In Vitro Colony Growth of Acute Myelogenous Leukemia

K. A. Dicke*
G. Spitzer
P. H. M. Scheffers
A. Cork
M. J. Ahearn
B. Löwenberg
K. B. McCredie

University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute
Houston, Texas 77025, USA.

Summary

Colony formation in vitro by marrow cells from patients with untreated acute myelogenous leukemia (AML) and from patients in AML relapse is infrequent using the standard Robinson assay. A newly developed culture system has been described in which marrow from AML patients in these disease stages form leukemic cell colonies. In this in vitro system, phytohaemagglutinin is the essential stimulator for colony formation. The leukemic origin of the colonies has been proven by ultrastructural morphology and cytogenetics. It appears that colony formation by leukemic cells in this system is predominantly independent from the leukocyte factor which is the main stimulator in the Robinson assay for growing colonies of marrow cells from haematologically normal individuals.

Bone marrow cells in untreated acute myelogenous leukemia (AML) demonstrate abnormal growth in vitro in the Robinson assay (Robinson et al., 1971; and Bull et al., 1973). Characteristically, there is a near total failure of colony formation; pre dominantly clusters are formed containing 20 cells or less (Bull et al., 1973; Greenberg et al., 1971; Moore et al., 1973 and 1974, and van Bekkum et al., in press). The absence of colonies has been shown to be due to a marked decrease of the normal myeloid precursor cell population in untreated AML. The small aggregate formation of AML cells has been attributed to the suboptimal response of leukemic cells to the leukocyte stimulation factor. Because this poor proliferation in vitro might not represent the maximal in vitro and in vivo proliferation potential of the leukemic cells, we studied a number of modifications of the in vitro culture system. A number of factors were studied which may have some influence on cell pro-

* Visiting Professor from the Radiobiological Institute TNO, Rijswijk, The Netherlands.
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liferation in general, notably phytohaemagglutinin (PHA), which induces lymphocyte colonies in vitro (Rozenszajn et al., 1974), and endotoxin which has been demonstrated to increase the labelling index of leukemic cells in vivo (Golde et al.).

In this paper an in vitro system is described in which marrow cells from untreated AML and AML in relapse were stimulated by phytohaemagglutinin (PHA) to form leukemic cell colonies in soft agar. These (similar) cells predominantly formed small aggregates (20 cells or less) in the presence of the normal leukocyte feeder layer alone. Moreover, in the course of the experiments, it appeared that by adding low concentrations of endotoxin to the cultures, the stimulating effect of PHA could be amplified.

Materials and Methods

Patients with acute myelogenous leukemia

The clinical diagnosis and the haematological findings of 13 patients with leukemia included in the study have been listed in Table I. Cases both untreated and

Table I: Differentials and Cytrochemistry of Marrow Cells from Untreated AML and AML in Relapse

Bone Marrow Differential*			Wright-Giemsa Staining			Cytochemistry		
Patient	Blast	Promye-locytes	Lympho-cytes	Mono-cytes	Auer Rods	Perox-idase	Es-terase**	P. A. S.
Ca	84	1	1	2		+	+	–
Fl	83	1	13	0.5		+		
He	84	0.8	6.5	0.2	+	+	–	–
St	75	3	5	0	+			
Tu	82	7	4	0	+			
Kr	53	6	1	0.2		+	–	–
Sal	70	0.4	5	0	+	+	–	–
San	6.1	52.4	11	0	+	+	–	–
Se	85	1	4.8	0		+	–	–
Ka	93	0.2	0.6	0.8		+	+	–
Ne	74	0.6	2.1	0	+	+	–	–
Pa	91	1	3	0.4		+	–	–
Po	72	2.8	3.2	0	+	+	–	–
Wo***	38.5	2	20	15.5		+	–	–
Be***	91	2	2	1		+	–	–
El***	55	2	15	0		+	–	–
Fr***	73	0	16	0		+	–	–

* 500 cells per slide counted.
** Non-specific esterase.
*** Patients in relapse.

in relapse had greater than 50 % leukemic blast cells on bone marrow differentials (Table I). The leukemia was diagnosed morphologically and cytochemically by the same criteria as described by Hayhoe et al. (1964). Leukemic relapses as presented in Table I, all occurred after previous treatment with combination chemotherapy plus immunotherapy according to the protocol documented by McCredie et al. (1975) and Gutterman et al. (1974).

