24/24 |
| | 1.062 | 20 | 13 | 77 | 1004 | 12 | 8/8 |

*This column gives the number of cells in colonies with $Ph^1$ chromosome per number of cells analysed.
c.o. = clusters only.
n.t. = not tested.
bc = blast crisis.

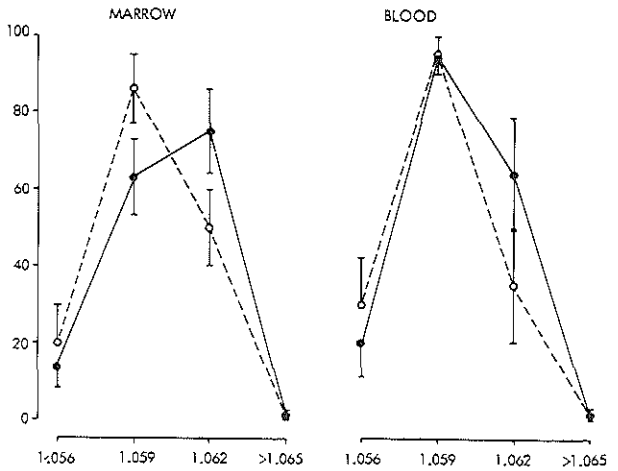


FIG. 1. Distribution of CFC in fractions obtained by density gradient separation and E-RFC depletion of bone marrow and blood cells from CML patients. O-----O Robinson CFC; ●——● $Ph^1$-positive, E-RFC-negative leukemic CFC growing in the PHA-l.f. assay. Data represent means ± S.E.M. of 12 marrow experiments for five different patients (three duplicates and two triplicates at different times of the disease in the same patients) and seven blood experiments for six different patients (one duplicate). In individual experiments distributions were normalized to the peak value (100%) and subsequently the means of all experiments were calculated. *x*-axis: specific density (g/ml); *y*-axis: per cent recovery.

apparent correlation between colony-forming cells and percentage of myeloblasts in the fractions of separate experiments.

*Density distributions of colony-forming cells (CFC)*

The mean density distributions of leukemic CFC in the PHA-l.f. assay and of CFC in Robinson cultures have slightly different profiles (Fig. 1). In both blood and bone marrow, the population of leukemic CFC grown in the PHA-l.f. assay extended into a higher density area than the Robinson CFC. Thus, on the average, PHA-l.f. CFC cover a somewhat more dense area.

Differences and similarities between the populations of colony-forming cells can be shown more precisely in individual experiments (Fig. 2). Experiments performed on samples from patients in the chronic phase of CML showed mainly congruency of the density profiles of both types of CFC. In contrast, in experiments performed with blood or bone marrow of patients in blast crisis the density distribution of each population of CFC appeared unpredictable, as shown in Fig. 2, experiments 2/1-79 and 17/6-79. The concentration in Robinson CFC remained the highest in the light density fraction (1.056 g/ml) but growth in the PHA-l.f. assay was drastically diminished (2/1-79) or displaced to higher density fractions (17/6-79).

It is to be noted that in two patients, tested during chronic phase, consistently different peaks of the density distribution profiles of both CFC populations were found in repeat experiments at different times (3/1-79, 11-79, 2-80 and 14/11-78, 12-79) the peaks of Robinson CFC were in fraction 1.059 g/ml, whereas the maxima of the curve of PHA-l.f. CFC were at density 1.062 g/ml.

*Colony morphology*

The morphological appearance of the cells in the colonies in both culture systems differed markedly. In chronic phase as well as BC experiments, the Robinson colony cells showed differentiation into mature granulocytes and macrophages. Colonies harvested from the PHA-l.f. cultures of the light, T lymphocyte depleted fractions were composed

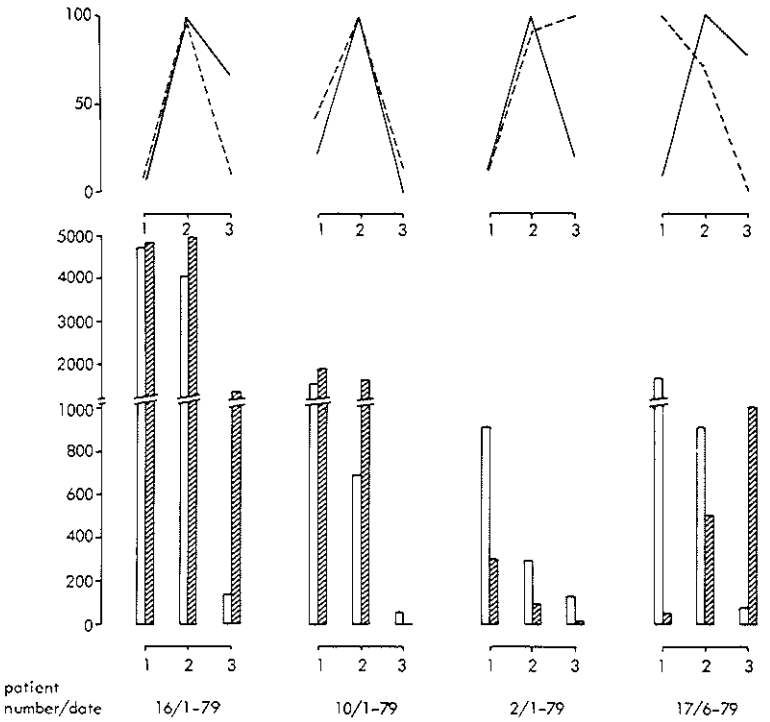


FIG. 2. Density distribution and concentration of CFC in two CML and two CML-BC patients. 16/12-78 and 10/1-79 chronic phase; 2/1-79 and 17/6-79 CML blast crisis. *Upper part*: distribution of Robinson CFC (·----·) and $Ph^1$-positive, E-RFC-negative leukemic CFC growing in the PHA-l.f. assay (·——·) expressed as per cent recovery relative to the peak value. *Lower part*: number of CFC/$10^5$ cells. □ = CFC in Robinson assay; ▨ = leukemic CFC in PHA-l.f. assay. *x*-axis: density fractions; 1 = 1.056 g/ml, 2 = 1.059 g g/ml, 3 = 1.062 g/ml.

of blast-type cells with only a small percentage (5–10%) of Sudan black or peroxidase-positive cells. No other features of differentiation were evident. PHA-l.f. colonies of the more dense fractions ($\geqslant$1.065 g/ml) were composed of lymphoblasts, which were invariably negative in Sudan black and peroxidase stainings.

## DISCUSSION

In culture techniques supplemented with PHA or PHA leukocyte conditioned medium [2, 4, 6, 7, 11], pure leukemic growth can be obtained only after removal of T lymphocyte precursors [6, 8]. In the present study leukemic CFC were concentrated in the light density fractions of a discontinuous albumin density gradient and T lymphocyte colony-forming cells (TL-CFC) were separated from these fractions by concurrent depletion of E-rosette forming cells (E-RFC). Cytogenetic analysis and E-rosette tests of PHA-l.f. colony cells revealed that in all experiments pure leukemic colonies were grown from the density fractions 1.056 and 1.059 g/ml as well as from the majority of the cultures from fraction 1.062 g/ml. Thus, using appropriate separation procedures, leukemic cells capable of extensive proliferation were cultured selectively in the PHA-l.f. colony technique. These results show the advantage of the applied separation procedure over previously reported methods, which had failed to preclude interference of T lymphocyte with leukemic colony growth in the same cultures [6, 13].

High numbers of CFC were found in (myelo)-blast enriched fractions (Table 3) which is in agreement with findings of Moore *et al.* for Robinson CFC [9]. In separate experiments, no strict correlation was apparent between the percentage of myeloblasts and the

number of CFC in the fractions. Robinson CFC and even more so PHA-l.f. CFC did not mimic the density profile of blast cells, indicating that only certain subsets of blast cells had the ability to proliferate into colonies in either assay.

The density profiles of PHA-l.f. CFC and Robinson CFC in chronic phase CML were not concordant (Figs. 1 and 2, Table 2). Although the distributions of PHA-l.f. and Robinson CFC appeared equal in fractions 1.056 and 1.059 g/ml, the results indicate higher recoveries of PHA-l.f. CFC in fraction 1.062 g/ml. Thus, PHA-l.f. CFC represent leukemic cells with a broader density range, which would suggest that they are not the same subpopulations as those proliferating in the Robinson technique. Morphological examination of PHA-l.f. colonies failed to show maturation, whereas Robinson CFC gave rise to differentiating progeny in colonies. This is indicative that the special features of the PHA-l.f. culture do not favour differentiation of colony-forming cells, and maturation capacity is probably not a prerequisite for precursors growing in these cultures. Dissimilarities between PHA-l.f.- and Robinson-type CFC were most clearly demonstrated in experiments in patients in blast crisis, in which numbers of CFC in separate density fractions, in direct comparison, were strikingly discrepant (Fig. 2).

The reported findings are consistent with the notion that the PHA-l.f. assay induces a subset of leukemic cells to proliferate, which differs from the population of Robinson CFC. Therefore, the PHA-l.f. technique is additive to conventional colony cultures of leukemic cells and may provide other insights into leukemia growth *in vitro*. In the myeloid leukemias clonal evolution is a frequent phenomenon. The formation of new clones may be associated with alterations of the clinical course. It would be desirable to have methods to evaluate the proliferative and differentiation capacities of cells belonging to separate clones. The availability of techniques which assess different classes of leukemic progenitors is of potential value for differentiating leukemic cell populations. Whether or not the cells, generating colonies in PHA-l.f. and Robinson assays, represent different stages of differentiation, remains uncertain. Nevertheless, the ability to culture a distinct class of leukemic cells in an assay, in which growth is not characterized by, nor dependent on, maturation, seems valuable for studying the proliferative leukemic cell population, particularly during transformation of CML into blast crisis.

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CHAPTER 7

# ACUTE MYELOID LEUKEMIA COLONY GROWTH IN VITRO: DIFFERENCES OF COLONY FORMING CELLS IN PHA-SUPPLEMENTED AND STANDARD LEUKOCYTE FEEDER CULTURES

K. Swart, A. Hagemeijer & B. Löwenberg, Blood **59** (1982), 816–821

# Acute Myeloid Leukemia Colony Growth In Vitro: Differences of Colony-Forming Cells in PHA-Supplemented and Standard Leukocyte Feeder Cultures

By Klaas Swart, Anne Hagemeijer, and Bob Löwenberg

**Bone marrow or blood of patients with acute myeloid leukemia was subjected to cell separation and the cells investigated for in vitro colony growth. Discontinuous albumin density gradient centrifugation and depletion of E-rosette-forming cells resulted in purified fractions of acute myeloid leukemia cells. From these fractions, growth of large leukemic colonies was obtained in the PHA–leukocyte feeder (PHA-LF) colony technique in 12 of 14 patients. The standard double agar layer technique with a leukocyte feeder for granulocyte-macrophage colony forming cells (GM-CFC) supported colony formation in only four cases. The PHA-LF leukemic colony-forming cells (CFC) were found to be of low buoyant density (always ≤ 1.062 g.ml$^{-1}$) when compared to normal marrow GM-CFC (peak at 1.065 g.ml$^{-1}$). The density profile of PHA-LF CFC paralleled the distribution of the nucleated cells in 8 cases, but in 4 patients, the CFC peak was found at a distinctly lower density; this suggested that a specific leukemic subpopulation had a colony-forming capacity. In three of the four patients with colony growth in the double layer agar technique, it was evident that these CFC had density properties different from those of PHA-LF CFC. These findings suggest that cells giving rise to large colonies in the PHA-LF and double layer agar assays represent distinct leukemic subpopulations.**

LEUKEMIC CELL proliferation can be investigated in vitro by clonogenic culture techniques.[1,2] The standard leukocyte-feeder method (double layer agar technique) devised by Pike and Robinson[1] has been shown to result in clusters from bone marrow and blood from patients with acute myeloid leukemia (AML), but no actual colonies were formed in the majority of cases.[3–6] More recently, colony techniques using phytohemagglutinin (PHA) or culture medium conditioned by PHA-stimulated blood cells have been introduced for obtaining better in vitro growth of leukemic colonies.[7–10] PHA-supplemented cultures have been reported to also support T-lymphocyte colony growth.[11,12] To estimate PHA-dependent leukemic colony-forming cells (CFC), contaminant T-lymphocyte colony formation must be avoided.[9,13] Depletion of E-rosetted cells as a single procedure was insufficient to separate T-lymphocyte colony-forming cells (TL-CFC) from acute leukemia CFC.[14] In studies in patients with chronic myeloid leukemia, discontinuous density gradient centrifugation and simultaneous depletion of T lymphocytes from the light density fractions permitted selective leukemia colony growth in PHA-supplemented cultures.[15] Here, we have used the same fractionation technique in patients with AML. We report on the density distribution of acute leukemia cells that form colonies in PHA-supplemented cultures and compare their density properties with those of leukemic cells forming colonies in the double layer agar technique.

## MATERIALS AND METHODS

### *Patients*

Fourteen adult patients (ages 19–73 yr) with acute nonlymphocytic leukemia were studied: 12 at the time of diagnosis and 2 in relapse. In patient 8, a second experiment was done at relapse. Cytologic variants of acute nonlymphocytic leukemia were classified according to the FAB nomenclature:[16] M1–M6. Leukemia subtypes and selected clinical characteristics of the patients are presented in Table 1.

