

# Granulocytic Stem Cells in Friend Leukemia<sup>1</sup>

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## SUMMARY

Friend virus induces a leukemia characterized by the proliferation of neoplastic hematopoietic cells believed to be erythroid precursors. *In vitro* studies were conducted with spleen cells from mice with terminal Friend leukemia in order to determine their capacity for leukocytic differentiation. Spleen cells were obtained from leukemic DBA/2 mice 1 to 2 days before anticipated death and cultured in the presence or absence of colony-stimulating activity (CSA). Growth in liquid culture in diffusion chambers was dependent on CSA and resulted in the generation of normally differentiated granulocytes and macrophages. Colony formation in agar was also dependent on CSA, and the cloning efficiency of leukemic spleen cells was found to be approximately 10 times normal. The colonies formed were composed of leukocytes, which appeared morphologically normal. Total *in vitro* colony-forming units per leukemic spleen exceeded normal by more than 300-fold, but cells elaborating CSA were decreased. Although it is uncertain whether the stem cells stimulated by CSA are "normal" or "leukemic," it is clear that Friend leukemia has profound effects on the proliferation and differentiation of nonerythroid stem cells.

## INTRODUCTION

Friend leukemia virus induces an erythroleukemia characterized by the proliferation of undifferentiated cells in the spleen, liver, and bone marrow (5, 18). The characteristic Friend leukemia cell is thought to be an erythroid precursor (25, 27). Although transplantable tumors derived from the leukemic tissue show no evidence of differentiation (6, 23), the erythroid nature of these tumor cells becomes apparent when they are established in tissue culture (7). Compelling evidence comes from studies on the enhancement of erythroid differentiation when the cells are grown in the presence of dimethyl sulfoxide. Heme, globin, and erythrocyte surface antigens are produced (8, 14, 21).

Based on the premise that there might be undifferentiated cells in the leukemic spleen with the capacity for leukocytic as opposed to erythroid differentiation, cells from mice in the terminal phase of Friend leukemia were cultured *in vitro* using the liquid diffusion and semisolid gel techniques. Under these conditions, a marked increase in the number of

cells in the leukemic spleen with the capacity for granulocyte-monocyte differentiation *in vitro* was revealed.

## MATERIALS AND METHODS

Young DBA/2 mice were used for all studies. The leukemia was initiated by injecting 0.1 ml of a 10% (w/v) crude splenic virus extract i.v. (5). Subsequently, it was maintained by passing viable leukemic spleen cells i.p. Leukemic mice were observed daily until we could predict with reasonable accuracy the period 24 to 48 hr prior to death. At this time, when they appeared moribund, there was massive splenic and hepatic enlargement. The spleen was removed aseptically, and a single-cell suspension was prepared by mincing the tissue and passing it repeatedly through a No. 25 needle. Similarly prepared spleen cells from normal DBA/2 mice served as controls.

Spleen cells were cultured in liquid suspension using the Marbrook *in vitro* diffusion chamber technique (9, 11, 24) and in semisolid gel with the agar plate method (10, 12, 22). McCoy's Medium 5A with 15% fetal calf serum, 100 units of penicillin, and 50  $\mu$ g of streptomycin per ml was used throughout. Three million spleen cells were cultured in liquid suspension in the Marbrook chambers. These cultures were set up with and without the addition of 0.1 ml PMU.<sup>2</sup> The PMU was prepared as previously described and served as the source of colony-stimulating activity (24). Liquid cultures were harvested at intervals up to 14 days for viable cell counts, for [<sup>3</sup>H]thymidine radioautography, and for morphological examination (9, 11). Cellular differentiation in these cultures was assessed histochemically using peroxidase as a marker for granulocytes,  $\alpha$ -naphthyl butyrase as a marker for cells of the monocyte-macrophage line (9), and benzidine for erythroid differentiation (16). Leukocyte phagocytic capacity was tested for with heat-killed *Candida albicans*. In some experiments, cells that had grown in liquid suspension for up to 7 days were transferred to agar to assess the number of CFU-C present (24).