Haemopoietic cell culture in vitro using a leukocyte feeder layer as source of stimulation (Robinson assay)

The technique used in these studies only differed in minor details from that described by Pike and Robinson (1970). The basic components of the Robinson system is a feeder layer of peripheral leukocytes and an overlay of human bone marrow cells. Peripheral leukocytes were obtained from peripheral blood from healthy volunteers, collected by venipuncture into heparinized tubes. Methyl cellulose (final concentration 0.1 %) was added to the blood sample and the red cells were allowed to sediment by gravity for 20 minutes. The buffy coat was then collected, and after centrifugation, the cells were resuspended in Dulbecco's modified Eagle's medium (MEM) to which 20 % serum plus agar was added (the final agar concentration was 0.5 %). The serum was a mixture of 1 volume fetal calf serum (FCS), 1 volume horse serum, and 1 volume 3 % trypticase soy broth. From this cell suspension 1 x 10^6 cells were pipetted into a 35 mm plastic petri dish (Falcon) (final volume: 1 ml agar medium) and the medium was allowed to gel. Bone marrow cells were obtained by aspiration from the posterior iliac crest and collected into heparinized tubes. After removing the erythrocytes by the buffy coat method (Dicke et al., 1969), the cells were incorporated into the same medium used for the feeder cells with the exception of using 0.25 % agar. One hundred thousand cells in a volume of 0.2 ml agar medium were then pipetted gently over the solidified feeder layer and allowed to gel. The plates were incubated at 37 °C in a humidified atmosphere of 7.5 % CO_2 in air. At the end of seven days, a cluster estimate was performed, and after 14 days of incubation, the plates were removed and the colonies scored visually. Aggregates containing 50 cells or greater were scored as colonies. Aggregates containing less than 50 cells were scored as clusters. All cultures were scored in triplicate.

The in vitro PHA + E assay

Basically, the technique consists of two phases: an initial liquid phase of 15 hours at 37 °C, and a semi-solid phase of seven days incubation at 37 °C. In the liquid phase 2 x 10^6 cells per ml medium (Dulbecco's MEM + 20 % serum) were cultured in pyrex glass tubes to which 0.05 ml PHA (Difco, PHAM) per ml medium and endotoxin (Difco, Lipopolysaccharide W. E. coli, 0111:B4) 10^7 g/m were added. After 15 hours of incubation, the cells were washed X 2 using HBSS (305 mOsm) and resuspended in agar medium (final concentration agar 0.25 %, medium Dulbecco's MEM + 20 % serum). After resuspension in agar medium, the cells (1 x 10/^{5} 0.2 ml per dish) were pipetted into Falcon plastic petri dishes containing 1 ml agar medium (final concentration agar 0.5 %, medium Dulbecco's MEM + 20 % serum)

to which 1 x 10^6 peripheral blood leukocytes from normal individuals were added using the method as detailed for the Robinson assay. Simultaneously, the cells were plated in petri dishes containing agar underlayers without leukocytes. After seven days of incubation in a 7.5 % CO_2 gas controlled humidified incubator at 37 °C, colonies were visible microscopically. These colonies were counted using an inverted microscope. Aggregates containing 50 cells or more were considered colonies. Aggregates containing 50 cells or less were considered to be clusters. The number of colonies and clusters presented in this paper is the mean value of triplicate petri dish cultures.

Electronmicroscopical procedure

Soft agar colonies for morphological observation were fixed in their petri dishes for 12 hours at 37 °C with 2.5 % Sorensen's buffered glutaraldehyde, pH 7.2, for 30 minutes each and then incubated in the dark with 33' diaminobenzidine tetrahydrochloride reagent for two hours at room temperature. The staining solution for endogenous peroxidase was removed with three additional rinses of Sorensen's phosphate buffer and postfixed in 1 % osmium tetroxide, pH 7.2, for one hour at 4 °C. Following three distilled H_2O rinses the agar disks were removed to flat covered glass dishes for the acetone dehydration and Epon infiltration steps. Final embedding of the agar disks was accomplished in foil weighing cups. After Epon polymerization at 80 °C, the aluminum foil was removed from the specimen, and the colonies of interest marked under a dissecting microscope. Those colonies selected for observation were cut from the specimen disk and mounted on plastic rods for ultramicrotomy. Alternate thick and thin serial sections were cut for light and electron microscopic study of the entire colony. Epon sections for light microscopy were stained with Paragon's stain for frozen sections. For ultrastructural observation, thin sections were stained with 0.5 % uranyl acetate and Reynold's Lead Citrate prior to their examinations with a Siemens' Elmiskop IA at 80 kV.