### *Bone Marrow and Blood Cells, Cell Separation, Cytogenetic Analysis and Cytology*

Previously described techniques were used for the collection of bone marrow and blood,[12] separation of nucleated cells,[12] density gradient separation combined with E-RFC depletion,[15,17] cytogenetic analysis of fresh bone marrow, blood samples, and colony cultures,[14,19,20] cytology, and E-rosette tests.[14]

In this study, the discontinuous bovine serum albumin (BSA) gradient contained the following density layers from top to bottom: 1.053 (not included in all experiments), 1.056, 1.059, 1.062, and 1.083 g.ml$^{-1}$. In one experiment (patient 1), only a small number of cells was harvested from fraction 1.056 and these cells were added to those of fraction 1.059 g.ml$^{-1}$.

### *Colony Assays*

A double layer agar technique was used to culture granulocyte-macrophage colonies.[1] In brief: bone marrow or blood cells ($1 \times 10^5$ or $2 \times 10^5$) were plated in 35-mm Petri dishes in 0.2 ml 0.3% agar medium on top of a 1-ml 0.5% agar underlayer containing $10^6$ normal peripheral blood leukocytes as the source of CSF. The culture medium was Dulbecco's modified Eagle's medium, supplemented with fetal calf serum, horse serum, trypticase soy broth, and a mixture of BSA, $Na_2SeO_3$, egg lecithin, and $\beta$-mercaptoethanol as previously described.[18] Cells from the density fractions were usually

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*0006–4971/82/5904–0017$1.00/0*

**Table 1. Hematologic Characteristics of 14 Patients With Acute Myeloid Leukemia**

| Patient Number | Age (yr) | Cytologic Subtype (FAB) | Peripheral Blood | | Marrow | | In Vitro Colony Growth | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | WBC × $10^3/\mu l$ | Percent Blasts | Percent Blasts | Karyotype | Source of Cells Studied | PHA-LF Assay | Leukocyte Feeder Assay |
| 1 | 66 | M2 | 123 | 56 | 48 | A | Marrow | + | + |
| 2 | 54 | M2* | 49 | 98 | 65 | A | Marrow | − | − |
| 3 | 63 | M4 | 100 | 73 | 75 | A | Marrow | + | − |
| 4 | 26 | M4 | 30 | 10 | 60 | N | Marrow | + | + |
| 5 | 65 | M5 | 39 | 1 | 60 | N/A | Marrow | + | + |
| 6 | 36 | M4 | 5 | 29 | 82 | N | Marrow | + | − |
| 7 | 67 | M2 | 101 | 81 | 95 | N | Marrow | + | − |
| 8[a] | 19 | M5 | 34 | 95 | 94 | A | Blood | + | + |
| 8[b] | | M5* | 0.6 | 10 | 87 | A | Marrow | + | + |
| 9 | 48 | M4* | 20 | 87 | 92 | A | Marrow | + | − |
| 10 | 29 | M4 | 37 | 68 | 46 | N | Blood | + | − |
| 11 | 38 | M4 | 48 | 68 | 95 | N | Blood | + | − |
| 12 | 60 | M5 | 207 | 86 | 90 | N | Marrow | + | − |
| 13 | 25 | M2 | 91 | 96 | 90 | N/A | Marrow | + | − |
| 14 | 46 | M1 | 13 | 83 | 87 | N/A | Marrow | + | − |

*Relapse.

(+) Colony growth present; in these cases colony numbers were always at least $50/10^5$ cells in the peak fraction. (−) No colony growth.

N, normal karyotype: 46, XY or 46, XX. A, abnormal karyotype: details are given in Table 2.

cultured in two concentrations: $0.1 \times 10^5$ and $0.5 \times 10^5$ per dish. The cultures were always done in triplicate and incubated at 37°C in a humidified 7.5% $CO_2$ atmosphere. Using an inverted microscope, colonies of 50 cells or more were counted between days 10 and 14. Normal values for GM-CFC in 15 experiments in our laboratory are $26/10^5$ cells (range 10–45).

Blast cell colony-forming cells were assayed in a culture method that has previously been designated as the PHA-leukocyte feeder (PHA-LF) assay and of which details have been published.[12,14,18] In brief: bone marrow or blood cells ($0.5 \times 10^5$ or $1 \times 10^5$) were plated in 35-mm Petri dishes in 0.4 ml liquid medium supplemented with 0.01 ml PHA (HA 15, Wellcome) on top of a 1-ml 0.5% agar underlayer containing $2 \times 10^6$ irradiated (2500 rad X rays) normal peripheral blood leukocytes. The culture medium was the same as in the granulocyte-macrophage colony assay.

Cells from the density fractions were cultured at a lower concentration, usually $0.1 \times 10^5$, $0.2 \times 10^5$, or $0.5 \times 10^5$ per dish. In cases of high colony numbers, only estimates from the lowest cell number plated were used. Cell titration experiments were done twice to confirm the linearity of the dose–response curves for density gradient separated cells. Triplicate cultures were incubated at 37°C in a humidified 7.5% $CO_2$ atmosphere. At day 7, colonies of 50 cells or more, growing at the surface of the agar underlayer, were counted by use of an inverted microscope.

## RESULTS

The results of colony growth from light density ($\leq 1.062$ g.ml$^{-1}$) E-RFC-depleted cell fractions of bone marrow and blood from patients with AML are summarized in Table 1. Three patterns of colony growth were recognized: colony growth in both assays (4 patients; 5 experiments), colony growth in the PHA-LF assay only (8 cases), and no colony growth in either of the assays (2 cases). Thus, in the PHA-LF assay, colonies were obtained in 13 of the 15 experiments. In all cases, these colonies were composed of blast cells; a small percentage (<5%) of myeloperoxidase-positive cells was present in most cases. It was verified by E-rosette tests that the colonies were not of T-lymphocyte origin. Colony cells from fractions 1.053, 1.056, and 1.059 g.ml$^{-1}$ always gave less than 5% and usually no E-RFC. Colony cells from fraction 1.062 contained 2%–15% E-RFC. In 6 experiments, cytogenetic abnormalities demonstrated the presence of the acquired leukemic karyotype in metaphases of colony cells (Table 2). Normal karyotypes were found only in colony cultures from patients who had a mosaicism of normal and cytogenetically marked cells (nos. 5, 13, and 14).

The mean numbers of PHA-LF colony-forming cells in the light density fractions are given in Table 3. Average concentrations of PHA-LF CFC were between 100 and $500/10^5$ cells, but the highest individual values were in the order of $2000/10^5$ cells; this corresponds to a maximum efficiency of 2 colony-forming cells/100 cells.

The density profiles of the leukemic CFC (PHA-LF assay) are shown in Fig. 1 (upper panel) and compared with the density distribution of nucleated cells (Fig. 1, lower panel). Density peaks of CFC were in fraction 1.056 in 2 experiments (Fig. 1A), in fraction 1.059 in 8 (Fig. 1B), in fraction 1.062 in 2 (Fig. 1C), and in one experiment, the peak of the profile was divided over fractions 1.059 and 1.062 g.ml$^{-1}$ (Fig. 1C). The density profile of the CFC usually paralleled the distribution of the nucleated cells (compare Figs. 1A–C with D–F, respectively), but in four experiments (exp. 6 in Fig. 1A and D, exp. 14 in B and E, and exps. 1 and 5 in C and F) leukemic CFC accumulated in a lighter fraction than did nucleated blast cells.

**Table 2. Data From Patients With Acute Myeloid Leukemia With Cytogenetic Abnormalities**

| Patient Number | Marrow and Blood Cells* | | Colony Cells From PHA-LF Cultures | |
|---|---|---|---|---|
| | No. of Cells Studied | Karyotype† | No. of Cells Studied | Karyotype† |
| 1 | 24 | 47, +8(12, 5%)/46, −X, +8(12, 5%)/48, +8, +8(75%) | 24 | 47, +8(9%)/48, +8, +8(91%) |
| 3 | 30 | 45, −7 | 24 | 45, −7(96%)/N(4%) |
| 5 | 32 | N(84%)/47, +8, t(1; 13)(p31; q11?)(10%)/48, +8, +mar(18q⁻)(6%) | 16 | N |
| 8[a] | 56 | 46, t(6; 11)(q27; q23)(12%)/52, +3, t(6; 11), +der(6), +19, +19, +21, +dm(83%)/53, +4, t(6; 11), +der(6), +10, +13, +18, +19, +21(5%)‡ | 15 | 46, t(6; 11)(40%)/52, +3, t(6; 11) +der(6), +19, +19, +21, +dm(60%) |
| 8[b] | 35 | N(10%)/46, t(6; 11)(90%) | 48 | 46, t(6; 11) |
| 9 | 32 | 46, −1, 1q⁺, 2p⁻, −5, +multiple abnormalities | | No yield of metaphases |
| 13 | 40 | N(15%)/47, +8(85%) | 8 | N(10%)/47, +8(90%) |
| 14 | 32 | N(7%)/47, +21(93%) | 8 | N(25%)/47, +21(75%) |

*Bone marrow and/or blood culture unstimulated with PHA.

†Total number of chromosomes and the different aberrations given for the aneuploid karyotypes; N stands for normal karyotype 46,XY or 46,XX; in parentheses, the percentage of cells with a given karyotype in cases of mosaicism.

‡This clone, containing 53 chromosomes, was found only in blood culture (−PHA) at diagnosis, see reference 20.

In four patients, large size colonies were also obtained in the double layer agar technique (Table 1). The morphology of the colony cells in these cultures was studied in experiments 8[a] and 8[b]. Most of these cells were monocytes and macrophages, whereas in the PHA-LF assay, the colonies were composed of undifferentiated blast cells. Cytogenetic analysis of the colonies in the double layer agar cultures was also done and showed an exclusively abnormal karyotype in 32 (8[a]) and 40 (8[b]) metaphases, respectively. The morphology and karyotyping of the colony cells in the double layer agar cultures of the other patients was not studied. The number of colonies from unfractionated and light density cells of these four patients and the ratios of double layer agar CFC/PHA-LF CFC numbers in each fraction are given in Table 4. The double layer agar technique mostly showed a higher plating efficiency than did the PHA-LF method. In one patient (no. 5), there was a constant relationship between the numbers of colonies in both culture techniques for the different fractions. This might indicate growth from the same colony-forming cell population. In the other 3 patients, this ratio varied and increased (patient 4) or decreased (patients 1 and 8) through fractions 1.056, 1.059, and 1.062 g.ml$^{-1}$. This varying numerical relationship between the two types of CFC from the same patients as a function of density suggests that subpopulations of leukemic cells with dissimilar density properties proliferated into colonies in the two assays.

**Table 3. Mean Number of PHA-LF Colony-Forming Cells in Light Density Cell Fractions in Acute Myeloid Leukemia***

| Density Fraction (g.ml$^{-1}$) | Number of Experiments† | Colonies/$10^5$ Cells Plated | |
|---|---|---|---|
| | | Mean | Range |
| 1.053 | 5 | 117 | 4–350 |
| 1.056 | 12 | 498 | 9–2,032 |
| 1.059 | 13 | 251 | 0–577 |
| 1.062 | 12 | 259 | 0–1,491 |

*Thirteen experiments in 12 patients with positive colony formation in PHA-LF culture.

†Density fraction 1.053 g.ml$^{-1}$ was included in only 5 experiments, i.e., patient nos. 8[b], 9, 11, 12, 13; in patient 1, fraction 1.056 contained a minimal cell number and these cells were added to those of fraction 1.059 g.ml$^{-1}$; in patient 9, colony growth in fraction 1.062 was not assessed.

## DISCUSSION

In this study we have employed a cell separation method that has proved to be satisfactory for the culture of chronic myeloid leukemia in the PHA-LF technique without T-lymphocyte colony contamination.[15] Light density cell fractions depleted of E-RFC were prepared from bone marrow and blood of patients with acute myeloid leukemia. Leukemic blasts accumulated in fractions 1.053 through 1.062 g.ml$^{-1}$ in high purity. Cells from these fractions gave rise to colonies in the PHA-LF assay in 13 of 15 experiments, i.e., in 12 of 14 patients. Cytogenetic markers of colony cells (when available), the cytologic appearance of the colony cells, and the absence of E-rosette-forming cells in colonies indicated that colony growth was always leukemic.

