Cytologie and Cytochemistry of Colony Cells in Soft Agar Gel Culture from Normal and Leukemic Bone Marrow

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Summary

In order to judge differentiation of cells in soft agar colonies, cytological and cytochemical classification of single cells within these colonies is necessary. In this study, 1,026 colonies from 15 normal and 95 leukemic bone marrows have been evaluated using cytological, cytochemical, and immunocytochemical techniques. In 180 colonies from 15 normal controls no segmented neutrophils have been observed. The colonies mostly consisted of monocytes and macrophages, rarely pure eosinophil colonies were observed. The number of monocyte/macrophage colonies in untreated AML and the percentage of pure eosinophil colonies in AML and ALL in remission are reduced, as compared to normal controls.

In 174 colonies from a total of 926 colonies derived from bone marrows of leukemic patients, plasma cells and in 20 colonies, blast cells have been observed. In contrast to normal colonies, growth of colonies containing blast cells does not depend upon the conditioned medium of the leukocyte feederlayer.

This investigation has demonstrated the necessity of cytological and cytochemical classification in addition to quantiative evaluation of soft agar colonies when studying the effect of factors on proliferation and differentiation of normal and leukemic stem cells.

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Introduction

Human bone marrow contains cells which form – dependent on specific interactions with diffusible factors – leukocyte and erythrocyte colonies in soft agar (1–6). On the base of published data, most authors assume that each colony arises from one single cell. Leukemic bone marrow cells exhibit a nearly total failure of colony formation in vitro (7–14), which is most probably due to the blocked differentiation. Recent results have shown that cells from untreated AML and from AML in relapse could be stimulated with PHA to form colonies in soft agar in the absence of diffusible factors, i. e. without feeder layer (8). GALLAGHER et al. reports proliferation and differentiation of AML cells in suspension cultures after stimulation with conditioned medium from embryonal tissues (15). These data suggest that some of the organizational and regulatory features of normal hemopoiesis persist in leukemic hemopoiesis. In order to judge differentiation of cells in soft agar colonies, a cytological and cytochemical classification of single cells within these colonies appears necessary. Simple quantitative evaluation of colony formation does not yield enough information regarding differentiation patterns. As a first step towards the evalutation of the effect of specific differentiation factors on normal and leukemic bone marrow cells in soft agar, more than 1,000 colonies from normal and leukemic bone marrows have been classified according to cytological and cytochemical criteria (16–19).

MaterialandMethods

a) Source of Material

Normal bone marrow was obtained by aspiration from the iliac crest or sternum, taken during the hematologic examination of 13 children and two adults. Six of these patients were defined as normal, and the other nine showed no evidence of hemoblastosis or granulopoietic abnormalities. For cytological and cytochemical classification of bone marrow cells the usual criteria were used.

ALL patients received the same chemotherapy described by PINKEL et al (20), including vincristine (VCR) and prednisone (PRED), followed by central nervous system (CNS) leukemia prophylaxis with CNS irradiation and intrathecal methotrexate (MTX). For maintenance a combination of cyclophosphamide, MTX, and 6-mercaptopurine (6-MP) was given. Patients with AML were treated with a combination of 6-thioguanine and cytosine arabinoside.

b) Cell Separation

For the separation of leukocytes the method of BÖYUM (21) was used. The resulting buffy coat was suspended in culture medium. The nucleated cells were counted with a hemocytometre. For the preparation of feeder layers, granulocytes and monocytes were counted. The cell layer was prepared by suspending two or three drops of aspirated bone marrow into the culture medium, followed by a hypertonic shock, repeated twice for lysis of erythrocytes, and by counting the number of mononucleated bone marrow cells, omitting non-deviding cells like metamyelocytes and polymorphs.