Cytogenetic procedure

Chromosome studies were performed on cells from both the liquid phase and from the colonies which had formed after seven days in culture. The cells obtained from the liquid phase, following the 15 hours incubation with PHA + E, were placed in 10 ml Ham's F 10 tissue culture medium supplemented with 20 % fetal calf serum and incubated overnight at 37 °C. The following day the cells were arrested in metaphases with 0.01 mg/ml Colchicine, submitted to hypotonic treatment with 0.075 M KC1 and fixed in methanol : acetic acid (3:1) mixture. Air-dry slide preparations were made, stained with Giemsa and scanned for well spread metaphases using a Zeiss microscope.

To obtain dividing cells from the colonies which were formed after seven days in culture, 0.1 µg colcemid was added to the petri dishes and incubated for an additional 3 hours. The colonies were collected with a fine Pasteur pipette and pooled in 0.2 ml Hank's Balanced Salt Solution (HBSS). The harvesting and slide preparations were completed as above and the slides scanned for analyzable metaphases.

Results

Colony formation in vitro

Colony formation by marrow cells from leukemic patients was obtained by exposing the cells to PHA and endotoxin (E). As has been mentioned already in materials and methods, the culture system consisted of two stages. An initial liquid phase (15 hrs), in which the cells were cultured in the presence of the two stimuli and a semi-solid phase (7 days) in which the cells were immobilized in an agar containing medium. In the second phase, the actual colony formation occurred. In the PHA + E assay used for analyzing the marrow of the patients, listed in Table I, on colony formation, PHA and endotoxin were only added in the liquid phase and were not present in the semi-solid phase.

Figure 1 shows a logarithmic plot of the relationship between the number of colonies and the number of cells, plated in dishes which contained agar without leukocytes. The 12 data points as depicted in Figure 1 were obtained from one individual in two experiments on two successive days. The data were consistent between experiments and so the data were combined. The calculated slope of the least

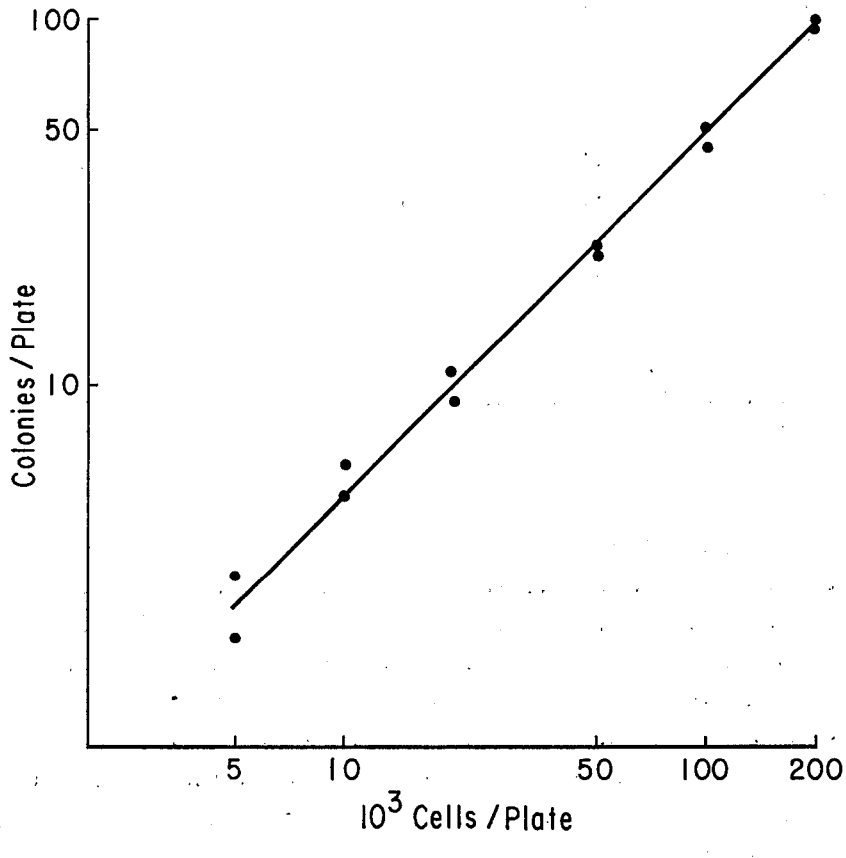


Fig. 1: Relationship between number of colonies per plate scored at seven days and number of cells cultured per plate. The data of two experiments were combined and have been depicted. Each point represents the mean value of colony number of 5–10 plates. The coefficient of variations at the different cell levels per plate ranged from 10–25 %. The stimulated slope of the least squares regression line is 0.98 ± 0.03, which is consistent with a slope of one.

squares regression line is 0.98 ± 0.03, which is consistent with a slope of one and shows that the number of colonies produced is proportional to the number of cells plated (Iscove et al., 1975). In these experiments, colony incidence was approximately 1 per 1,640 bone marrow cells.