The agar culture technique has been described in detail previously (10, 22). Spleen cells (usually  $2 \times 10^5$  in 1 ml) were plated in 0.3% agar with and without the addition of 50  $\mu$ l PMU in 0.5% agar underlayers. The plates were incubated at 37° in a humidified atmosphere of 7.5% CO<sub>2</sub>, and colonies were counted with a dissecting microscope at 7 to 10 days. Only colonies containing greater than 50 cells were scored. Selected colonies were picked from the agar cultures with a

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<sup>2</sup> The abbreviations used are: PMU, pregnant mouse uterus extract; CFU-C, colony-forming unit (culture); CFU-S, colony-forming unit (spleen).

fine pipet and smeared on slides for morphological examination with Giemsa and acetoorcein stain.

**RESULTS**

**Liquid Culture.** Cell suspensions prepared from the leukemic spleens contained more than 97% undifferentiated cells (Fig. 1). These cells did not contain peroxidase-positive material and had no hemoglobin detectable by benzidine stain. The mean leukemic spleen wet weight was approximately 30 times control and total cell counts showed a similar distribution. The pattern of leukemic spleen cell growth in liquid culture is illustrated in Chart 1. During the 1st 3 days of culture, viable cell counts decreased precipitously. By Day 6 *in vitro*, cultures containing PMU showed marked proliferative activity reflected by a rise in cell counts and [<sup>3</sup>H]thymidine labeling (Table 1). The proliferating cells included undifferentiated blasts and immature cells showing peroxidase-positive granules forming in the Golgi zone (Table 2; Fig. 2). After 8 days in liquid culture, viable cell counts and [<sup>3</sup>H]thymidine labeling indices fell. There was poor cell viability in cultures not containing PMU.

Cells with morphological, histochemical, and functional evidence of maturation along the granulocytic and mononuclear pathways were predominant in culture after Day 5 (Table 3; Fig. 3). Leukopoietic differentiation was largely dependent on the presence of PMU. Granulocytes had typical ring nuclei, were phagocytic, and contained peroxidase-positive granules. The mature macrophages were phagocytic, had characteristic morphology, were peroxidase negative, and stained heavily for  $\alpha$ -naphthyl butyrase (4, 9). The mature leukocytes were indistinguishable from cells grown from bone marrow or spleen of normal mice. Erythrocytic progenitors were not observed in the liquid cultures.

**Cloning in Semisolid Gel.** Leukemic spleen cells did not form colonies in agar cultures without the addition of PMU. Spontaneous colony formation at high cell densities (auto-stimulation) is thought to be due to cells elaborating colony-stimulating activity in the culture plate (17). Even at high cell concentrations, cells from leukemic mice did not form colonies. With normal spleen cells, colony formation was observed at cell densities of  $5 \times 10^6$ /plate.



Chart 1. Growth of leukemic spleen cells in liquid culture. Viable cell counts were determined at intervals up to 10 days. Data represent mean ± S.E. of 7 experiments. —, cultures with added PMU; ---, control cultures.

Table 1  
[<sup>3</sup>H]Thymidine labeling in liquid cultures of leukemia spleen cells (mean ± S.E. of 7 experiments)

Days in culture	With PMU		Without PMU	
	Mean labeling index (%)	Total cells labeled ( $\times 10^5$ )	Mean labeling index (%)	Total cells labeled ( $\times 10^5$ )
0	40	12	40	12
1	25	7.5	23	5
6	40	7.0 ± 1	13	<0.5
8	26	4.5 ± 2	12	<0.5
10	6	1.0 ± 1	0.5	<0.5

Table 2  
Peroxidase reaction in liquid cultures of leukemic spleen cells

Days in culture	Peroxidase-positive cells			
	With PMU		Without PMU	
	$\times 10^3$	%	$\times 10^3$	%
0	0	0	0	0
3	0	0	0	0
6	187 ± 43	11	0	0
8	459 ± 135	27	12 ± 4	4
10	168 ± 58	14	8 ± 4	4