c) Culture Technique

Agar cultures were prepared using the double layer agar technique of PIKE and ROBINSON (22). McCoy's 5A medium containing 15 % fetal calf serum and supplemented with amino acids and vitamins, was mixed in a 9:1 ratio with boiled 5 % agar (Difco). After addition of the appropriate number of leukocytes (1.5 – 1.8 x 10^6), 1 ml of this agar cell medium mixture was pipetted into 35 mm plastic Petri Dishes (Falcon Plastics). These prepared feeder layers were stored at 37 °C in a humidified incubator continously flushed with 8 % CO_2. The washed bone marrow cells were mixed with culture medium and boiled 3 % agar, in a ratio of 9:1; of this, 1 ml aliquots were then pipetted onto the feeder layer. The final concentration of the cell layer was 1 x 10^5 mononucleated bone marrow cells per ml. Following this preparation, no aggregates, clumps, or tissue fragments were found in bone marrow suspensions or in agar. The dishes were incubated for three weeks. During this time, at intervals of 12 to 14 days, the number of colonies was counted with an inverted microscope (Diavert) at 40x magnification. Only those colonies containing 50 or more cells were counted. For each experiment, at least four plates with, und two plates without feeder layer were examined; the counts were expressed as the mean result of these plates. The number of colonies in two plates never varied more than 10 % for bone marrow cells, and maximally 100 % for peripheral blood leukocytes.

d) Source of Colony Stimulating Factor

A feeder layer of peripheral blood leukocytes was used as source of colony stimulating factor (CSF). Since the induction of proliferation of colonies depends on the age of the feeder layer, the feeder layer was used during the day of preparation or only few days later. After seven days of incubation, the feeder layer had lost about 50 % of its original stimulating activity (23).

Mature granulocytes inhibit the proliferation of colonies (24). Therefore the number of granulocytes in the feeder layer was not allowed to exceed 1 x 10^6 cells per ml (23). At this concentration of granulocytes the feeder layer contained nearly 1–2 x 10^5 monocytes per ml.

c) Cytologic Analysis of Colonies

For cytologic analysis, colonies were picked out of the agar under the inverted microscope with an angled (ca 110°) micro-hematocrit. They were put on slides and incubated for 10 minutes in a humidified chamber wit a 1 % solution of agarase (Calbiochem., Los Angeles). The colonies were prepared according to the method of TESTA and LORD (25). After incubation, the agarase was drawn off carefully, and the colonies were fixed according to the cytochemical reaction necessary (10 % formalin alcohol for peroxidase reaction, 60 % cold acetone for acid phosphatase reaction). Fixation solution was dropped onto the slide. Then a coverslip was placed on top of the drop. This in turn was covered with a piece of filter paper and gently pressed. The slide was frozen on dry ice for 10 minutes, the cover slip was removed quickly, and the slide immediately dried. The colony cells were stained with May-Grünwald-Giemsa, respectively for peroxidase (16), or acid phosphatase (26).

f) Demonstration of Immunoglobulins

To demonstrate immunoglobulin within plasma cells, some colonies were fixed as described above, stained for 30 minutes with FITC conjugated anti-human immunoglobulins (Table 2), and then washed three times with PBS.

The colonies were investigated with a Leitz ortholux microscope equipped with an Opak-Fluor vertical illuminator.