Table II: Comparison between Number of CFU-c in the Robinson Assay and the PHA+E Assay of Marrow from Untreated Acute Myeloid Leukemics and Normals*

	Patient Name	Leukocyte Feeder	Agar**	Leukocyte Feeder	Agar**
	Knt	1***	n. d.****	75	n. d.
	San	0	n. d.	68	n. d.
	Sal	0	n. d.	290	n. d.
	Se	0	0	280	440
AML	He	0	0	29	30
	Ka	0	0	59	43
	Ne	0	0	50	54
	Pa	0	0	14	22
	Po	0	0	22	16
	1	30	n. d.	25	n. d.
	2	25	n. d.	30	n. d.
Normal	3	40	1	35	0
	4	44	2	34	0
	5	32	1	22	0

* See materials and methods.
** Agar underlayer without leukocytes.
*** Figure represents number of colonies per 10^5 cells plated.
**** n. d. = not done.

In Table II, the results of experiments have been listed in which bone marrow cells from untreated AML patients and from haematologically normal individuals were cultured in the Robinson assay, and the PHA + E assay. It can be noted that in the Robinson system, no colonies were present when marrow from untreated AML patients were cultured, which is in contrast to the presence of colonies in the PHA + E assay. Even in those cases in which no leukocytes had been added to the agar underlayer on which the bone marrow cells were plated after the liquid phase in the PHA + E assay, a distinct number of colonies could be grown from AML marrow cells. In the majority of cases, this number was equivalent to or exceeded the number of colonies present in cultures to which leukocytes were added, which clearly demonstrates that colony formation of leukemic marrow in the PHA + E system is independent of the presence of leukocyte feeder layers. This is in contrast with the results obtained with marrow cells from normal individuals which show that colony formation by normals is clearly leukocyte dependent, both in the Rob-

inson system and the PHA + E assay. Moreover, no additional effect of the PHA + E on colony growth by normal marrow has been obtained.

Table III: Comparison between Number of CFU-c in the Robinson Assay and the PHA+E Assay of Marrow from AML in Relapse and from AML in Remission

	Patient Name	Robinson Assay Leukocyte Feeder	Agar**	PHA + E Assay* Leukocyte Feeder	Agar**
	Wo	2***	n. d.****	52	0
AML	Be	0	0	150	130
Relapse	El	0	0	160	103
	Bo	17	n. d.	8	1
	We	36	n. d.	20	0
AML	Ja	24	0	20	0
Remission	Ba	44	0	46	0
	Br	6	0	0	0
	Mo	5	0	0	0

* See materials and methods.
** Agar underlayer without leukocytes.
*** Figure represents number of colonies per 10^5 cells plated.
**** n. d. = not done.

In Table III, the results of growth of marrow cells from AML relapse and from AML remission have been listed. It can be observed that the behavior of marrow cells from AML relapse in the two in vitro systems was identical to that of marrow cells from untreated AML patients, whereas the in vitro growth characteristics of marrow cells from patients in complete remission resembled that of haematologically normal marrow.

In order to make sure that colony formation was not an artifact due to clumping of cells by phytohaemagglutinin in the liquid phase of the PHA + E assay, the total number of colonies and clusters per plate were counted. It was reasoned that colony formation is unlikely to be caused by clumping in experiments in which the total number of colonies and clusters in the PHA + E assay is equivalent to or exceeds the number of aggregates in the in vitro systems to which PHA + E was not added. In Table IV, results have been documented of cultures in which the total number of aggregates were counted. It is evident from these results that the total number of aggregates in the cultures to which PHA + E were added to the liquid phase was highly increased. Furthermore, close observation at the time of plating in the semi-solid phase, after 15 hours of PHA + E incubation, did not reveal any higher degree of clumping than that observed in the cell suspension used for plating in the Robinson system which did not involve previous PHA incubation.