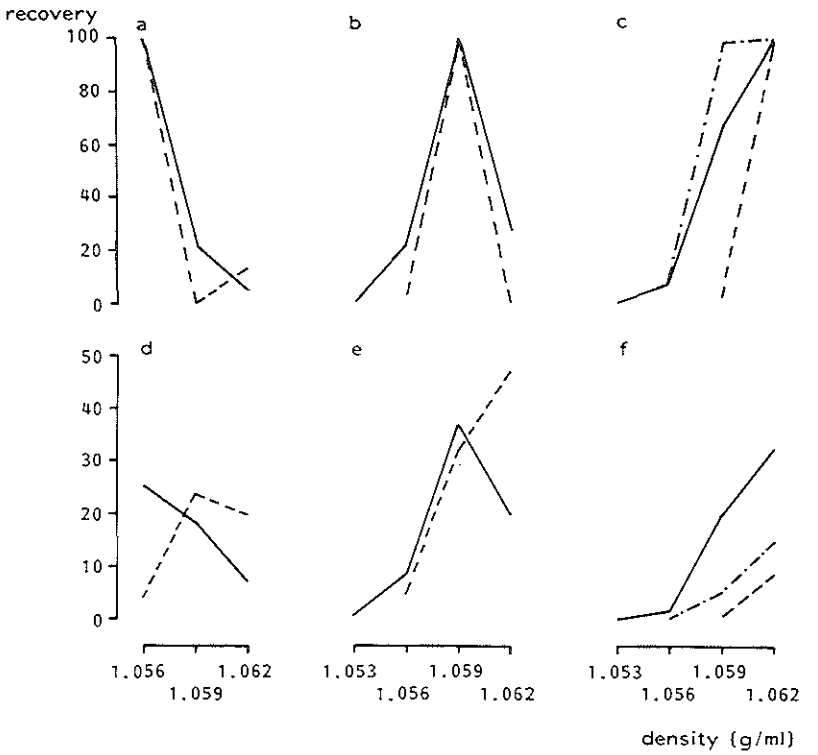


Fig. 1. Density profiles of leukemic colony-forming cells (PHA-LF assay) and recoveries of nucleated cells in 12 patients (13 experiments) with acute myeloid leukemia. Upper panel: CFC recoveries relative to the peak value (100%). Lower panel: cell recoveries as percentages of the number of cells subjected to gradient fractionation. Peak CFC at 1.056: A and D, patients 3 (——) and 6 (– – –). Peak CFC at 1.059: B and E, patients 4, 7, 8ª, 8ᵇ, 9, 11, and 13 (peak nucleated cells at 1.059), mean values (——); and patient no. 14 (- - - -) (peak nucleated cells at 1.062). Peak CFC at 1.062: C and F, patients 1 (– – –), 5 (- - - - - -), and 12 (——).

Leukemic CFC had peaks at light buoyant densities: 1.056 g.ml$^{-1}$ (2×), 1.059 g.ml$^{-1}$ (8×), 1.059 plus 1.062 g.ml$^{-1}$ (1×), and 1.062 g.ml$^{-1}$ (2×). These values are very low as compared to those of normal bone marrow GM-CFC, which have a peak density at 1.065 g.ml$^{-1}$.[18] This difference from normal GM-CFC is similar to that obtained by others for leukemic cluster-forming cells in the double layer leukocyte feeder technique.[4,21]

Large size leukemic colonies appeared in the double layer leukocyte feeder technique, without PHA stimulation, in 4 of the 14 patients. This figure is somewhat higher than the 12%–17% incidence reported by Moore and coworkers,[21] Knudtzon,[6] and Hiraoka et al.,[22] but our series comprises a small number of patients. In these four cases the density profiles of the CFC were shifted to the least dense fractions, just as reported above for CFC in the PHA-LF assay. In three patients (nos. 1, 4, 8), notable discrepancies were evident in the quantitative relationships between the colony-forming capacities of cells from different density fractions in standard leukocyte feeder and PHA-LF assays. In one of these patients (no. 4), the ratio of double layer agar CFC/PHA-LF CFC increased with increasing density; in two others (nos. 1, 8) a decrease in this ratio was noted as a function of density (Table 4). This suggests that in these instances different populations of leukemic cells each with specific

**Table 4. Colony Growth From Unfractionated and Light Density Cells in Standard Leukocyte Feeder and PHA-LF Assays in Four Patients With Acute Myeloid Leukemia**

| | Patient 1 | | Patient 4 | | Patient 5 | | Patient 8* | | Patient 8ᵇ | |
|---|---|---|---|---|---|---|---|---|---|---|
| Cells | Leukocyte Feeder | PHA-LF | Leukocyte Feeder | PHA-LF | Leukocyte Feeder | PHA-LF | Leukocyte Feeder | PHA-LF | Leukocyte Feeder | PHA-LF |
| Unfractionated | 503 | † | 397 | † | 54 | † | 37 | † | 43 | † |
| Fraction 1.053 | - | - | - | - | - | - | - | - | 1,607 | 360(4.6) |
| 1.056 | - | - | 250 | 1,073(0.2) | 707 | 191(2.7) | 229 | 9(25.4) | 1,596 | 585(2.7) |
| 1.059 | 4,625 | 341(13.6) | 610 | 488(1.0) | 583 | 153(3.8) | 166 | 186(0.9) | 936 | 577(1.6) |
| 1.062 | 5,380 | 1,491(3.6) | 257 | 30(8.6) | 217 | 55(3.9) | 34 | 79(0.4) | 780 | 469(1.7) |

Figures represent numbers of colonies per $10^5$ cells plated; the ratios of leukocyte feeder CFC/PHA-LF CFC are indicated in parentheses.
†From unfractionated material, numerous admixed T lymphocyte and leukemic colonies were grown in the PHA-LF assay.
*Not obtained separately (included with the cells of the subsequent fraction).

buoyant density characteristics gave rise to colonies in the two assays. In one patient (no. 5), the numbers of colonies in the density subfractions in double layer agar culture and the PHA-LF assay showed a constant ratio (Table 4); this suggests that in some cases identical cells are induced to form colonies in both assays. Further evidence for the proliferation of different subsets of leukemic progenitor cells in the PHA-LF and standard leukocyte feeder assays has been gathered previously by our group.[20] In one patient (no. 8 in this study), the leukemic cells showed karyotypically distinct cell populations (Table 2). Evidence was obtained here that the double layer leukocyte feeder technique selectively induced colonies from cells of the minor clone, while the PHA-LF assay demonstrated colony growth from the same subpopulations as in the noncultured cell material.[20] Differences in the buoyant density of PHA-LF and double layer agar CFC have been noted earlier in CML blast crisis patients.[15] Considered together, these data indicate that leukemic PHA-LF CFC and leukemic double layer agar CFC are not identical in most if not all of the cases.

Our observations suggest heterogeneity of cells of acute myeloid leukemia with respect to growth requirements for colony formation in vitro. Apparently the leukemic cells respond to granulocyte-macrophage colony-stimulating activity in the double layer agar cultures in a minority of patients. On the other hand, blast cell colony formation was obtained in most patients in PHA-LF cultures. The factors in the PHA-LF method necessary for leukemia cell colony growth are unknown at present. The variation in culture results between patients may be explained by differences in maturation of leukemic cells and, hence, differences in requirements for stimulation. The concept of variable maturation in acute leukemia as related to in vitro growth is so far supported by rare evidence. Hiraoka et al.[22] showed that expression of the Ia-like antigen in ANLL correlated with in vitro colony-forming capacities. Chang et al.[23] demonstrated heterogeneity with respect to the self-renewal capacity of leukemic colony-forming cells. They suggested that this could reflect diversity of differentiation between hierarchical subsets of leukemic blast cells. A similar heterogeneity may account for the different requirements of growth in the two colony culture methods in this report. Subsets of leukemic cells have also been distinguished by cytogenetic studies.[19,24] In patient no. 8 of this study,[20] cytogenetically distinct subpopulations required different growth conditions, as was evident from the selective outgrowth of the ancestral cell line in one of the two culture methods.

It may be concluded that the PHA-LF method is an assay for a specific subset of leukemic progenitors and is therefore of interest for further in vitro studies on proliferating blast cells, their growth requirements and therapeutic susceptibility.

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CHAPTER 8

# KARYOTYPICALLY DISTINCT SUBPOPULATIONS IN ACUTE LEUKEMIA WITH SPECIFIC GROWTH REQUIREMEMTS

B. Löwenberg, A. Hagemeijer & K. Swart, Blood **59** (1982), 641-645

# Karyotypically Distinct Subpopulations in Acute Leukemia With Specific Growth Requirements

By B. Löwenberg, A. Hagemeijer, and K. Swart

**Bone marrow and blood cells of a patient with acute monoblastic leukemia with subclones marked with specific karyotypic abnormalities were investigated. In order to more precisely evaluate the proliferative abilities of these populations, leukemic cell enriched fractions were prepared and incubated in two colony assays. Colony forming cells of the disparate clones had growth advantages in different systems which shows that their proliferation depended on the presence of selective stimulatory factors in culture. In one assay, at diagnosis, colonies from the minor clone were demonstrated exclusively. It is suggested that the assays measured distinct cellular stages of myeloid differentiation and the findings indicate that prior to diagnosis the neoplasm had evolved into subsets with progressive dedifferentiation. Differences of growth in vitro correlated with the different roles of these clones in the clinical history of the disease. Approaches based on differential cloning of tumor stem cells as in this example, may be useful for discriminating biological properties of heterogeneous subpopulations within neoplasms, and may facilitate the cytogenetic recognition of minimal clones among composite malignant cell specimens.**

THE BONE MARROW of patients with acute myeloid leukemia frequently shows acquired cytogenetic abnormalities[1,2,3] and, when present, these karyotypic changes provide a tool to follow the evolution of the disease.[4,5] New clones of cells derived from the original one, may present themselves with disease relapse but in some cases karyotypic evolution has taken place before diagnosis, as indicated by the presence of multiple clones at first presentation. In chronic myeloid leukemia the emergence of additional cytogenetic abnormalities is a frequent phenomenon and may herald the transformation to blastic phase.[6,7,8] The clinical role of a new clone is usually evident only from retrospective evaluation, and therefore in vitro assays for testing the proliferative abilities of separate clones would be helpful to assess their clinical significance. To date, in vitro tests have not been shown to successfully discriminate subclones of individual tumors.

In vitro colony assays are utilized to investigate the proliferative properties of leukemic progenitors. The leukocyte feeder colony cultures, initially employed for nonleukemic myeloid precursor cells,[9] did usually result in only abortive (so called cluster) growth when cells from patients with acute myeloid leukemia were cultured.[10,11] Culture methods supplemented with phytohaemagglutinin (PHA) or medium conditioned by PHA stimulated lymphocytes did not stimulate normal myeloid colony growth but proved successful in supporting the proliferation of acute leukemia stem cells.[12,13,14,15] The application of more than one colony assay to bone marrow and blood cell specimens from patients with hematological malignancies, coupled to cytogenetic analysis, may permit the differentiation of coexisting clones in single patients.

Here we report the cytogenetic and colony culture results in a newly diagnosed patient with acute monoblastic leukemia who carried a mixture of related subclones with distinguishable karyotypes. By using two culture systems and leukemic purified cell fractions, it could be demonstrated that leukemic cells belonging to cytogenetically different populations and with different roles in the clinical course of the disease, had different growth requirements in vitro.

## MATERIALS AND METHODS

### *Colony Assays*

Nucleated cells from bone marrow and blood were harvested following sedimentation of the erythrocytes in 0.1% methylcellulose. Our techniques for cell collection, the leucocyte feeder method of colony formation in culture and the PHA–leucocyte feeder assay (PHA–l.f. assay) have all been described.[13,14,16]

Leukemic colony formation in the leucocyte feeder and in the PHA–l.f. assays were estimated in triplicate cultures for unfractionated marrow and blood cells and for different density fractions of cells obtained as described below. Morphological examination and cytogenetic analysis of colony cells were also performed.

### *Cell Fractionation Procedure*

Blood or bone marrow cells were separated by a combination of E-rosette sedimentation and discontinuous albumin gradient fractionation. Nucleated cells ($10 \times 10^6$/ml) were incubated with an equal volume of a 1% (v/v) neuraminidase treated sheep red blood cells suspension as described.[17] The sedimented cells were carefully resuspended in bovine serum albumin (BSA) of density 1.050 g/ml, and pipetted on top of the gradient. The gradient was prepared of the following layers from top to bottom: 1.056 g/ml, 1.059 g/ml, 1.062

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g/ml, and 1.083 g/ml. BSA processing and preparation of the fractions have been reported.[17]

### *Cytogenetic Analysis*

Cytogenetic analysis was done according to standard procedures, slightly adapted to the different materials which were examined. Total bone marrow, blood and the different density fractions were cultured for 24 or 48 hr in the absence of PHA stimulation; the technique using methotrexate treatment and the harvesting procedure have been reported earlier.[18] Remission blood was cultured once for 72 hr with PHA stimulation in order to verify the karyotype of (nonleukemic) lymphocytes. Colony cells were derived from 7 day PHA–l.f. cultures and from 12 to 14 day leucocyte feeder cultures. The night before harvesting, medium containing methotrexate ($10^{-7}$M) was added to the dishes. The following morning colony cells were collected from the culture plates with a pasteur pipet, pooled in a sterile plastic tube, and spun down; fresh medium containing either TdR ($10^{-5}$M) or BrdU ($10^{-5}$M) was added to the pellet and the culture was continued for 5 to 6 hr at 37°C. Further processing was similar to bone marrow or blood cultures. Banding techniques were used to identify the chromosomes: QFA (Q–banding by fluorescence using atebrine), RFA (reverse–banding by fluorescence using acridine orange), GTG (G–banding by trypsin–Giemsa technique) and CBG (C–banding by barium hydroxide using Giemsa) according to the Paris Conference (1971) supplement 1975.[19]

### *Case History*

In November 1979 a 17 yr old man was admitted to the hospital with high fever (39°C) and a painful throat. The previous history was unremarkable. For several days he felt fatigue and was feverish. Gums hypertrophy and bilateral cervical lymphadenopathy were present. Liver and spleen reached 3 cm below the costal margins. A diagnosis of acute monoblastic leukemia (FAB–classification: M5)[20] was made. Leukocytes were 64.6 x $10^9$/1 with 1% neutrophils, 4% lymphocytes and 95% monocytes and monoblasts. A bone marrow aspirate showed excess (94%) blast cells with monocytoid appearance which stained α–naphtyl acetate esterase positive. Blasts were negative with Sudan black and periodic acid Schiff reagents. Bone marrow cells were aspirated for cytogenetic analysis and colony cultures. Selected hematological data at presentation and during patient follow up are given in Table 1.