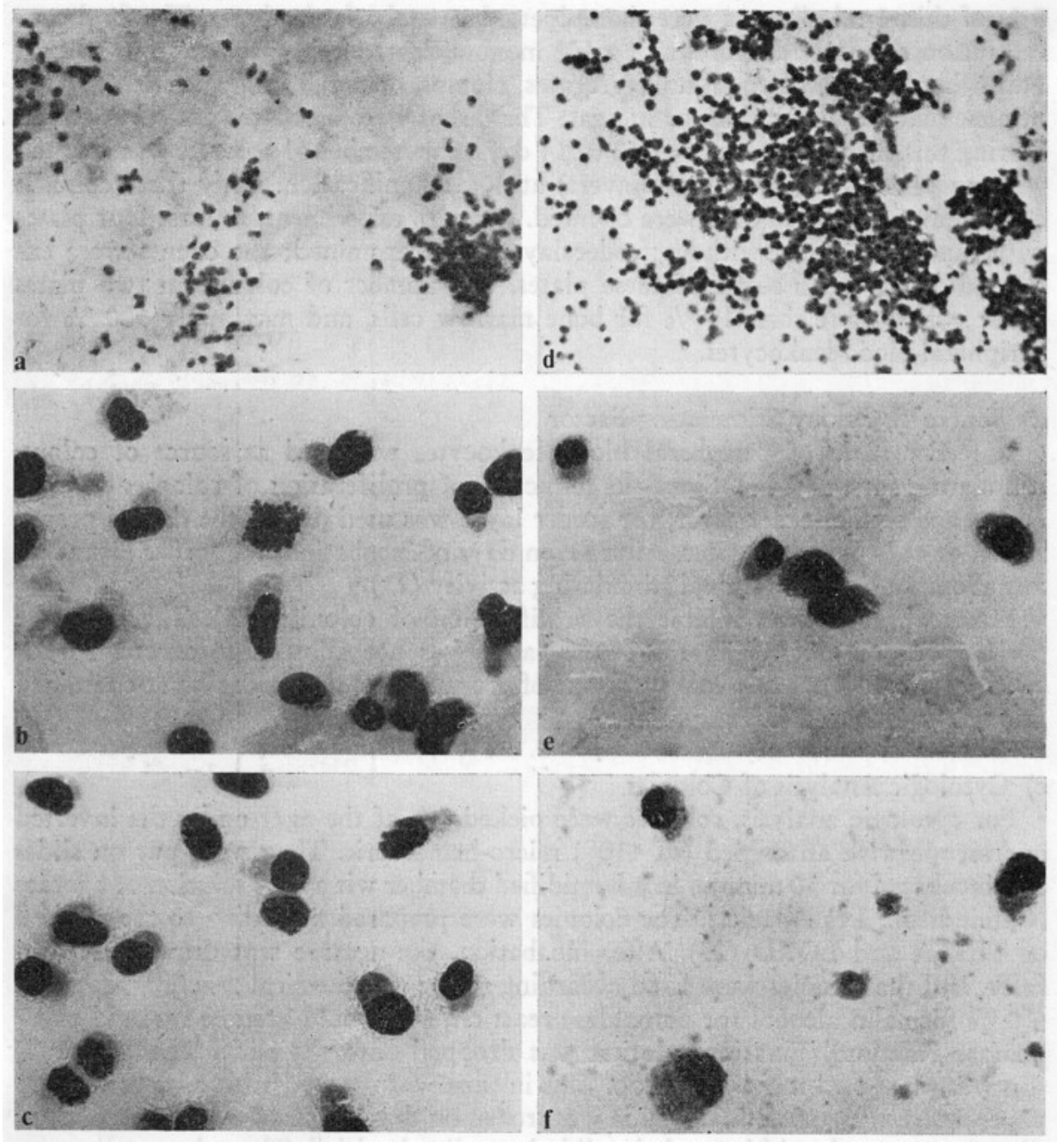

Fig. 1: Peroxidase negative monocyte colony (a) and peroxidase positive eosinophil colony (d), counterstained with Giemsa stain.
b, c: Monocytes in mitosis,
e, f: peroxidase positive eosinophils.

Results

1,026 colonies from 15 normal and 95 leukemic bone marrows have been evaluated using cytochemical and cytological techniques (Table 1). Quantitative analysis of colony formation confirmed the well-known reduction of colony formation in untreated ALL and AML and the increased colony formation in CML as compared to normal controls (4, 5, 7, 10–14). Cytological and cytochemical methods allow further differentiation of colony formation by analyzing the cellular composition of these colonies (Fig. 1–3). In untreated AML or AML in relapse there is a significant reduction of monocyte/macrophage colonies as compared to ALL and an increase of pure eosinophil colonies (Table 1). During remission, fewer pure eosinophil colonies are observed in both, ALL and AML, as compared to nor-