A summary of the results has been listed in Table V, in which the mean values

Table IV: Total Number of Aggregates in the Robinson Assay and the PHA+E Assay of Bone Marrow Cells from Untreated AML

	Robinson Assay		Without PHA+E*		With PHA+E**	
Patient	Leukocyte Feeder	Agar	Leukocyte Feeder	Agar	Leukocyte Feeder	Agar
Tu	0***/330****	0/0	0/45	0/0	166/590	144/530
Fl	0/827	0/1867	0/840	14/885	80/1800	300/3200
He	0/157	0/3	0/3	0/3	29/220	30/226
Ca	0/20	0/20	0/90	0/140	66/280	60/360
St	0/360	0/0	0/47	0/0	38/460	20/440

* Incubation of cells in liquid medium without PHS and endotoxin before plating on leukocyte feeders and agar underlayers without leukocytes.
** See materials and methods; PHA+E assay.
*** Figure represents number of colonies per 10^5 plated marrow cells.
**** Figure represents number of clusters per 10^5 plated marrow cells, the size of the clusters is predominantly smaller than 20 cells.

of the number of colonies per 10^5 plated cells from marrow obtained from AML and haematologixally normal patients has been documented. As has been already mentioned, no increase in number of colonies produced by normal marrow has

Table V: Mean Number of Marrow Colonies from AML Patients and Haematologically Normal Individuals in the Robinson Assay and the PHA+E Assay

	Robinson Assay	PHA+E Assay*		Ratio**	
Bone Marrow***	Leukocyte Feeder	Leukocyte Feeder	Agar Feeder	PHA+E Leukocyte Feeder	Robinson Assay
Haematologically Normal	34**** (25-44)****	29 (22-35)	0 (0)		0.85
Remission AML	22 (5-44)	16 (8-46)	0.16 (0-1)		0.72
Untreated AML	0.0 (0-1)	99 (14-290)	113 (22-440)	1187	
Relapse AML	0.5 (0-2)	125 (52-160)	95 (0-130)	250	

* See materials and methods.
** Ratio between number of CFU-c in the PHA+E assay with leukocyte feeders, and in the Robinson assay.
*** Normals are listed in Table II. AML patients are listed in Tables I and II.
**** Mean number of CFU-c per 10^5 marrow cells plated. Figures in parentheses represent the range of colonies obtained.

been observed by adding PHA + E to the system, resulting in a ratio of the number of colonies between the PHA + E assay and the Robinson assay of < 1. The same ratio holds for marrow from AML in remission. These results are in contrast with the results obtained from marrow cultured from AML in relapse and from untreated AML patients. In those cultures, the colony ratio between the two in vitro systems is markedly increased, demonstrating the need for PHA and endotoxin in the liquid phase for colony formation in vitro.

The effect of PHA+E in vitro

Specific experiments were carried out to study the effect of PHA and endotoxin on colony formation by the leukemic cell population when these substances were added separately to the cultures. The results of such an experiment which is representative of three other experiments have been listed in Table VI. It can be noted that leukemic bone marrow cells without being stimulated in a liquid system did not give rise to colonies in an agar system as listed in Table VI, even when

Table VI: Comparison of Effect of PHA and Endotoxin added Separately and in Combination to the Cultures, on Colony Formation of Marrow Cells from a Relapse (AML (E l))

	Semi-solid Phase							
	Agar Underlayer				Agar + Leuk. Underlayer			
No Liquid Phase	– * 0	Endotoxin 0	PHA** 0	(PHA+E***) 0	– * 0	Endotoxin 0	PHA 0	(PHA+E***) 0
Liquid Phase								
– ****	0	0	0	0	0	0	0	0
Endotoxin	0	0	0	0	0	0	0	0
PHA	138*****	145	178	248	124	180	145	205
PHA+E***	155	160	171	228	150	170	217	300

* Agar underlayer without PHA, endotoxin.
** PHA = phytohaemagglutinin; 0.05 ml/ml medium.
*** E = endotoxin: 10^{-7} g/ml medium.
**** Liquid phase in which $2x10^6$ cells have been cultured for 15 hours in medium without PHA, endotoxin.
***** Mean number of colonies from triplicate cultures. Colony number per 10^5 plated cells. The colony incidence in the individual plates do not differ more than 15 % of the mean.

PHA + E were added to the semi-solid phase. When cells were incubated in a liquid system, only PHA could induce colony formation. Addition of endotoxin to the liquid phase had no effect on the untreated colonies generated by PHA, whereas endotoxin added to the semi-solid phase had an amplifying effect on colony formation induced by PHA. Therefore, in the PHA + E in vitro assay as describ-

ed in the materials and methods, PHA is the essential stimulator for the leukemic cell population.