In agar culture with PMU, leukemic spleen cells had a cloning efficiency approximately 10 times that of spleen cells from normal mice. Leukemic cells exhibited a mean CFU-C incidence of 39 per  $2 \times 10^5$ , whereas normal spleen cells had a mean of 4 per  $2 \times 10^5$ . Colony formation was found to be linearly related to the number of cells plated (Chart 2). Since the leukemic spleens were slightly more than 30 times normal size, these observations imply that colony-forming cells (CFU-C) in the leukemic spleen exceeded normal by about 300-fold. The generation of CFU-C in liquid culture was also dependent on the presence of PMU (Table 4). When the cells were transferred to agar at 4 and 7 days, CFU-C had increased by almost 10-fold over the numbers present at the initiation of culture, suggesting that committed stem cells proliferated in response to PMU. All of the colonies formed appeared morphologically normal and were of both granulocytic and monocytic types.

**DISCUSSION**

Leukemia induced by the anemic strain of Friend virus is characterized by the proliferation of undifferentiated cells within the hematopoietic organs of the host. In the terminal phase of the disease, there is massive enlargement of the liver and spleen and infiltration of the bone marrow with resultant failure of normal hematopoiesis (5, 18). Cell lines derived from these leukemic cells show erythroid differentiation which is greatly enhanced in the presence of dimethyl sulfoxide (8). There is considerable evidence suggesting that the "target cell" for Friend virus is a committed erythroid progenitor (13, 15, 18, 25, 27).

Table 3  
Total differential cell counts in liquid culture ( $\times 10^3$ )

Days in vi- tro	PMU	Blasts	Granulocytes	Macrophages	Lymphocytes
0		2940	30	0	30
3-4	+	385 $\pm$ 135	160 $\pm$ 48	266 $\pm$ 114	11 $\pm$ 5
	-	40 $\pm$ 9	25 $\pm$ 5	336 $\pm$ 85	
5-6	+	880 $\pm$ 165	208 $\pm$ 40	480 $\pm$ 90	32 $\pm$ 6
	-	18 $\pm$ 9	20 $\pm$ 10	130 $\pm$ 35	0
7-8	+	255 $\pm$ 75	510 $\pm$ 150	935 $\pm$ 275	0
	-	14 $\pm$ 7	19 $\pm$ 8	168 $\pm$ 84	0
9-10	+	180 $\pm$ 45	276 $\pm$ 72	744 $\pm$ 195	0
	-	10 $\pm$ 5	20 $\pm$ 10	170 $\pm$ 80	0

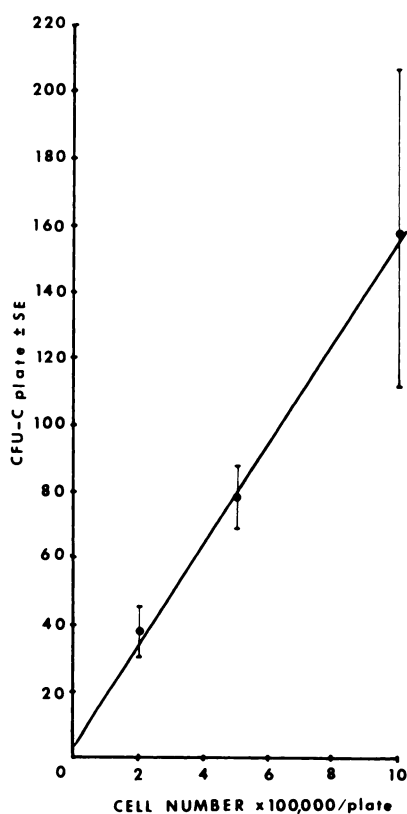


Chart 2. Granulocyte-monocyte colony formation by leukemic spleen cells in agar. Cloning was linearly related to the number of cells plated between  $2 \times 10^5$  and  $1 \times 10^6$ .