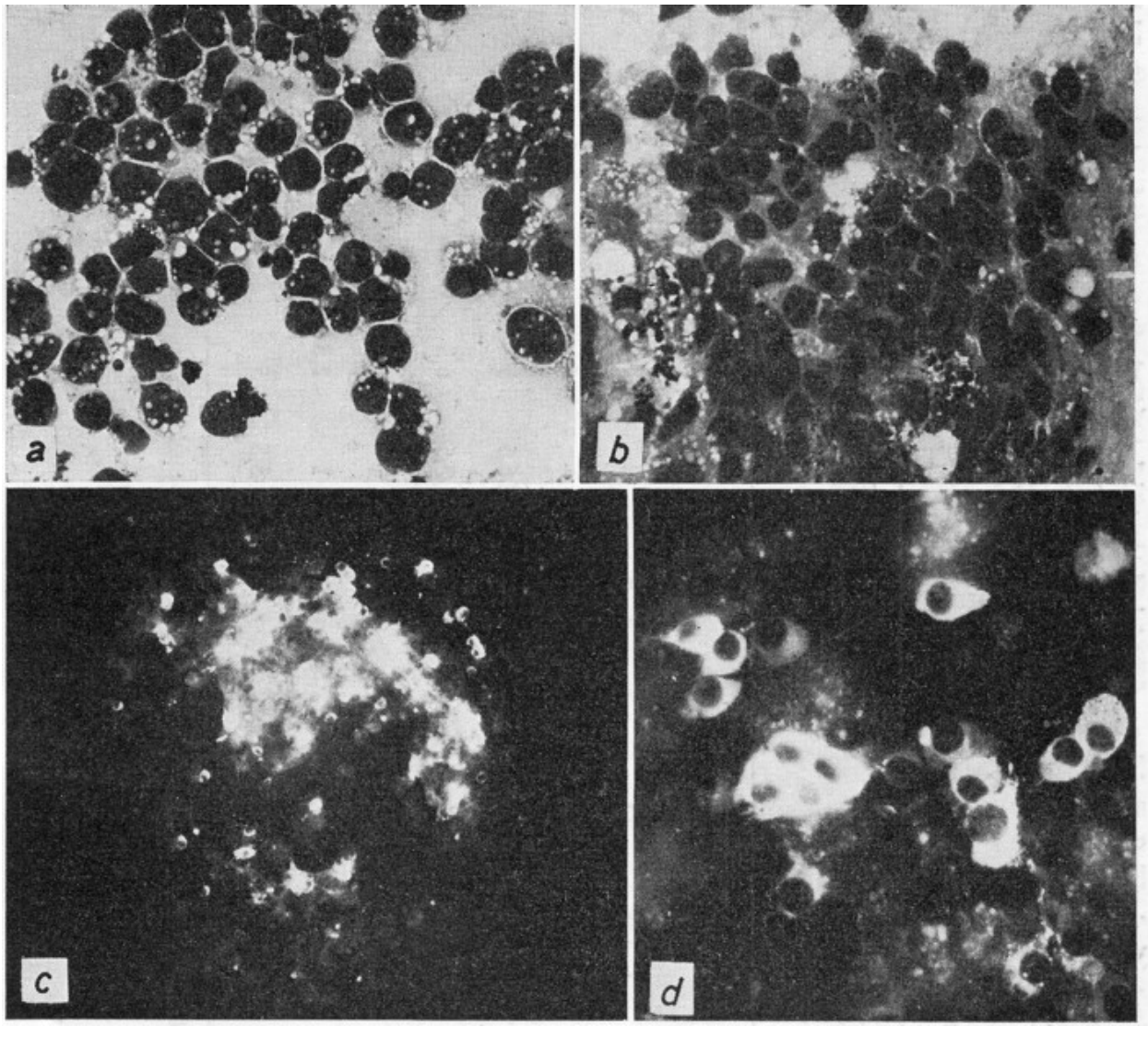

Fig. 2: Cells of isolated colonies from patients with AML.
a: blast cells of a peroxidase negative colony, counterstained with Giemsa stain,
b: a group of macrophages and plasma cells, Giemsa stain,
c: plasma cells of a colony with positive fluorescence in the cytoplsma, spread between several negative cells,
d: plasma cells with positive fluorescence, the positive background reaction is a result of immunoglobulin phagocytosed by macrophages.