Analysis of cell type in the in vitro colonies

Evidence of colony formation by the leukemic cell population in the marrow from several leukemics in the PHA + E system was obtained by morphology and chromosome analysis. In several cases (3), May-Grünwald staining of several colonies clearly demonstrated the blastic cell population, predominantly present, whereas the more differentiated cells of the myeloid series were scarce. Since we are aware of the limited value of the May-Grünwald staining procedure as a proof for the leukemic origin of a colony, morphological studies of the colonies were

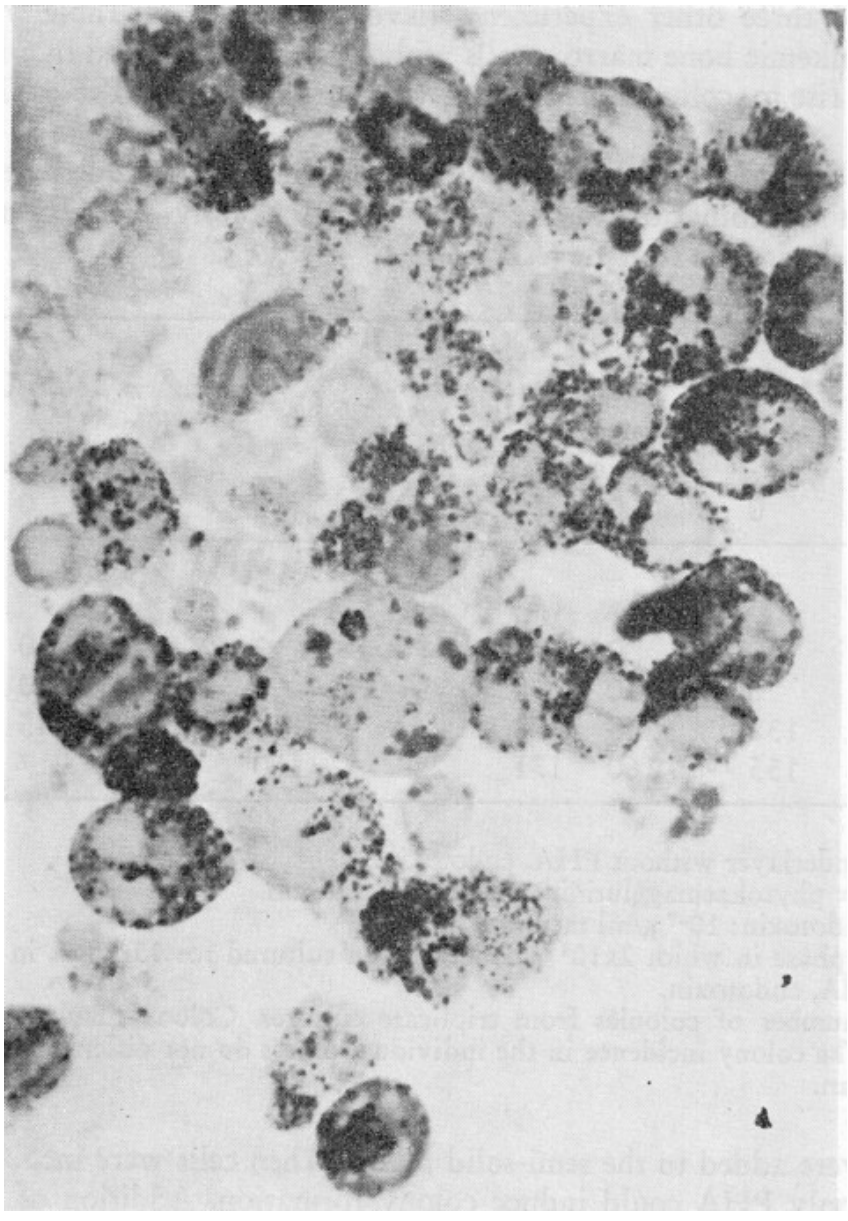
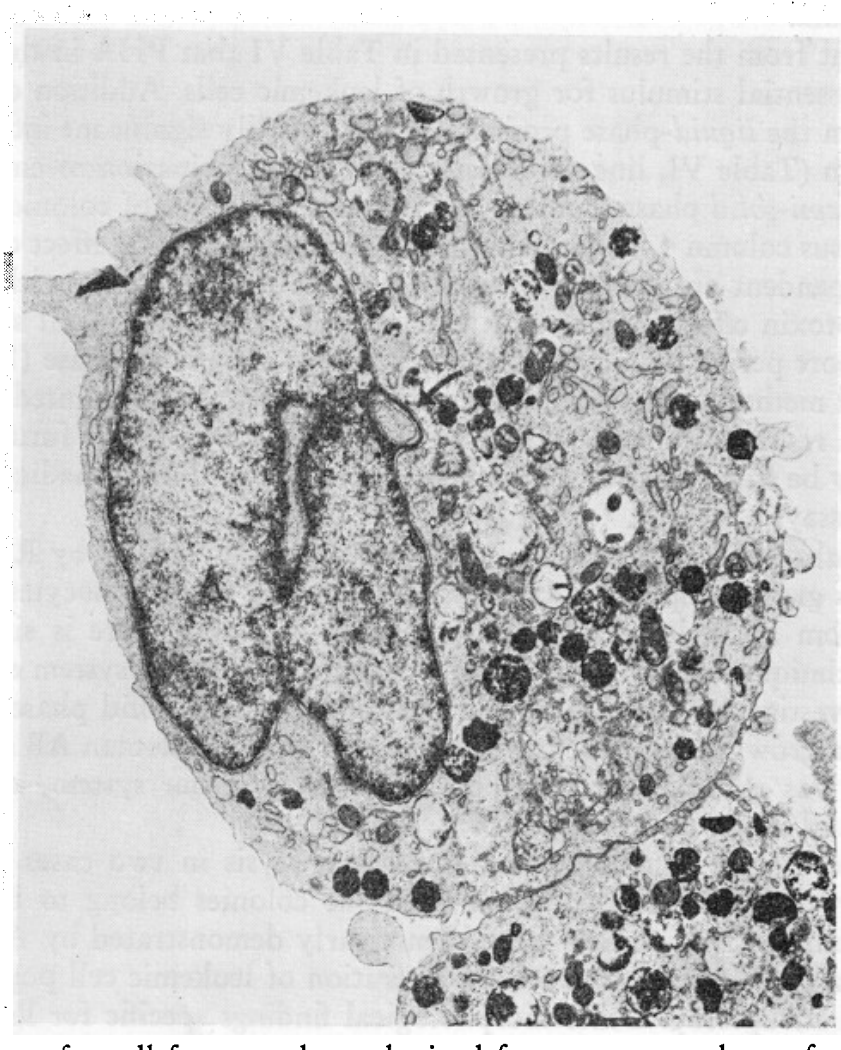