Remission induction therapy was attempted repeatedly. Several trials which all failed to induce complete remission, included the following agents: cytosine–arabinoside, vincristin, daunorubicin, hydroxydaunomycin, 6–thioguanin, high dose methotrexate with leukovorin rescue and VP-16. At best a partial remission was obtained (Table 1). In April 1980, progressive leukemia with severe granulocytopenia and thrombocytopenia was evident. The patient, refractory to various forms of cytotoxic therapy, died in July 1980.

## RESULTS

Chromosome analysis at diagnosis revealed three abnormal karyotypes, respectively designated as clones A, B, C (Table 1). Clone A, (Fig. 1A) showed an apparently balanced translocation (6;11) (q27;q23). Clone B, (Fig. 1B) the major clone at diagnosis stemmed from A, and showed 51 or 52 chromosomes with different trisomies in addition to the t(6;11) : 51, XY,t(6;11), +der(6), +3,+19,+19,+21 and in the majority of the cells one pair of double minutes (dm) which stained only with Q– and G–banding, not with R–banding and showed no positive C–bands. Clone C observed in blood culture at diagnosis, showed a divergent evolution from the presumed parental clone A: 53,XY,t(6;11),+der(6),+4,+10,+13,+18,+19, +21. A PHA stimulated blood culture yielded lymphocytic metaphases with a normal constitutional karyotype: 46,XY.

Following treatment, during partial remission (Feb. 6, 1980), the bone marrow yielded mostly cells with the normal karyotype, a few cells of clone A but none of clone B and C. Remarkably, at the same time colonies

**Table 1. A Selection of Hematological and Cytogenetic Data of Patient With Acute Monoblastic Leukemia**

| | First Presentation* (Nov., 1979) | | Partial Response (Feb., 1980) | Relapse (April, 1980) |
|---|---|---|---|---|
| Blood | | | | |
| WBC (x $10^9$/1) | 64.6 | | 2.4 | 0.6 |
| blast cells† (%) | 95 | | 0 | 11 |
| Marrow | | | | |
| M/E ratio | 99/1 | | 2/1 | 12/1 |
| blast cells† (%) | 99 | | 25 | 87 |
| Cytogenetics | | | | |
| | Marrow | Blood | Marrow | Marrow |
| No. of cells analysed | 38 | 18 | 58 | 50 |
| with normal karyotype‡ | 0 | 0 | 50 | 3 |
| with karyotype A§ | 4 | 3 | 8 | 47[7] |
| with karyotype B‖ | 34 | 12 | 0 | 0 |
| with karyotype C¶ | 0 | 3 | 0 | 0 |

*Bone marrow taken before treatment

†Includes (mono)blasts and all immature and abnormally looking cells with monocytoid appearance.

‡Normal karyotype : 46,XY.

§Clone A : 46,XY,t(6;11) (q27,q23).

‖Clone B : 51 or 52,XY,t(6;11),+der(6)+3,+19,+19,+21±dm.

¶Clone C : 53,XY,t(6;11),+der(6)+4,+10,+13,+18,+19,+21.

One cell showed an additional translocation : 46,XY,t(6;11),t(3;5) (p12;q35). Blood culture with PHA was done on December 18, after one course of chemotherapy and revealed a normal lymphocyte karyotype in 16 cells.

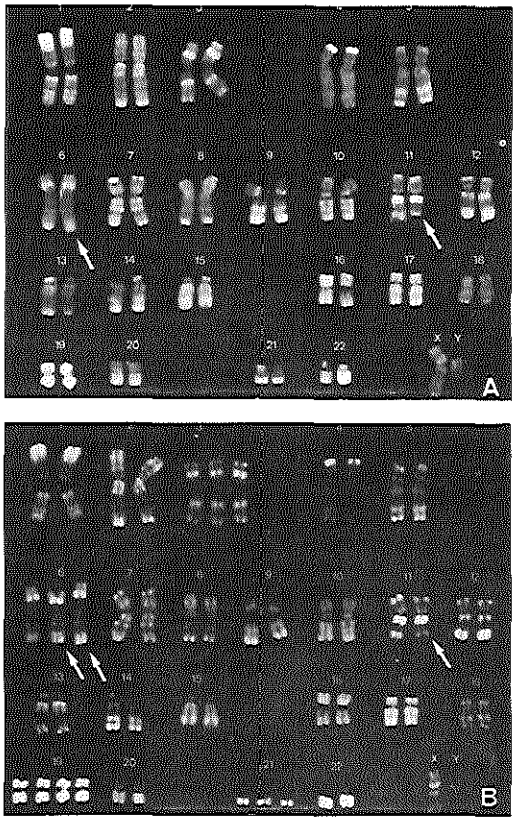

Fig. 1. Karyotypes observed in bone marrow cells of the patient at diagnosis. Upper section : 46,XY,t(6;11) (q27;q23) representative of clone A. Lower section : 51,XY,t(6;11), +der (6),+3,+19,+19,+21 representative of clone B. In this metaphasis one of the der (6) is slightly contracted. R-bands with acridine orange.

grown from unfractionated bone marrow (leucocyte feeded culture) showed only clone A metaphases (n = 17). On April 23, 1980, during florid relapse, three normal and 47 clone A karyotypes were identified.

*Cell Fractionation Studies*

Fractionation studies by the combined techniques of E-rosette forming cell depletion and discontinuous albumin gradient centrifugation require a large number of cells. They were applied to blood at diagnosis and to bone marrow at the time of relapse. Results of colony growth and cytogenetic analysis of the different blood cell fractions, before and after culture are given in Table 2. At diagnosis, numerically, clone B appeared the major one in fractions 1.056 and 1.059 g/ml. Grossly the same repartition between clones A and B were observed in colony cells in the PHA–l.f. assay. At the same time, leucocyte feeder colonies were grown in large numbers from fractions 1.056 and 1.059 g/ml. They exclusively showed type A metaphases (n = 32), indicating their formation from the minority clone only.

Morphological studies of the cell fractions before culture did not reveal apparent differences between the cells from fractions 1.056 through 1.065 g/ml. The colony cells in the leucocyte feeder assay were mostly monocytic cells and macrophages. In the PHA–l.f. assays undifferentiated blast cells were grown and cytochemistry was inconclusive.

Similar cell fractionation was performed on bone marrow at the time of relapse (Table 3) and an even lighter density fraction 1.053 g/ml was isolated. Colony growth in both techniques was numerous. Chromosome analysis showed a monotonous karyotype of clone A in all metaphases before and after culture in both assays.

## DISCUSSION

This study of one patient with acute monoblastic leukemia demonstrates that combined cytogenetic analysis and colony cultures may disclose within the neoplasm subclones with different karyotypes, growth requirements and therapeutic responses. The PHA–l.f. assay has been designed for acute leukemic blast colony growth and supports leukemic cell growth in about 80% of cases. The standard leucocyte feeder assay is suitable for normal myeloid stem cell proliferation rather than leukemia. In patients with AML usually no colony or minimal colony formation in the presence of large numbers of clusters is observed.[21,22,23,24] Only rarely will large numbers of colonies arise from AML bone marrow.

In the case reported here, colonies were grown in both assays. The PHA–l.f. assay did not show selectivity of growth for different leukemic subpopulations and clones A and B were represented in colony cells approximately in the same proportions as in the cell suspensions before culture. In the conventional leucocyte feeder method on the other hand, only cells of clone A with the minimal chromosome rearrangement formed colonies. The selectivity was striking because colony cells showed merely clone A metaphases whereas cytogenetic analysis had revealed that 85% of blood and bone marrow metaphases belonged to clone B at diagnosis (Table 1 and 2) or to normal stem cells at partial remission (Table 1).

Morphological examination of these colonies suggested that the cells from clone A were still capable of some maturation along the monocytic cell lineage. The findings that cells of the ancestral clone with the minimal and apparently balanced chromosomal rearrangement (karyotype A) were still able to grow in the

Table 2. Cytogenetic Analysis on Blood Cell Fractions Prior to and After Culture in Conventional Leucocyte Feeder and PHA-Leucocyte Feeder Colony Assays (Nov. 21, 1979—at diagnosis)

| | 1.056 g/ml | | | 1.059 g/ml | | | 1.062 g/ml‡ | | 1.065 g/ml§ |
|---|---|---|---|---|---|---|---|---|---|
| Fraction | Before Culture | Leucocyte Feeder Colonies | PHA-l.f. Colonies | Before Culture | Leucocyte Feeder Colonies | PHA-l.f. Colonies | Before Culture | PHA-l.f. Colonies | Before Culture |
| Number of colonies† | — | 229 | 10 | — | 165 | 185 | — | 79 | — |
| number of cells with karyotype‖ | | | | | | | | | |
| clone A | 2/8 | 16/16 | 1/5 | 0/12 | 16/16 | 2/6 | 1/3 | 3/4 | 6/8 |
| clone B | 6/8 | 0/16 | 4/5 | 12/12 | 0/16 | 4/6 | 2/3 | 1/4 | 2/8 |

*fractions obtained with concurrent E-rosette cell depletion and discontinuous albumin density gradient centrifugation.
†number of colonies per $10^5$ cells plated.
‡$65/10^5$ colonies were scored in the leucocyte feeder assay; these were not karyotyped.
§$26/10^5$ leucocyte feeder and $80/10^5$ PHA-l.f. type colonies were counted, but not karyotyped.
‖number of metaphases with the clone A or B karyotype as a ratio of the total number of cells karyotyped.

leucocyte feeder assay and that the derived clones B and C were not, fit in with the concept of progressive dedifferentiation of subclones during malignant evolution.

While cells of clone B did not grow in the leucocyte feeder system, they produced large numbers of blast cell colonies when tested in the PHA–l.f. assay. It indicates that the cells of clone B were capable of expanding in vitro, when given specific growth stimulating factors.

Cells from clone C were not recognized in the cell fractions, neither before nor after culture. This is not too surprising. From their karyotypic changes one could expect these cells to behave similarly as the cells from clone B. The number of cells analyzed from each fraction was probably too small to recover metaphases from this minor subclone. In any case, one can safely conclude that the fractions studied were not enriched in cells from clone C and that the culture techniques used were not selectively growing these cells.

It is noteworthy that clones A and B, characterised by specific karyotypes, and typical requirements of colony growth in vitro had seemingly different fates and roles in the course of the disease. Before diagnosis clones B and C had overgrown the parental clone A and, in this sense, demonstrated faster growth. Clones B and C were successfully eradicated following cytotoxic treatment and were not identified again, which is consistent with a complete remission of these components of the leukemia. Clone A cells persisted and were responsible for therapy failure, i.e., only partial remission. During partial remission, a cytogenetic survey demonstrated 50 normal karyotypes and eight clone A cells among 58 metaphases, and at the same time the leucocyte feeder colonies (from unfractionated marrow) were exclusively of clone A karyotype. Meanwhile, in spite of further therapy, clone A grew out and progressively replaced normal marrow elements, which had transiently regenerated following elimination of B and C cells.

It has been proposed that some myeloid leukemias can be induced to revert from malignant to a nonmalignant phenotype by induction of differentiation.[25] In this case we have an example of a leukemic population (clone A), still capable of some maturation in vitro (and possibly in vivo) that proved less amenable to treatment than the undifferentiated clone B. Differences in growth rates of both clones may possibly

Table 3. Cytogenetic Analysis on Bone Marrow Cell Fractions Prior to and After Culture in Conventional Leucocyte Feeder and PHA-Leucocyte Feeder Colony Assays. (April 23, 1980—at relapse)

| | 1.053 g/ml | | | 1.056 g/ml | | | 1.059 g/ml | | | 1.062 g/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fraction | Before Culture | Leucocyte Feeder Colonies | PHA-l.f. Colonies | Before Culture | Leucocyte Feeder Colonies | PHA-l.f. Colonies | Before Culture | Leucocyte Feeder Colonies | PHA-l.f. Colonies | Before Culture | Leucocyte Feeder Colonies | PHA-l.f. Colonies |
| No. of colonies† | — | 1606 | 350 | — | 1596 | 585 | — | 936 | 577 | — | 780 | 459 |
| No. of cells with karyotype A‡ | n.t. | 16/16 | 16/16 | 16/16 | 8/8 | 16/16 | 16/16 | 8/8 | 16/16 | 16/16 | 8/8 | n.t. |

*fractions were obtained by concurrent E-rosette cell depletion and discontinuous albumin gradient centrifugation.
†no. of colonies per $10^5$ cells plated.
‡number of metaphases with the clone A karyotype as a ratio of the total number of cells karyotyped
n.t. = not tested.

explain these different therapeutic susceptibilities: investigations along this line have not been made.

One important point which emerges from the findings in this patient is that in vitro assays can recognize subsets with qualitatively different proliferation/maturation capacities in human leukemia. We realise that we could make these observations on karyotypes related to growth abilities due to the unique circumstances in this patient. This limits the possibility to generalize from these findings at present. Nevertheless, if confirmed in situations of frequent karyotypic evolution (e.g. in CML), colony assays with varying selectivity may prove useful to characterise disparate cell populations among neoplasms for clinical monitoring. The principle of using assays, in which distinct cell clones exhibit preferential growth, could also potentially be applied to increase the sensitivity of cytogenetic analysis for minimal cell clones. The demonstration of pure clone A colonies from the minor clone A, as shown in our patient, is essentially in line with this assumption. One might suppose, that this approach could e.g., permit earlier detection of the emergence of new clones in hematological diseases (CML) which are prone to transform into acute leukemias.