Table I: Cytological Classifikation of Colonies (%)

diagnosis	number of patients	number of colonies analyzed	mon	mac	mon mac	eos	mon eos	mac eos	mon mac eos	mon mac eos neu	pc mon mac	pc mon mac eos	pc bc mon mac	bc mon mac
ALL untreated	21	164	70,5	8.	12	2	2	–	0,5	–	4	–	0,5	0,5
All CR	33	331	53	9	6	2	4,5	4	5	3	7	6	0,5	–
ALL PR	5	97	44	10	4	–	4	–	1	–	29	1	6	1
AML untreated	12	74	18,5	–	–	16,5	5	1	5	4	27	14	6,5	2,5
AML CR	5	76	64	5	2,5	2,5	6,5	–	–	–	–	17	2,5	–
AML PR	7	21	58	4,5	4,5	–	–	–	–	–	9	24	–	–
CML remission	2	83	59	2,5	21	1	2,5	–	–	–	5	10	–	–
normal bone marrow	15	180	76	2,5	6	5,5	5	1	4	–	–	–	–	–
	n = 110	n = 1,026												

CR = complet remission, PR = partial remission, mon = monocyt, mac = macrophag, eos = eosinophil, neu = neutrophil, pc = plasma cells, bc = blast cells

mal controls while the sum of pure eosinophil colonies and mixed colonies with eosinophils is of the same order as in normal bone marrow (Table 1).

The most remarkable observation is the presence of plasma cells in 174 colonies and of blast cells in 20 colonies of a total of 926 colonies derived from the bone marrows of leukemic patients. Neither plasma cells nor blast cells were observed in any of 180 colonies from normal bone marrow. Using immunofluorescence techniques we were able to demonstrate that these plasma cells produce immunoglobulins in vitro (10). Plasma cells in isolated colonies exhibited positive immunofluorescence after incubation with goat FITC-anti-human-globulin (Fig. 2, Table 2). In colonies from five patients, additional labelling was carried out with specific antisera against IgG, IgA, and IgM heavy chains. Some plasma cells showed positive immunofluorescence with only one, others with all three antisera used. In addition, plasma cells from these colonies were incubated with anti-kappa and anti-lambda sera. The plasma cells of all colonies studied were labelled by both antisera (Table 2).

In order to study the effect of diffusible factors, produced by the feeder layer, on proliferation of leukemic cells, we investigated colony formation with and without feeder layer in various leukemias during remission and relapse. Bone marrows from patients in complete remission yield fewer colonies as compared to

Table II: Demonstration of Immunoglobulins in Plasma Cells from Soft Agar Colonies

patient	diagnosis	anti-Ig	anti-IgG	anti-IgM	anti-IgA	anti-kappa	anti-lambda
D.	ALL untreated	nt	+ (2)	nt	nt	+ (1)	+ (2)
B.	ALL partial remission	+ (1)	+ (1)	nt	– (2)	+ (6)	+ (6)
Z.	AML partial remission	nt	+ (3)	+ (1)	+ (3)	+ (3)	+ (3)
H.	AML partial remission	+ (5)	– (2)	– (2)	nt	nt	nt
	complete remission	nt	nt	– (1)	+ (1)	+ (1)	+ (1)
Wa.	AML complete remission	+ (4)	nt	nt	nt	+ (1)	+ (1)
We.	AMML untreated	+ (4)	+ (2)	– (1)	nt	nt	nt
O.	AMML untreated	nt	nt	nt	nt	nt	+ (1)

demonstration of immunoglobulins using FITC coupled anti-human-immunoglobulin (goat), and anti-IgG, anti-IgM, anti-IgA (H-chain specific; rabbit), anti-kappa, anti-lambda (rabbit)
+ = positive immunofluorescence / / – = negative immunofluorescence / / nt = not tested / / ( ) = number of colonies studied