Fig. 2: a. Cross-section of a colony from a seven day marrow culture from patient (El) in AML relapse. Magnification 1700x. The diamino benzidine technique for peroxidase activity clearly shows enzyme activity at the level of granule formation.

performed at the ultrastructural level. Figure 2a is a thick Epon cross-section through a colony grown from an untreated leukemic marrow. The dark granules heavily stained in the cytoplasm of these cells represent the peroxidase positive granules demonstrating the myeloid origin of these cells. Figure 2b represents the typical ultrastructural morphology of one of the colony cells seen in Figure 2a. The cell displays a pocket or bleb on its nuclear surface. This structural abnormality in bone marrow cells has been associated with leukemia and lymphoma by a number of investigators (Achong et al., 1966; Anderson, 1966; Ross et al., 1969). More recently, a correlation between the presence of a high frequency of these nuclear blebs and aneuploidy in acute leukemia has been demonstrated (Ahearn et al, 1974). This particular case (E1) was aneuploid and exhibited a complex chromosome aberration which has been described in several other cases of acute leukemia (45, X, –Y, –C, +D, +E, –G) (Trujillo et al., 1974). This abnormal chromosome pattern could be demonstrated in cells collected from the in vitro colonies of this study and from the liquid cultures incubated with PHA and endotoxin for 15 hours. Twenty-five metaphases were counted of which 19 clearly demonstrated the chromosome abnormality. The remaining six metaphases exhibited a diploid karyotype.



b. Cross-section of a cell from a colony obtained from marrow cultures from patient (El) suffering from AML relapse. Magnification 300x. The nuclear bleb, indicated by arrow, is clearly visible.

Discussion

It has been clearly demonstrated that leukemic cells can form colonies in vitro in the absence of a feeder layer of leukocytes. This is in contradiction with the data of Moore et al. and Metcalf et al. who observed dependence on CSF* in vitro for leukemic cell proliferation (Moore et al., 1974; Metcalf et al., 1974). An explanation for this discrepancy between our results and the Australian data might be that phytohaemagglutinin stimulates subpopulation(s) of the leukemic cell pool which are different from the leukocyte factor dependent leukemic cells.

In the PHA + E system, aggregates of different sizes are present as can be noted from the results presented in Table IV. This spectrum of aggregate size might be indicative of different sensitivity to the PHA stimulus by the leukemic cell population, reflecting the heterogeneity of the blast cell population. More extensive investigation with respect to the number of clusters (aggregates less than 50 cells) in relation to the number of colonies has to be performed in the future. Just like in the Robinson system, the colony cluster ratio as well as the size of the colonies and the clusters might be of significance for further classification of acute myelogenous leukemia.