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CHAPTER 9

# FEEDER CELL REQUIREMENTS FOR LEUKEMIA CELL COLONY FORMATION IN PHA SUPPLEMENTED CULTURES

K. Swart & B. Löwenberg, Submitted for publication

CHAPTER 9

# Feeder cell requirements for leukemia cell colony formation in PHA supplemented cultures

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Summary

The use of PHA supplemented colony cultures has recently offered new opportunities for studying acute myeloid leukemia (AML) cell growth in vitro. The active stimulator cells for AML colony forming cells have not been identified, although this could be important for optimal application of the technique and for elucidating differences in growth between normal and leukemic progenitor cells. In this study, feeder layers were prepared from subpopulations of normal peripheral blood leukocytes which were obtained by centrifugation through Ficoll Isopaque, E-rosette sedimentation and adherence separation. Underlayers containing lymphocytes (B, T or B+T) or adherent monocytes failed to stimulate AML colony formation. The colony stimulation capacity of total mononuclear cells was significantly decreased following depletion of T lymphocytes. The highest AML colony numbers were obtained when adherent monocytes and T lymphocytes in combination were added to PHA containing cultures. Stimulation of AML colony formation depended on the quantitative interrelationship of monocytes and T lymphocytes in the cultures. Thus, AML colony forming cells, unlike normal marrow GM-CFC, do not respond to monocyte stimulation alone and require for their proliferation an inducing factor derived from PHA exposed T lymphocytes and monocytes.

## Introduction

In vitro colony formation by acute myeloid leukemia (AML) cells in PHA containing cultures has become an accepted procedure for studying neoplastic cell proliferation in patients with AML. These cultures are of potential value in the analysis of the leukemia

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progenitor cell compartment and interesting results of the features of proliferation and differentiation of leukemia progenitor cells are accumulating; e.g. cell cycle characteristics (1), self-replication capacities (2), buoyant density properties (3) and cytogenetic abnormalities (3, 4) of leukemia progenitor cells have been studied and recent investigations have demonstrated specific growth abilities of karyotypically distinct subpopulations (4) and induction of cellular differentiation in culture (5). The colony formation techniques utilized by different groups vary in their detailed design. As stimulators, PHA (6) or PHA and leukocyte feeder cells (7) have been directly incorporated into the cultures, or a medium conditioned by leukocytes exposed to PHA (PHA-LCM) has been supplemented (8, 9).

Proliferation of AML cells usually does not occur or is limited to cluster formation (10, 11, 12, 13) in a leukocyte feeder colony assay without PHA (14). This indicates a positive role of the lectin in leukemia colony formation. It is unclear whether the effect of PHA is direct or whether it is mediated through an indirect mechanism on the leukemic colony forming cells, e.g., by stimulating normal leukocytes to release essential factors into the medium. The findings that the stimulatory activity of PHA-LCM for leukemia colony growth significantly exceeds that of conventionally prepared LCM plus an appropriate amount of PHA (15, Swart and Löwenberg, unpublished results) are in favour of an indirect function of the lectin.

Elucidation of the stimulative role of the cellular components incorporated into a feeder layer or used as the source of the conditioned media would seem to be important for understanding differences in stimulation of normal and leukemic cells, for clarifying differences between the activities of separate batches of stimulating materials and for standardizing the preparation of these materials. The experiments reported here were carried out to identify the active subset of feeder layer cells in leukemia colony formation. Peripheral blood leukocytes were separated by centrifugation through Ficoll-Isopaque, E-rosette sedimentation and adherence to tissue culture plastic. Each fraction of cells was tested for stimulation of the formation of AML colonies in culture in the presence of PHA.

Materials and Methods.

Bone marrow and blood cells were obtained from patients with acute myeloid leukemia (AML) at diagnosis or in relapse. Previously described techniques were used for cell collection and harvesting of nucleated cells (16). Only pure leukemia blast cells which had been obtained by density gradient separation combined with E-rosette depletion as previously described (17, 18) were cultured. These cells were used as fresh preparations or following cryopreservation in 7.5 % DMSO and 20 % fetal calf serum employing a controlled freezing apparatus and storage in liquid nitrogen (19).

Colony assay.

Leukemic colonies were grown in the PHA-leukocyte feeder (PHA-l.f.) assay as reported (16, 17). Cells were plated in 35 mm petri dishes in 0.4 ml liquid medium supplemented with 0.01 ml PHA (HA 15, Wellcome) at a concentration of $0.1 \times 10^5$ - $1.0 \times 10^5$ per dish. Triplicate cultures were incubated at 37°C in a humidified 7.5 % $CO_2$ atmosphere. At day 7, colonies of 50 cells or more growing at the surface of the underlayer were counted by use of an inverted microscope.

Feeder layers containing various sources of cells were prepared. The following cell concentrations of peripheral blood leukocytes or their fractions were used: 1) mononuclear cells, $1 \times 10^6$/dish; 2) E-RFC depleted mononuclear cells, $0.2 \times 10^6$ - $0.4 \times 10^6$/dish; 3) E-RFC enriched mononuclear cells, $0.4 \times 10^6$ - $0.8 \times 10^6$/dish; 4) non-adherent mononuclear cells, $0.5 \times 10^6$ - $1.0 \times 10^6$/dish; 5) adherent mononuclear cells, adherent cells from $1 \times 10^6$ mononuclear cells/dish or from $0.4 \times 10^6$ E-RFC depleted mononuclear cells/dish. These concentrations of fractionated cells were based on the yield of the fractions in the separation procedures and matched to the numbers of the same cell type in the unfractionated mononuclear cell suspension. Feeder layers with $1 \times 10^6$ mononuclear cells (plateau of dose response curve) were taken as the 100 percent stimulation value. Agar underlayers without feeder cells were used in control cultures. These gave negligible numbers (usually zero) of colonies.

Cell separation procedures for feeder layers.

To prepare feeder layers of varying fractions of peripheral blood leukocytes, the following separation procedures were performed. Mononuclear cells were obtained by centrifugation (30 min, 400 g)

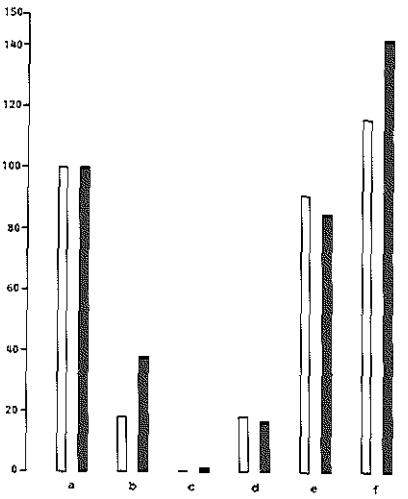
through Ficoll-Isopaque with a density of 1.077 $g.ml^{-1}$. Rosette forming and non-rosette-forming cells were separated by a second centrifugation on Ficoll-Isopaque after the cells (10 x $10^6$/ml) had been incubated with neuraminidase treated (18) or AET treated (20) sheep red blood cells to form E-rosettes. The E-RFC depleted and E-RFC enriched fractions were collected separately and washed two times. Sheep red blood cells in the E-RFC enriched fraction were lysed with autologous plasma (15 min) (21) and the remaining nucleated cells were washed two times. Mononuclear cells (1 x $10^6$) and E-RFC depleted mononuclear cells (0.4 x $10^6$) were incubated in medium (Hanks Balanced Salt Solution) with 2 % fetal calf serum in plastic tissue culture petri dishes (35 mm) for 1½ hours at 37°C in a 7.5 % $CO_2$ atmosphere to collect adherent and nonadherent cells. The non-adherent cells were harvested for use. The dishes were then washed twice with the same medium/serum mixture and agar containing Dulbecco's modified Eagle's medium was poured into these dishes to prepare underlayers of the adherent cells.

Results.

Pure AML blast cells were plated in cultures which, besides PHA, contained different fractions of mononuclear cells as stimulators. These cell fractions were evaluated for their capacity to support leukemia colony growth. The comparative AML colony numbers are given in Table I. The various fractions all had reduced capacities for stimulating leukemia colony formation as compared with the unfractionated mononuclear cells. To exclude the possibility of loss of stimulation capacities of monocytes due to the adherence procedure, reconstitution experiments in which nonadherent and adherent cells were recombined in feeder layers were carried out. In these cultures, colony formation was restored to 80 % - 100 % of control values (two experiments).

In another series of experiments, feeder layers of adherent cells were supplemented with increasing numbers of T lymphocytes (Fig 1). Stimulation of leukemia colony formation was restored following the addition of T lymphocytes which as a single population were unable to stimulate colony growth (Table I). Full recovery of stimulation was dependent on the number of T lymphocytes and the highest number of T lymphocytes stimulated colony formation to above reference values.

*Fig. 1. AML colony growth in the PHA assay with adherent monocytes and T lymphocytes or their combination, as feeder cells (compared to unfractionated mononuclear cells).*




*Horizontal axis: cells in feeder layer.*

- *a = control, 1 x $10^6$ peripheral blood mononuclear cells (MNC)*
- *b = T-depleted MNC, 0,4 x $10^6$*
- *c = adherent cells from 0.4 x $10^6$ T-depleted MNC*
- *d = c + 0.2 x $10^6$ T cells*
- *e = c + 0.4 x $10^6$ T cells*
- *f = c + 0.8 x $10^6$ T cells.*

*Vertical axis: number of colonies relative to the control cultures (set at 100 %).*

□ *and* ■ *= two separate experiments.*
*Unstimulated cultures (no cells in feeder layer) were always run paralel and gave no colonies.*

Discussion.

Leukemic blast cell proliferation in leukocyte feeder or LCM cultures in the absence of PHA is restricted to cluster formation and only rarely is normal sized colony growth apparent from AML bone marrow or blood in these cultures. When AML cells are cultured in the presence of PHA-LCM or PHA and leukocyte feeder cells, the colony forming efficiency is significantly increased. This higher efficiency can be explained by: a) the release of a stimulating factor different from GM-CSA in the presence of PHA to which the leukemic cells respond by proliferation; b) a shift in the dose response to normal GM-CSA due to a cofactor which is produced following PHA stimulation; or c) a direct stimulating effect of PHA on leukemic cells. It is unlikely that enhanced colony formation is due to mainly a direct effect of PHA, because cultures with only PHA

*Table I. Feeder layers prepared from various blood cell fractions and tested for stimulation of leukemia colony formation.*

| procedure | cell fraction | predominant cell type | mean stimulation capacity (percent ± SD)* | number of experiments |
|---|---|---|---|---|
| Ficoll | interphase | T+B+monocytes | 100 † | 7 |
| Ficoll + adherence | nonadherent | T+B | 15 ± 22[a)] | 5 |
| | adherent | monocytes | 10 ± 12[a)] | 5 |
| E-rosette ficoll | interphase | B+monocytes | 60 ± 33[b)] | 7 |
| | sediment | T | 16 ± 18[a)] | 7 |
| E-rosette ficoll + adherence | interphase nonadherent | B | n.d. | |
| | interphase adherent | monocytes | 1 ± 1 | 2 |

* AML colony growth in the PHA assay with different leukocyte fraction supplements; only pure leukemia blast cells (see Materials and Methods) were plated.

† Colony numbers set at 100 %; the other data are expressed as relative values.

a) and b) statistically significant at p-values of 0.001 and 0.01, respectively (two-tailed, paired t-test).

as stimulator and LCM stimulated cultures supplemented with PHA gave poor colony growth (15; Swart and Löwenberg, unpublished results). The fact that others have used PHA-LCM in leukemia colony assays (8, 9) which contained only minimal concentrations of PHA in the final cultures is also evidence in favour of an indirect effect of PHA. Whether this effect depends on a cofactor of GM-CSA or on a separate stimulating factor cannot be concluded with certainty. The substance responsible for leukemia colony stimulating or enhancing activity in PHA-LCM cultures has been purified by Price and McCulloch (22) and Fauser and Messner (23). Preliminary data suggest that this leukemia colony stimulating factor has a molecular weight of 44,000 daltons with an active subunit of 27,000 daltons (22). These values are distinct from those known for GM-CSA preparations. Also by isoelectric focusing, the activity for leukemic blast cell progenitors appeared to be partially separable from factors active on normal hematopoietic precursors (23). Therefore, it has been suggested that a distinct stimulating factor is involved in the formation of leukemia colonies. The experiments reported here support this suggestion. In vitro growth of AML blast cell progenitors required feeder cells which differ from those needed for proliferation of normal GM-CFC. Peripheral blood monocytes are active sources of colony stimulating activity for normal bone marrow GM-CFC (24, 25). When incorporated into the feeder layer of the PHA colony system, however, purified adherent monocytes were unable to stimulate leukemia colony growth. Similarly, T lymphocytes failed to induce the formation of leukemic colonies following appropriate incubation. The combined addition of these two cellular components, on the other hand, provided significant stimulation. This suggests that a combination of monocytes and T lymphocytes as well as PHA is required for the production of a factor by which leukemic progenitor cells are induced to proliferate into colonies. This effect was shown to be dependent on the actual numbers of stimulating cells in the cultures.

Various groups have indicated that a wide spectrum of stimulating activities is produced in cultures supplemented with PHA and leukocytes, e.g., GM-CSA (26, 27), erythroid burst feeder/promoting activity (28, 29), an activity that stimulates colony forming units-spleen (CFU-s) (30, 31) and a factor required for the proliferation of mixed granulo-erythropoietic colony forming cells (CFU-GEMM) (32). It is unknown whether the growth factor required for leukemia colony formation is identical to one of these. It appears of interest to define the leukemia growth factor in relation to the

series of regulators for normal hematopoietic progenitors. The results of the experiments described in this paper may be useful for optimalization of the cellular conditions for cultures of human leukemic colony formation.