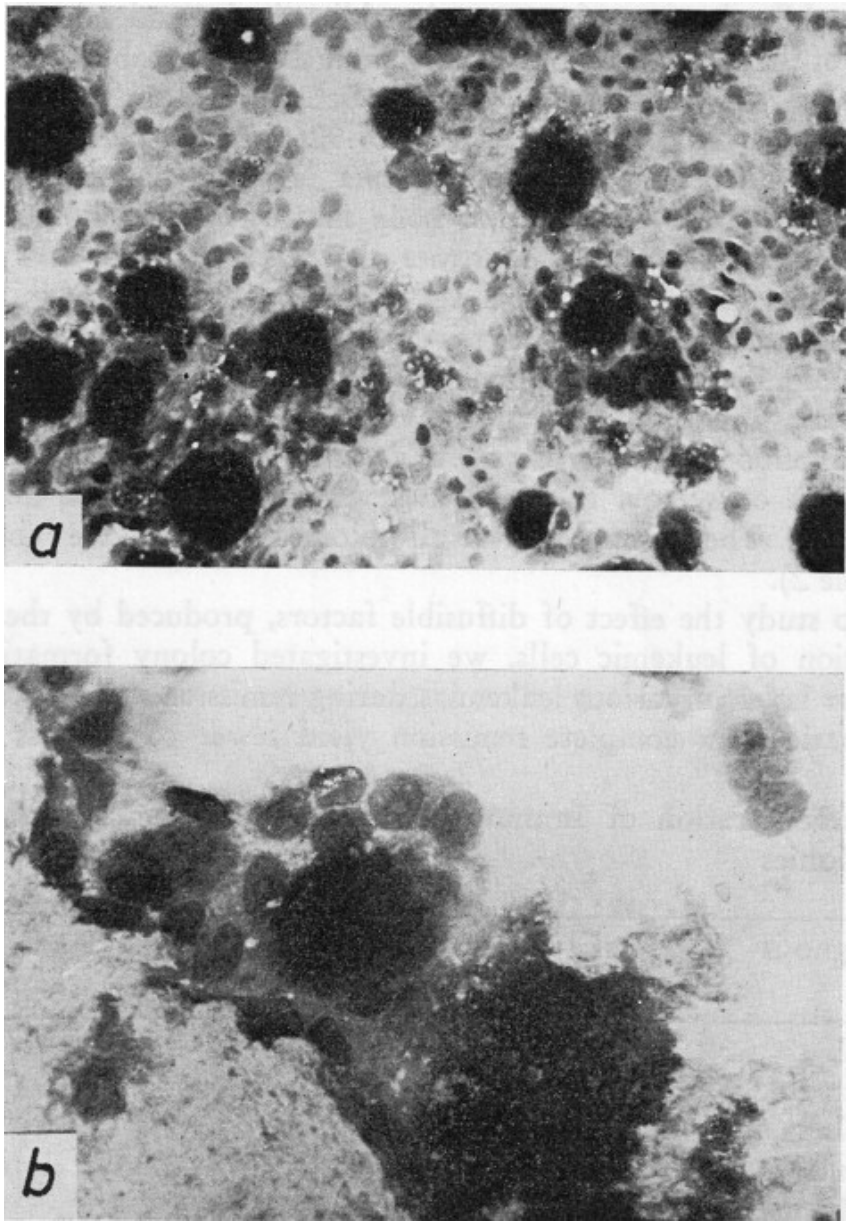

Fig. 3: Mixed colonies of macrophages, blast cells, and plasma cells (acid phosphatase reaction). Strongly positive reaction in macrophages (a, b), granular positive reaction in plasma cells (b), negative reaction in blast cells (a, b).

normal controls with and without leukocyte feeder layer (Table 3). While hardly any normal colonies have been observed without feeder layer, colonies consisting of blast cells only or of blast cells in combination with normal cells (Fig. 2, 3) have been detected in equivalent quantities with and without feeder layer.

Discussion

The results described (Table 1) demonstrate that under the culture conditions used (of Materials and Methods), normal bone marrow cells mainly produce colonies consisting of monocytes and macrophages. In addition, pure eosinophil colonies and mixed colonies of monocytes and eosinophils are observed. In all 180 colonies from 15 normal controls investigated so far, no segmented neutrophils