It is evident from the results presented in Table VI that PHA in the PHA + E assay is the essential stimulus for growth of leukemic cells. Addition of endotoxin to the PHA in the *liquid*-phase produced no statistically significant increase in colony formation (Table VI, line 4 versus line 5). The combination of endotoxin and PHA in the *semi-solid* phase markedly increased the number of colonies (Table VI, column 3 versus column 4, and column 7 versus column 8). The effect of endotoxin in vivo is dependent on previous incubation of the leukemic cells with PHA. The in vitro endotoxin effect might be an enhancement of leukemic cell sensitivity to PHA. The short period of incubation with PHA in the liquid phase (15 hours, see materials and methods) does not produce cell clumping as was pointed out already in the section results (Table IV). Because of the simplicity, in the future, leukemic cells will only be stimulated by PHA without endotoxin during the liquid phase of the in vitro assay.

PHA was also used as stimulus in an in vitro assay described by Rozenszajn et al. (1974), to grow lymphocyte colonies. As a source of lymphocytes, peripheral leukocytes from normal individuals were used. Although there is similarity between our technique and the assay used by Rozenszajn, in the system employed by the Israeli investigators, PHA was added also to the semi-solid phase, which was obligatory for growing lymphocytes. Moreover, the use of human AB serum in the liquid phase was essential for lymphocyte cultures. In our system, a mixture of horse serum and fetal calf serum was used.

Electronmicroscopic studies and cytogenetic analysis in two cases (El and Fe, Table I), revealed that cells collected from the colonies belong to the leukemic cell population. Nuclear pockets have been clearly demonstrated by Ahearn et al. (1974) to be a consistent ultrastructural alteration of leukemic cell populations associated with aneuploidy. Other morphological findings specific for leukemic cells such as asynchronous nuclear cytoplasmic development, nuclear bodies, and bundles

* CSF = colony stimulation factor from leukocytes.

of cytoplasmic fibrils were also found in the cells from the in vitro colonies. These latter findings mark the leukemic cell population in case no abnormal chromosome marker is present in the leukemic cell population. Only a limited number (10) of colonies had been analyzed by electronmicroscopical methods; therefore, these studies do not exclude the possibility that cell types other than leukemic cells proliferate in the PHA + E assay.

Cytogenetic analysis revealed the presence of the aneuploid line in the colonies. The origin of the diploid cells also found in the cultures is still unclear. Possibilities to be considered include the following sources: lymphoid cells, normal immature marrow cells (stem cells?), diploid leukemic elements, etc. This conventional Technique is limited by the fact that only a small number of available metaphases can be recovered. It is possible that the specificity of the PHA stimulation on leukemic cells may be further explored by culturing pure populations of malignant and non-malignant cells using cell separation techniques (Dicke et al., 1973; 1975, in press).

The mode of action of PHA on the leukemic cell population is not known. Colony formation might be a consequence of a humoral factor released by a second cell population stimulated by PHA. Experiments of Aye et al. (1974) support this concept. These investigators observed proliferation of leukemic cells in short term liquid cultures induced by conditioned medium which was prepared from cultures of human peripheral blood cells to which was added PHA. Haematologically normal individuals and patients with acute leukemia were used to prepare these conditioned media. Their data were not conclusive in proving the need of a humoral factor for leukemic cell proliferation due to the fact that residual amounts of PHA were still present in the conditioned medium. Moreover, labelling index and the rate of ^{3}H-TdR incorporation were used as parameters for response of the leukemic cells to the stimulus, which markedly differs from colony formation as measured in our experiments. At the present time, we favor the direct effect of PHA on the leukemic cell population. For the time being, only the linear correlation between the number of leukemic cells plated and the number of colonies per dish as demonstrated in Figure 1 support the above mentioned concept. The value of these results, however, is limited due to the fact that the data were generated from cultures of marrow cells from a single patient. In the future, it will be investigated as to whether this linear relationship is a consistent phenomenon in cultures of marrow from untreated AML and from patient in relapse.

So far, PHA appears to be remarkably specific in stimulating only leukemic cells to form colonies. No effect by PHA was observed on haemopoietic cells in remission. Although the fluctuation of the leukemic sub-populations sensitive to PHA with chemotherapy is not yet understood, it will be of interest to determine whether or not the in vitro phenomenon described in this paper can be used for detection of residual leukemic cells in the remission phase of AML.

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