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CHAPTER 10

# GENERAL DISCUSSION

# CHAPTER 10
# General dicussion

In vitro clonogenic culture techniques have been introduced to study the different cell lineages in hematopoiesis. At present, colony assays are available for granulocyte-monocyte, erythroid, megakaryocyte and B and T lymphocyte progenitor cells (see e.g. Metcalf 1977; Cline and Golde, 1979). Granulocyte-monocyte colony growth has been extensively used to study hematopoiesis in health and disease and has revealed multiple abnormalities in the early commited stem cells in chronic myeloid and acute non-lymphocytic leukemia. Several investigators have used the granulocyte-monocyte colony assay (standard leukocyte feeder technique) to study leukemic hematopoiesis in vitro. In acute non-lymphocytic leukemia these studies were hampered by the lack of real colony growth. Therefore, at different laboratories attempts were undertaken to develop colony culture techniques which would enable leukemic progenitor cells to grow, i.e. to stimulate to proliferation of those leukemic cells which have the potential for extensive proliferation and presumably are closely related to leukemia stem cells. We have introduced a colony method which might fulfil this criterium. This new method was compared with the standard technique for myeloid colony formation (CHAPTERS 4, 5, 6, 7) and it was investigated whether these techniques could be employed to differentiate different subclasses of progenitor cells in the leukemic neoplasm (CHAPTERS 6, 7, 8). Unfortunately, the initial experiments revealed frequently admixture of T lymphocyte colonies to leukemia colonies in the PHA-l.f. technique (Löwenberg and Hagemeijer, 1978; CHAPTERS 4, 5). Subsequent investigations,utilizing improved cell separation methods, showed selective leukemia colony growth in the PHA-l.f. assay and allowed a direct comparison of progenitor cells which gave rise to colonies in different culture methods (CHAPTERS 6, 7, 8). In patients with CML, colony formation in the standard leukocyte feeder cultures as well as in the PHA-l.f. method was derived from comparatively light cells. Thus, CML colony forming cells (CHAPTERS 5, 6) were distinguishable from normal GM-CFC (CHAPTER 3) by their low buoyant density; the same phenomenon had been shown by Moore et al. (1973) using the other, standard leukocyte feeder culture technique. The following characteristics appeared from a comparison

of aspects of leukemia colony formation in the standard leukocyte feeder method and in the PHA-l.f. assay. Firstly, colonies in both culture systems arise from cells which become concentrated in the lightest fractions following density gradient centrifugation (CHAPTERS 5, 6). Secondly, in a number of patients the density profiles of progenitor cells which give rise to colonies in the different culture methods, were not identical (CHAPTER 6). This finding was most prominent in CML-BC patients. Thirdly, cells of colonies in the standard leukocyte feeder method showed normal myeloid differentiation to mature granulocytes and monocytes/macrophages whereas colonies in the PHA-l.f. assay were composed of blast cells (CHAPTERS 4, 6). We concluded that a) the two colony methods allowed for a quantitative and qualitative analysis of progenitor cells in CML and CML-BC and b) the results were in favor of the idea that colonies in the different culture systems were produced by different progenitor cells. The latter conclusion warrants a few comments. Density profiles of the two types of colony forming cells were discrepant only in part of the patients (CHAPTER 6). However, the differences were found to be consistent in repeat experiments at different times in the same patients. In cases with no apparent differences between the density profiles of these two populations of colony forming cells two considerations should be taken into account; firstly, cells with divergent functional properties could still have identical physical (i.e. density) properties and, secondly, the resolution capacity of discontinuous density gradients could be too limited to allow the detection of small density differences. We feel that other approaches, directed at the functional features of progenitor cells, may be helpful to define the properties of the two distinct populations of colony forming cells, their interrelationship in terms of position in maturation hierarchy, and their fate and role in the evolution of the disease. This point will be discussed in more detail below.

Our results in acute non-lymphocytic leukemia are essentially a confirmation of the data obtained in CML. In vitro colony growth of leukemic cells was obtained from the least dense cell fractions (CHAPTER 7) and ANLL colony forming cells appeared of lower buoyant density than normal GM-CFC (CHAPTER 3). Analysis of the culture results revealed the absence of progenitor cells capable of giving rise to colonies in the standard leukocyte feeder method in the majority of patients. On the other hand, leukemic colonies were grown in the PHA-l.f. assay from blood or bone marrow cells of all but two in a group of 14 patients. Thus, the difference between the

two types of colony forming cells in most ANLL patients is that PHA-l.f. responding colony forming cells are present whereas their counterparts assayed in the standard leukocyte feeder colony method are not demonstrable. In four patients, however, colony growth was obtained in both culture techniques. Analogous to the results in CML,density differences between both types of colony forming cells were observed in three patients, in one of them at presentation as well as in relapse. The evidence for the proliferation of distinct subsets of progenitor cells in the different colony assays was even more strengthened following cytogenetic analysis of colony cells in one patient (CHAPTER 8). The leukemia in this patient happened to include subclones with specific karyotypic abnormalities. These subclones were different with respect to growth potential in standard leukocyte feeder and PHA-l.f. colony assay and thus, expressed different in vitro growth requirements. Subsequently, it became evident that these subclones were differently responsive to chemotherapeutic treatment. These observations raise the question whether subsets of leukemic progenitor cells with potentially divergent roles in the course of the disease, can be distinguished prospectively by in vitro colony culture methods. To answer this question more knowledge of the properties of the complex, proliferating cell compartment in leukemia is required. If we assume a hierarchical structure in a leukemic cell population, only certain cells are responsible for the maintenance of the cell population, whereas others, probably the majority of cells, differentiate and loose proliferative capacity. This model has been solidly established in normal hematopoiesis and CML but is less obvious in CML-BC and ANLL. Colony culture data presented in this thesis (CHAPTERS 4 - 9) and those reported by others (see e.g. Dicke et al., 1976; Park et al., 1977; McCulloch and Till, 1981),however, would be compatible with such a concept in acute leukemia as well. The great majority of leukemic cells has not retained the stem cell capacities of proliferation and, hence, has no part in the maintenance and/or evolution of the neoplastic clone. The expansion of the neoplastic clone depends on self replication of cells within the stem cell compartment. Leukemic colony forming cells may be considered as representatives of the leukemic stem cell compartment. Experiments by Buick et al. (1979) and Chang et al. (1980) have provided evidence for self renewal of acute leukemia colony forming cells. These investigations also revealed heterogeneity of leukemia colony forming cells with respect to self renewal capacity, suggesting hierarchical stages among the population of colony

forming cells. Heterogeneity with respect to buoyant density (CHAPTERS 6, 7) and in vitro growth requirements (CHAPTER 8) of distinct populations of colony forming cells could be in line with this concept. Furthermore, our experiments (CHAPTER 8) revealed that, comparing karyotypically distinct clones, progenitor cells of the cytogenetically least effected, ancestral clone were still capable of giving rise to colonies in the standard technique for normal GM-CFC, whereas a daughter clone with more severe abberrations required the specific conditions of the PHA-l.f. culture method for in vitro growth. Thus, it is suggested that progressive dedifferentiation in a leukemic cell population may correspond with loss of the capacity to proliferate in vitro under conditions designed for normal myeloid progenitor cells. Whether maturation sequences within a leukemic cell population can be detected by other means is still unclear. Preliminary experiments by Marie et al. (1981) suggest cell marker (antigenic and cytochemical) differences between functionally different leukemic cells. The availability of cytochemical staining methods and even more of monoclonal antibodies directed against a broad variety of differentiation markers on hematopoietic cells, may become highly important for investigations along this line. These techniques, in particular when combined with colony culture methods, are of great interest to correlate proliferative properties with phenotypic characteristics of leukemic cells. This approach might contribute data to a better understanding of (the lack of) control of proliferation and differentiation in leukemia. Sachs (1978, 1980), on the basis of experimental animal studies, has put forward a model in which proliferation and differentiation are considered to be linked, i.e. counteracting processes in leukemia. He showed that a block of differentiation was associated with more prominant proliferative abilities and that, if differentiation was promoted, proliferation capacities were lost. Although this model is attractive, it would be important to verify whether similar changes occur in the stem cell compartment of human leukemias following differentiation induction. Is, e.g., the regulation of proliferation of leukemia stem cells dependent on the degree of differentiation of their progeny? Recently, McCulloch and Till (1981) have suggested that the blast cell population in leukemia is an independent lineage rather than a stage in one or more of the pathways of myelopoietic differentiation. Hence, leukemic cell proliferation may be independent of normal feedback regulation. Francis and coworkers, however, have proposed a model in which leukemic clonogenic cells correspond with normal progenitor cells in a genuine position of

the granulocyte-monocyte maturation hierarchy (Francis, 1979; Francis et al., 1981c). The observed decreased sensitivity of ANLL clonogenic cells to CSA (Francis et al., 1979; 1981b) has been suggested to be the consequence of a predominance of rather undifferentiated progenitor cells. In vitro colony culture studies combined with analysis of cellular differentiation markers can be of use to test the validity of aspects of the above mentioned models. To date, our studies are in line with the assumption of cellular heterogeneity and favor a hierarchical structure within a leukemic cell population. In normal hematopoiesis density properties of progenitor cells are ranked according their degree of maturation, with the most immature progenitor cell having the lowest density(Bol et al., 1979; Francis et al., 1981c). A similar relationship between PHA-l.f. responding and standard leukocyte feeder responding leukemic colony forming cells is not apparent from our studies. In some patients colony forming cells in the PHA-l.f. assay had a lower buoyant density than colony forming cells in the standard leukocyte feeder cultures, whereas in others the reverse was observed (CHAPTERS 6, 7). This inconsistency of the rank order of the buoyant density of the two types of leukemic progenitor cells might be explained by the fact that the relationship between physical and functional properties of progenitor cells in leukemia has been lost, probably due to divergent abnormalities in the differentiation programs in different cases of leukemia. In one case of proliferation of distinct subsets of progenitor cells in standard leukocyte feeder and PHA-l.f. cultures, the two types of colony forming cells did represent different stages in the hierarchy of genesis as evidenced by a parental karyotype in one clone and a daughter karyotype in the other (CHAPTER 8). It is possible that different maturation stages also occur in leukemias in the absence of karyotypic markers and, hence, are more difficult to identify, but may be disclosed by the application of colony culture techniques in combination with e.g. antigenic marker analysis.

It should be realized that normal myeloid progenitor cells may also produce colonies in the standard leukocyte feeder cultures as well as in the PHA-l.f. assay. This can particularly be the case when a considerable hematopoietic proliferation activity of normal cells has remained in the presence of leukemia. Residual normal progenitor cells may obscure the identification of different subpopulations of leukemic progenitor cells. When leukemia specific cytogenetic markers are absent, one would depend on the distinct density properties and on different membrane markers (e.g. anti-

genic) for distinguishing between leukemic and normal colony forming cells and to elucidate whether the leukemic cell compartment comprises one or more subpopulations.

Proliferation of leukemic progenitor cells with different maturation stages in certain culture systems is dependent on the availability of the appropriate stimulation factors. Monocytes are the major source of GM-CSA in the standard leukocyte feeder cultures (Chervenick and LoBuglio, 1972; Golde and Cline, 1972). Leukemia colony growth in the PHA-l.f. assay required T lymphocytes in addition to monocytes as feeder cells (CHAPTER 9). These increased requirements for stimulation suggest the involvement of other factors, either as stimulating factors as such, or as enhancing factors acting in cooperation with GM-CSA. Purification and characterization of the growth factors required by leukemic colony forming cells would be helpful to relate these factors to the spectrum of regulators for normal hematopoietic progenitors. It is also anticipated that investigations along this line may clarify differences in control of proliferation between distinct subclones within a leukemic cell population and between leukemic progenitor cells and normal hematopoietic precursors.

# SUMMARY

# Summary

In the past decade, in vitro colony culture methods have been developed to study cellular proliferation and differentiation in hematopoiesis. The application of these culture methods in human myeloid leukemia revealed several disturbances in the hematopoietic stem cell compartment of these neoplastic diseases. It has been the general finding that acute leukemia cells lack the ability to produce colonies in the culture systems designed for the growth of progenitor cells in normal hematopoiesis. Our investigations intended to develop a colony culture system specific for leukemic progenitor cells and to characterize these cells in order to get some insight into pathophysiological events in leukemia.

In the introduction in CHAPTER 1 the development of clonogenic in vitro culture methods for hematopoietic progenitor cells is briefly reviewed and the current status of regulation in normal granulo- and monocytopoiesis is summarized. In the myeloid leukemias several abnormalities in the hematopoietic progenitor cell compartment have been detected utilizing the standard culture technique for normal granulocyte-monocyte colony forming cells (GM-CFC). A description of the abnormalities relevant to the studies in this thesis shows the value of the application of in vitro culture methods to understand proliferation and differentiation defects in myeloid leukemias. The lack of colony growth in most cases of acute non-lymphocytic leukemia (ANLL) and blast crisis of chronic myeloid leukemia (CML-BC) has raised the question whether other culture devices would allow the in vitro proliferation of leukemic progenitor cells. Different laboratories succeeded in developing culture techniques in which leukemic cells give rise to colonies and thus methods became available to study leukemic progenitor cells in vitro. The colony culture method developed in our laboratory was designated the PHA-leukocyte feeder (PHA-l.f.) assay.