Table III: Dependence of Normal and Leukemic Colony Formation on CSF

patient	diagnosis	number of colonies[1] / 1 x 10^5 mononuclear bone marrow cells with feeder layer normal	with feeder layer pathologic	without feeder layer normal	without feeder layer pathologic
P.	ALL	1,3	0,3	ϕ	ϕ
D.	ALL				
	untreated	0,8	9	ϕ	14
	untreated	0,8	0,3	ϕ	ϕ
W.	AMML				
	untreated	ϕ	1	ϕ	2
O.	AMML				
	untreated	ϕ	0,4	ϕ	1
B.	ALL				
	PR	78	0,7	ϕ	ϕ
H.	AML				
	PR	96	1,8	ϕ	2
	CR	95	0,5	ϕ	0,3
Z.	AML				
	PR	7	1,5	ϕ	0,3
	PR	31	1	ϕ	1,5
	CR	55	1,2	2	0,5
D.	AML				
	PR	224	0,2	ϕ	0,3
K.	AMoL				
	PR	238	1,5	ϕ	1,3
H.	AMoL				
	PR	1,5	1,3	ϕ	2,5
W.	AML				
	CR	59	1	ϕ	1

[1] = mean of five plates
CR = complete remission – PR = partial remission

have been observed. These data are in good agreement with the only other systematic cytologic study of colony formation in soft agar we are aware of. This study has been carried out by SHOHAM et al. (27), who used spleen cells as source of conditioned medium.

The reduced number of monocyte/macrophage colonies in untreated AML and AML in relapse is probably a result of the reduced number of normal myeloid precursor cells during the active stages of AML. The relatively high percentage of pure eosinophil colonies would then represent the differentiated stages of a residual population of normal stem cells. Cytochemical analysis using the N-ASD-chloracetate reaction, which shows a positive reaction in leukemic eosinophils only (28, 29), as well as comparative cytogenetic studies in these colonies from leukemias with

chromosomal aberrations will give some important information regarding this hypothesis. Since macrophages are regarded to play an important role in the afferent limb of the immune system, the reduced number of monocyte/macrophage colonies in AML as compared to ALL and normal bone marrow might be of relevance for the clinical course of the disease. The relatively low percentage of pure eosinophil colonies from bone marrow of patients with AML and ALL in remission as compared to normal controls could be the result of a persisting differentiation defect in these cells, since eosinophils have to be regarded as the most differentiated myeloid cell in soft agar colonies. This hypothesis would also explain the increased number of mixed monocyte/macrophage/eosinophil colonies in these patients. Further studies are necessary to investigate the role of pure eosinophil colonies as a marker of marrow differentiation capacity and the possible correlation with the clinical course of the disease.

The presence of plasma cells in colonies derived from leukemic patients and their absence in normal colonies cannot be explained so far. These plasma cells could be present accidentally in a colony containing blast cells. A specific interaction between immunologically competent B cells and blast cells would then explain the persistence of plasma cells in abnormal colonies only. Due to the limited supply of plasma cells as yet no investigations have been carried out regarding the specificity of the antibodies produced, e. g. against leukemic blast cells. The alternative explanation would be that macrophages, monocytes, eosinophils and plasma cells, are derived from one pluripotent stem cell in presence of leukemic blast cells. The demonstrated presence of both, kappa- and lambdachains, in colonies containing plasma cells does not favour the hypothesis of a monoclonal origin of these plasma cells since the simultaneous presence of both, kappa- and lambda-chains, is observed in 1–2 % of all plasma cells only. The demonstration of colonies containing blast cells derived from leukemic bone marrows suggest the presence of factors inducing proliferation and in some cases even differentiation of blast cells under our cultural conditions. The experiments described by GALLAGHER (15) and DICKE (8, 30) also prove that these factors do exist. Our data, however, demonstrate that these factors do not originate from the leukocyte feeder layer, since there was the same number of pathologic colonies with and without feeder layer, while normal colonies were almost completely missing (Table 3). If it were possible to enhance growth of pathologic colonies from AML remission bone marrow with PHA, this technique allowed better evaluation of the residual leukemic stem cells during clinical remission.

Our investigation has demonstrated the necessity of cytological and cytochemical classification in addition to quantitative evaluation of soft agar colonies when studying the effect of factors on proliferation and differentiation of normal and leukemic stem cells. Differentiation patterns should only be interpreted after careful cytological and cytochemical identification of the cell.

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