In CHAPTER 2 the objectives of the studies are described. The investigations were carried out,

1) to establish the value of the PHA-l.f. colony assay for the analysis of progenitor cells in human myeloid leukemia
2) to characterize the buoyant density of leukemic cells forming colonies in the PHA-l.f. cultures in comparison with normal GM-CFC
3) to compare leukemic progenitor cells which give rise to colonies

in the PHA-l.f. cultures with those producing colonies in the standard leukocyte feeder culture method without PHA

4) to analyse whether in vitro colony culture techniques could be helpful to disclose distinct subpopulations of progenitor cells within a leukemic cell population
5) to establish the growth requirements for leukemic progenitor cells to produce colonies in vitro.

The first paper (CHAPTER 3) deals with an analysis of T lymphocyte colony forming cells (TL-CFC) in bone marrow and blood of hematologically normal individuals. These studies were carried out because preceding experiments had revealed the admixture of T lymphocyte colonies in leukemic colony formation in the PHA-l.f. assay. Investigations were undertaken to characterize some properties of TL-CFC which would be neccessary for designing procedures for the depletion of these interfering colony forming cells from blood and bone marrow of leukemia patients. In blood as well as in bone marrow TL-CFC were shown to be E-rosette positive. Following density gradient centrifugation blood and bone marrow derived TL-CFC appeared different. TL-CFC in the bone marrow were found to comprise a cell population more dense than normal GM-CFC and had the peak of the density profile at 1.071 g/ml. Blood TL-CFC appeared less dense cells with the peak of the density distribution at 1.065 g/ml. The results of these experiments suggested that cell fractionation procedures utilizing E-rosette depletion or density gradient centrifugation could serve to separate TL-CFC from the other blood and bone marrow cells of leukemia patients.

In CHAPTER 4 experiments are described that provide evidence for the growth of leukemia colonies in the PHA-l.f. assay, even in cases where the standard leukocyte feeder method failed to do so. It is also shown that, without cell separation prior to culture, the admixture with T lymphocyte colonies is frequently considerable. Application of the E-rosette ficoll method to remove E-RFC improved the selectivity of the PHA-l.f. assay for leukemia colony growth. However, pure leukemia colony formation was not obtained in all experiments. It was concluded that a better separation procedure of TL-CFC and leukemic CFC was desirable.

CHAPTER 5 reports on the application of albumin density gradient centrifugation to purify leukemia colony forming cells in CML. The experiments were also directed to establish the density properties of leukemic cells which grow colonies in the PHA-l.f. assay in comparison with those forming colonies in the standard leukocyte feeder technique. In nearly all experiments pure leukemic colonies

were cultured from the lightest cell fractions (1.059 g/ml and/or 1.056 g/ml). It was also clear from these experiments that, despite the relatively high density of TL-CFC, the density profiles of leukemia CFC and TL-CFC overlapped. This phenomenon was more evident in blood than in bone marrow, which is in good agreement with our observation that bone marrow TL-CFC were recovered from fractions of slightly higher density than blood TL-CFC (CHAPTER 3). Due to the admixture of leukemia colonies with T lymphocyte colonies in the density fraction 1.062 g/ml and in experiments with blood, also in fraction 1.059 g/ml, we could not yet reliably estimate the density properties of the leukemic colony forming cells in the PHA-l.f. assay. The experiments confirmed the finding of other investigators that CML colony forming cells have a lower buoyant density than normal GM-CFC.

CHAPTER 6 deals with an improved separation procedure applied to CML blood and bone marrow cells to further analyse the characteristics of the population of colony forming cells in the PHA-l.f. assay in direct comparison with the cells giving rise to colonies in the standard leukocyte feeder culture method. In these experiments the basic separation procedure was discontinuous density gradient centrifugation. By permitting T lymphocytes in the cell samples to form E-rosettes prior to gradient centrifugation we achieved an almost complete depletion of TL-CFC from the light fractions up to 1.062 g/ml. Thus, colony growth by leukemic cells covering a density range of 1.056 to 1.062 g/ml could be analysed in the two culture systems without annoying admixture of T lymphocyte colonies. It was demonstrated that the density profiles of precursor cells giving rise to colonies in two different culture systems, the PHA-l.f. assay and the standard leukocyte feeder culture method, were not identical in several patients. The most significant differences were apparent in CML-BC patients. These results suggested that blast cell colonies in the PHA-l.f. assay and colonies of differentiated cells in the standard leukocyte feeder cultures originated from different precursor cells and thus, that both methods could be used as additive, not as duplicate, means in the analysis of the proliferating cell compartment in myeloid leukemia.

In CHAPTER 7 the PHA-l.f. assay was applied to bone marrow and blood from a series of patients with acute non-lymphocytic leukemia. To obtain pure leukemic blasts, the discontinuous density gradient centrifugation method with simultaneous depletion of T lymphocytes was used. These cells were also cultured in the standard leukocyte

feeder colony method to allow a direct comparison of the in vitro growth abilities in both culture systems. Only in a few cases colonies were formed when the cells were plated in the standard leukocyte feeder cultures without PHA. In contrast, colony growth in the PHA-l.f. assay was obtained in 13 of 15 experiments. This occured in the absence of T lymphocyte colonies. The leukemic origin of the colonies was documented by cytogenetic analysis of colonies in all cases when the leukemia had a specific karyotypic marker. Leukemic colonies in the PHA-l.f. assay were generated by cells of unusually low buoyant density and, thus, were found to differ from normal GM-CFC. In some patients the density profile of colony forming cells was different from the overall density profile of the leukemic blasts. This was indicative that colony forming cells represented a certain subset of the leukemic cells in general. Only in those cases in which leukemic colony growth appeared in the standard leukocyte feeder cultures as well, a comparison of the density properties of these colony forming cells could be made with those growing in the PHA-l.f. method. Density profiles of standard leukocyte feeder responding CFC were different from density profiles of PHA-l.f. CFC in the same patients, suggesting that different subclasses of leukemic cells proliferated in the two culture systems. While the standard leukocyte feeder cultures had proved to support the growth of ANLL colonies in exceptional instances, these experiments thus demonstrated that the PHA-l.f. assay permits investigations on leukemic blast progenitor cells in the majority of ANLL patients. The PHA-l.f. assay therefore, provides a practical method to gain insight in the processes of proliferation and differentiation in ANLL cell populations. Our investigations revealed also the potential value of monitoring simultaneously ANLL growth in the standard leukocyte feeder colony assay, because a distinct population of progenitor cells gave rise to colonies in this culture system.

The theoretical possibility that the application of different colony assays might disclose subsets of leukemic progenitor cells with divergent roles in the malignant process was corroborated by experimental data summarized in CHAPTER 8. This paper describes detailed cytogenetic and colony culture investigations in a case of acute monoblastic leukemia (M5;FAB-classification). The leukemia cell population in this patient included karyotypically distinct subpopulations, thus providing a tool to analyse the in vitro growth potential of cells belonging to the separate clones in the different colony culture methods. Numerous colonies were grown in

the leukocyte feeder cultures without PHA, however, they appeared representatives of the minor, parental clone only. This indicated the selectivity of this culture method for the subpopulation of leukemic cells with the least complex, i.e., the presumably originally arisen abnormal karyotype. Thus, these leukemic cells were still able to proliferate in the leukocyte feeder method, as do normal GM-CFC, while the cells with the more complex karyotype had lost this capacity. Cytogenetic analysis of colonies obtained from the PHA-l.f. cultures revealed a predominance of the major clone. It demonstrated the non-selective outgrowth of progenitor cells of both clones in this colony method. In the course of the disease, the separate clones had clearly different roles with respect to their growth advantages in vivo and their therapeutic susceptibility. These observations showed that karyotypically distinct subpopulations of one neoplasm with different fates and roles in the course of the disease had different growth requirements and their proliferation depended on the supply of selective stimulatory factors in vitro. The analysis of progenitor cells that give rise to colonies in different culture methods offers an approach to detect the emergence of minimal subpopulations during clonal evolution of leukemias and could thus in theory have value in early diagnosis of leukemic transformation (e.g., blast crisis in CML).

CHAPTER 9 deals with experiments which were carried out to establish the function of leukocyte feeder cells in the stimulation of leukemia colony growth in the PHA-l.f. assay. It is of particular interest to characterize the in vitro conditions for leukemia cell proliferation and to compare these conditions to those required for normal hematopoietic progenitor cell proliferation. Leukemia colony formation in the PHA-l.f. method was shown to be dependent on the combined presence of T lymphocytes and monocytes in the feeder layer. Separately, lymphocytes (B, T and B+T) and monocytes were unable to stimulate colony formation. The quantitative inter-relationship of monocytes and T lymphocytes determined maximum stimulation. Undoubtly, these results are important for explaining differences between batches of stimulative material (feeder cells or conditioned medium) and for standardizing the technique for leukemia colony formation. In addition, the experiments suggested that the function of the lectin PHA in leukemia colony growth is mainly indirect, i.e., PHA serves to induce essential growth factor production by feeder cells. These investigations indicated that leukemia colony forming cells respond to other growth factors than do normal colony forming cells which can be induced to proliferate by adherent monocytes in the absence of T lymphocytes and PHA.

# SAMENVATTING

# Samenvatting

In het laatste decennium zijn in vitro koloniekweekmethoden ontwikkeld om de proliferatie en differentiatie te bestuderen van cellen welke behoren tot het hematopoietisch (bloedcelvormend) systeem. Met behulp van deze kweekmethoden werden diverse afwijkingen aangetoond in het hematopoietisch stamcel-compartiment van menselijke myeloïde leukemieën. In de kweekmethoden die in gebruik zijn voor de groei van voorlopercellen van de normale hematopoiese bleken acute leukemiecellen geen kolonies te kunnen vormen. In het hier beschreven onderzoek werd daarom een nieuw kweeksysteem toegepast waarin leukemische voorlopercellen kunnen uitgroeien tot kolonies. De studies werden verricht om in dit kweeksysteem selectief leukemische koloniegroei te verkrijgen en om een aantal eigenschappen van de leukemische kolonievormende cellen vast te stellen. De onderzoekingen beogen een basis te bieden om langs experimentele weg meer inzicht te verkrijgen in de pathofysiologie van leukemie.

In HOOFDSTUK 1 is de ontwikkeling van klonogene in vitro kweekmethoden voor hematopoietische voorlopercellen samengevat. Hier is ook een overzicht gegeven van het huidige model voor de regulatie van de vorming van granulocyten en monocyten. Enkele belangrijke waarnemingen, verricht met behulp van de standaard kweekmethode voor normale GM-CFC (granulocyten - monocyten kolonievormende cellen), zijn vermeld om aan te geven dat de toepassing van in vitro kweekmethoden waardevol is voor het begrip van defecten in cel-proliferatie en -differentiatie in myeloïde leukemieën. Het ontbreken van koloniegroei in de meeste gevallen van acute niet-lymfocytaire leukemie (ANLL) en blasten crisis van chronische myeloïde leukemie (CML-BC) heeft de vraag doen rijzen of wijzigingen in de kweekomstandigheden konden worden aangebracht waardoor leukemische voorlopercellen wel in staat zouden zijn uit te groeien tot kolonies. Dergelijke kweeksystemen werden ontwikkeld in verschillende laboratoria en daarmee kwamen methoden beschikbaar om leukemische voorlopercellen in vitro te bestuderen. De in ons laboratorium ontwikkelde koloniekweekmethode werd genoemd de "PHA-leukocyten feeder (PHA-l.f.) assay".

In HOOFDSTUK 2 zijn de doelstellingen van de onderzoekingen beschreven. De studies werden uitgevoerd om:

1) de waarde vast te stellen van de PHA-l.f. koloniekweekmethode ten behoeve van de analyse van voorlopercellen in humane myeloïde leukemie ;

2) de zweefdichtheid (in albumine dichtheidsgradienten) te bepalen van de leukemische cellen die kolonies vormen in de PHA-l.f. kweekmethode en deze zweefdichtheid te vergelijken met die van GM-CFC in normaal beenmerg;
3) leukemische voorlopercellen die kolonies voortbrengen in de PHA-l.f. kweekmethode te vergelijken met die welke in staat zijn kolonies te vormen in de standaard leukocyten feeder kweekmethode zonder PHA;
4) te onderzoeken of in vitro koloniekweekmethoden van nut kunnen zijn om afzonderlijke subpopulaties van voorlopercellen binnen een leukemische celpopulatie van elkaar te onderscheiden;
5) de specifieke behoeften van leukemische voorlopercellen voor het uitgroeien tot kolonies in vitro vast te stellen.

In HOOFDSTUK 3 is een onderzoek van T lymfocyten kolonievormende cellen (TL-CFC) in normaal beenmerg en bloed beschreven. Dit onderzoek werd uitgevoerd omdat uit voorgaande experimenten was gebleken dat in de PHA-l.f. kweekmethode naast leukemische kolonies ook T lymfocytenkolonies werden gevormd. Enkele eigenschappen van TL-CFC werden bepaald om aan de hand hiervan methoden te kunnen ontwikkelen die gebruikt kunnen worden voor het verwijderen van deze storende kolonievormende cellen uit bloed en beenmerg van leukemiepatienten. TL-CFC in bloed, zowel als in beenmerg, bleken E-rozette positief te zijn. Dichtheidsgradient centrifugatie toonde verschillen aan tussen TL-CFC afkomstig uit bloed en uit beenmerg. De piek van het profiel van de dichtheidsverdeling van beenmerg TL-CFC werd gevonden bij 1.071 g/ml, hetgeen betekent dat deze celpopulatie een hogere zweefdichtheid heeft dan normale GM-CFC. Het profiel van de dichtheidsverdeling van bloed TL-CFC vertoonde de piek bij 1.065 g/ml. De laatste kolonievormende cellen zijn dus iets lichter dan beenmerg TL-CFC.
De resultaten van deze experimenten duidden erop dat celscheidingsmethoden, die gebruik maken van het verwijderen van E-rozettes of van dichtheidsgradient centrifugatie aangewend kunnen worden om TL-CFC te scheiden van de andere cellen in bloed en beenmerg van leukemiepatienten.

HOOFDSTUK 4 beschrijft experimenten die aantonen dat in de PHA-l.f. methode leukemische kolonies worden gevormd, ook in gevallen waar geen koloniegroei werd waargenomen in de standaard leukocyten feeder kweken zonder PHA. Echter, zonder celscheiding voorafgaand aan de kweek, bleek de verontreiniging met T lymfocyten kolonies vaak aanzienlijk te zijn. De selectiviteit van de PHA-l.f. methode voor de groei van leukemische kolonies werd vervolgens verbeterd

door de E-rozette vormende cellen (E-RFC) te verwijderen met behulp van een E-rozette ficoll scheidingsprocedure. Echter, niet in alle experimenten werd zuiver leukemische koloniegroei verkregen. Naar aanleiding van deze experimenten werd geconcludeerd dat een betere procedure voor de scheiding van TL-CFC en leukemische CFC noodzakelijk is.

In de experimenten die beschreven zijn in HOOFDSTUK 5 is albumine dichtheidsgradient centrifugatie toegepast om leukemische CFC in bloed en beenmerg van patienten met CML te zuiveren. Deze proeven beoogden tevens om de dichtheidseigenschappen vast te stellen van de leukemische cellen die uitgroeien tot kolonies in het PHA-l.f. kweeksysteem en om deze eigenschappen te vergelijken met die van de leukemische cellen welke kolonies vormen in de standaard leukocyten feeder kweekmethode. Uit de lichtste celfracties (1.059 g/ml en/of 1.056 g/ml) werden in bijna alle experimenten zuivere leukemische kolonies gekweekt. Uit deze proeven werd evenwel duidelijk, dat ondanks de relatief hoge zweefdichtheid van TL-CFC, de dichtheidsverdelingen van leukemische CFC en TL-CFC elkaar overlapten. Deze situatie was ernstiger in bloed dan in beenmerg, hetgeen in overeenstemming is met onze eerdere bevindingen dat beenmerg TL-CFC een hogere zweefdichtheid hebben dan bloed TL-CFC (HOOFDSTUK 3). Tengevolge van de bijmenging van T lymfocyten kolonies in kweken van de dichtheidsfractie 1.062 g/ml en, in experimenten met bloed ook in kweken van de fractie 1.059 g/ml, waren we niet in staat om een betrouwbare uitspraak te doen over de dichtheidseigenschappen van de leukemische CFC die kolonies vormen in de PHA-l.f. kweekmethode. De proeven bevestigden evenwel de bevindingen van andere onderzoekers dat CML kolonievormende cellen een lagere zweefdichtheid hebben dan normale GM-CFC.

In de experimenten die beschreven zijn in HOOFDSTUK 6 is een verbeterde scheidingstechniek toegepast op CML bloed- en beenmergcellen. Dit gaf de mogelijkheid om een nauwkeuriger analyse te doen van de populatie kolonievormende cellen in de PHA-l.f. assay en deze direkt te vergelijken met de cellen die tot kolonies uitgroeien in de standaard leukocyten feeder kweekmethode. De celscheidingsmethode bestond opnieuw uit een discontinue dichtheidsgradient centrifugatie. Door, voorafgaand aan de gradient centrifugatie, de T lymfocyten in de celmonsters E-rozettes te laten vormen, werd een praktisch volkomen depletie van TL-CFC in de lichte fracties tot en met 1.062 g/ml bereikt. Hierdoor werd het mogelijk om de groei van leukemische cellen, variërend in dichtheid van 1.056 tot 1.062 g/ml, te analyseren in beide kweeksystemen zonder verstorende bijmenging

met T lymfocyten kolonies. In het celmateriaal van verschillende patienten werd gevonden dat de profielen van de dichtheidsverdelingen van voorlopercellen die uitgroeien tot kolonies in de twee verschillende kweeksystemen (i.c. PHA-l.f. assay en standaard leukocyten feeder kweektechniek) niet gelijk aan elkaar waren. De duidelijkste verschillen werden gevonden in patienten met CML-BC. Deze resultaten maakten aannemelijk dat kolonies van blastcellen in de PHA-l.f. assay en kolonies van gedifferentiëerde cellen in de standaard leukocyten feeder kweekmethode door verschillende voorlopercellen werden gevormd. Omdat de verschillende kolonietechnieken dus niet-identieke voorlopercellen detecteren, werd geconcludeerd dat de kweeksystemen als elkaar aanvullende methoden elk een bijdrage kunnen leveren in het onderzoek van de prolifererende cellen in myeloïde leukemie.

HOOFDSTUK 7 omvat een serie experimenten waarin de PHA-l.f. assay is toegepast op beenmerg- en bloedcellen van patienten met acute niet-lymfocytaire leukemie. Met behulp van de discontinue dichtheidsgradient centrifugatie methode met gelijktijdige depletie van T lymfocyten werden celfracties van zuiver leukemische blasten verkregen. Deze cellen werden tevens in de standaard leukocyten feeder methode gekweekt om een direkte vergelijking te kunnen maken van het in vitro groeivermogen in beide kweeksystemen. Slechts in enkele gevallen werd koloniegroei waargenomen in de standaard leukocyten feeder methode zonder PHA. Daarentegen werd in dertien van de vijftien experimenten koloniegroei verkregen in de PHA-l.f. assay en deze kweken waren vrij van T lymfocyten kolonies. De leukemische herkomst van deze kolonies werd in alle gevallen waarin de leukemie een specifieke cytogenetische merker had, aangetoond met behulp van cytogenetische analyse van de koloniecellen. Leukemische kolonies in de PHA-l.f. assay waren afkomstig van cellen met een buitengewoon lage zweefdichtheid en deze cellen onderscheiden zich hierin dus van normale GM-CFC. Het profiel van de dichtheidsverdeling van kolonievormende cellen en dat van de totale blastcelpopulatie was verschillend in het celmateriaal van enkele patienten. Dit kan betekenen dat kolonievormende cellen een bepaalde subpopulatie vormen binnen de totale populatie van leukemische cellen. Slechts in die gevallen waar leukemische koloniegroei tevens in de standaard leukocyten feeder kweekmethode werd verkregen kon een vergelijking gemaakt worden van de dichtheidseigenschappen van deze kolonievormende cellen met die van de kolonievormende cellen in de PHA-l.f. assay. De dichtheidsprofielen van de cellen die kolonies vormden in de standaard leukocyten feeder kweekmethode waren verschillend van de

dichtheidsprofielen van de PHA-l.f. CFC in dezelfde patienten. Dit gaf aanleiding tot de veronderstelling dat in de twee kweeksystemen verschillende subklassen van leukemische cellen uitgroeien tot kolonies. Terwijl koloniegroei in de standaard leukocyten feeder kweken slechts in enkele gevallen werd geconstateerd, laten deze experimenten duidelijk zien dat het onderzoek van de voorlopercellen van de leukemische blasten in de meerderheid van de ANLL patienten mogelijk wordt met behulp van de PHA-l.f. assay. Daarom kan de PHA-l.f. assay beschouwd worden als een praktische kweekmethode die aangewend kan worden om meer inzicht te verkrijgen in de proliferatie- en differentiatieprocessen in acute leukemie celpopulaties. Omdat in de standaard leukocyten feeder kweekmethode een andere populatie van voorlopercellen tot kolonies uitgroeit, kan verwacht worden dat gelijktijdige analyse van de koloniegroei in beide kweeksystemen bijdraagt tot het begrip van de opbouw van een leukemische celpopulatie.

HOOFDSTUK 8 bevat experimentele gegevens die de theoretische mogelijkheid bevestigen dat de toepassing van verschillende koloniekweekmethoden kan leiden tot het onderkennen van subklassen van leukemische voorlopercellen die elk een andere rol vervullen in het kwaadaardige proces. In dit hoofdstuk zijn gedetailleerde gegevens vermeld van cytogenetische onderzoeken en koloniekweekresultaten in een geval van acute monoblastaire leukemie (M5; FAB-klassificatie). De leukemische celpopulatie in deze patient omvatte karyotypisch verschillende subpopulaties. Daardoor werd het mogelijk de in vitro groeicapaciteit te onderzoeken van cellen die tot de afzonderlijke klonen behoorden. Grote aantallen kolonies werden gekweekt in de standaard leukocyten feeder methode zonder PHA, maar deze bleken afkomstig van uitsluitend de kloon die de minderheid vormde in het oorspronkelijke celmateriaal. Hiermee werd aangetoond dat, tenminste in dit geval, deze kweekmethode selectief was voor de subpopulatie van leukemische cellen met het minst complexe, en vermoedelijk het eerst ontstane, afwijkende karyotype. Deze leukemische cellen waren dus nog in staat, net als normale GM-CFC uit te groeien tot kolonies in de leukocyten feeder kweekmethode. Daarentegen hadden de cellen met het meer afwijkende karyotype dit vermogen verloren. Cytogenetische analyse van de kolonies die werden verkregen in de PHA-l.f. kweekmethode toonde aan dat hier de meerderheidskloon overheerste. Dus in dit koloniekweeksysteem was sprake van niet-selectieve groei van voorlopercellen van de twee klonen. In het verloop van de ziekte vertoonden de verschillende klonen elk een andere rol wat betreft het groeivoordeel in vivo en de gevoeligheid voor therapeutische behandeling. Deze bevindingen tonen aan dat karyotypisch verschillende

subpopulaties van een neoplasma, die elk een ander lot ondergaan en een verschillende rol spelen in het verloop van de ziekte, tevens verschillende groeibehoeften kunnen hebben en dat hun proliferatie in vitro afhankelijk kan zijn van de aanwezigheid van selectieve stimulerende factoren. De mogelijkheid om verschillende subpopulaties van leukemische cellen afzonderlijk te onderzoeken kan van bijzondere betekenis zijn voor het herkennen van nieuwe, nog zeer kleine subpopulaties tijdens klonale evolutie van leukemieën. In theorie kan dit een rol spelen in de vroeg-diagnostiek van leukemische transformatie, bijvoorbeeld in blastencrisis van CML.

In HOOFDSTUK 9 zijn experimenten beschreven die werden uitgevoerd om vast te stellen welke functie de leukocyten feeder cellen hebben in de stimulatie van leukemische koloniegroei in de PHA-l.f. kweekmethode. Het werd van bijzonder belang geacht om de condities, waaronder proliferatie van leukemische cellen in vitro mogelijk is, te bepalen en deze condities te vergelijken met die welke vereist zijn voor de proliferatie van normale hematopoietische voorlopercellen. Leukemische koloniegroei in de PHA-l.f. methode bleek afhankelijk te zijn van de gezamenlijke aanwezigheid van T lymfocyten en monocyten als feedercellen. Lymfocyten (B, T en B+T) en monocyten afzonderlijk waren niet in staat kolonievorming te stimuleren. De kwantitatieve verhouding van monocyten en T lymfocyten was bepalend voor maximale koloniestimulatie. Deze resultaten zijn ongetwijfeld van belang voor het verklaren van verschillen tussen preparaten van kolonie stimulerende aktiviteit (afkomstig van feeder cellen of van geconditioneerd medium) en voor het standaardiseren van de methode voor leukemische kolonievorming. Deze experimenten suggereerden tevens dat de rol van het lectine PHA met betrekking tot leukemische kolonievorming voornamelijk indirekt is, dat wil zeggen PHA speelt een rol in het aanzetten van de produktie van noodzakelijke groeifaktoren door feeder cellen.
Uit deze bevindingen werd geconcludeerd dat leukemische kolonievormende cellen andere groeifactoren nodig hebben voor proliferatie in vitro dan normale kolonievormende cellen. De laatste kunnen tot proliferatie aangezet worden door monocyten zonder de aanwezigheid van T lymfocyten en PHA.

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# Curriculum Vitae

Klaas Swart werd geboren op 11 januari 1951 te Ferwerd en behaalde in 1969 het H.B.S.-B diploma aan het Christelijk Lyceum "Oostergo" te Dokkum. In datzelfde jaar begon hij zijn studie aan de Landbouwhogeschool te Wageningen, waar hij de studierichting Zoötechniek volgde. Het doctoraalexamen werd in juni 1977 behaald (met lof) met als hoofdvakken de Erfelijkheidsleer en de Gezondheids- en Ziekteleer der huisdieren, en als bijvak de Veeteelt.

Vanaf 1 augustus 1977 tot 1 augustus 1981 was hij als wetenschappelijk medewerker verbonden aan het Instituut Hematologie van de Erasmus Universiteit Rotterdam. Hij verrichtte zijn werkzaamheden in het kader van een researchproject van het Koningin Wilhelmina Fonds. Gedurende deze periode werd het in dit proefschrift beschreven onderzoek verricht. Het hierop volgende jaar werd onder andere besteed aan het bewerken van dit proefschrift.

Vanaf 1 september 1982 is hij als wetenschappelijk medewerker verbonden aan de Vakgroep Erfelijkheidsleer van de Landbouwhogeschool te Wageningen met als onderzoeks- en onderwijstaak de microbiële genetica.