In this study, the capacity for *in vitro* leukocytic differentiation of spleen cells from mice with terminal Friend leukemia was examined. Growth of these cells in liquid culture was largely dependent on a source of colony-stimulating activity, a normal granulopoietic-stimulatory material (12). In these suspension cultures, there was a prominent generation of leukocytic stem cells (CFU-C) and development of morphologically normal mature granulocytes and macrophages. The leukemic spleen appeared deficient in cells with the capacity to stimulate colony formation in agar as

Table 4

Generation of CFU-C in liquid culture of leukemic spleen cells

Days in culture	PMU	Total CFU-C/culture <sup>a</sup>
0		590 (550-630)
4	-	547 (385-770)
4	+	5020 (4075-5964)
7	-	483 (360-606)
7	+	3640 (3323-3960)

<sup>a</sup> Mean and range of 2 experiments.

evidenced by lack of autostimulation in high-cell-density cultures. Assays for colony-forming cells in PMU-supplemented cultures, however, showed leukemic spleen cells to have a cloning efficiency approximately 10 times normal. The grossly enlarged leukemic spleen therefore contained over 300 times the number of granulocyte-monocyte precursors (CFU-C) found in the normal spleen. These CFU-C appeared normal with respect to their growth characteristics in semisolid gel culture.

The observation that the leukemic spleen contains a strikingly increased number of functionally normal CFU-C leads to several possible lines of interpretation. It may be, as has been reported in leukemic AKR mice, that bone marrow infiltration causes a migration of leukopoietic stem cells to the spleen (3). Normal CFU-C resident in the leukemic spleen may continue to proliferate as part of a compensatory phenomenon or other response to the presence of the leukemia. Another possibility is that some Friend virus-infected cells are not yet committed along the erythroid pathway and retain the potential for leukopoietic differentiation under appropriate stimulation. If this were the case, it would be analogous to the nature of pluripotent stem cell (CFU-S) involvement in Friend leukemia. Splens from mice with Friend leukemia contain increased numbers of CFU-S with the capacity to form normally differentiated clones in the spleen colony assay (19, 26). An analogy may also be drawn with Rauscher virus-induced leukemia in which CFU-S in the spleen are also greatly increased and have near-normal repopulating capacity when injected into lethally irradiated hosts (1, 2). Although such stem cells are func-

tionally similar to normal, they are infected with virus since CFU-S in Rauscher leukemia may be inactivated with appropriate antibody (1, 2, 20).

The present experiments do not distinguish between the possibilities discussed and several mechanisms may be operative. Most of the granulocyte-monocyte stem cells, however, do not appear to undergo maturation within the leukemic spleen, inasmuch as there is little evidence for granulopoiesis or macrophage genesis in this organ during the terminal phase of Friend leukemia. The lack of mature macrophages in these spleens may explain the deficit in colony-stimulating cells (cells elaborating colony-stimulating activity).

The finding of markedly increased numbers of granulocyte-monocyte progenitor cells in the spleens of mice with Friend leukemia may have important implications regarding the pathogenesis of the disease. Although it is uncertain whether these stem cells are "normal" or "leukemic," it is clear that Friend leukemia has profound effects on the proliferation and differentiation of nonerythroid stem cells.

## REFERENCES

- Brommer, E. J. P. The Role of the Stem Cell in Rauscher Murine Leukemia. Thesis, Rotterdam, 1972. Rijswijk, Z. H., The Netherlands: Radiobiological Institute of the Organization for Health Research TNO.
- Brommer, E. J. P., and Bentvelzen, P. The Haematopoietic Stem Cell in Rauscher Virus-induced Erythroblastosis of BALB/c Mice. *European J. Cancer*, 10: 827-833, 1974.
- Chevalier, C., Gaillard, N., and Frindel, E. The Number of *In Vitro* Stem Cells in AKR Leukemic Mice. *Blood*, 44: 743-750, 1974.
- Cline, M. J., and Sumner, M. A. Bone Marrow Macrophage Precursors. I. Some Functional Characteristics of the Early Cells of the Mouse Macrophage Series. *Blood*, 40: 62-69, 1972.
- Friend, C. Cell-free Transmission in Adult Swiss Mice of a Disease Having the Character of a Leukemia. *J. Exptl. Med.*, 105: 307-318, 1957.
- Friend, C., and Haddad, J. R. Tumor Formation with Transplants of Spleen or Liver from Mice with Virus-induced Leukemia. *J. Natl. Cancer Inst.*, 25: 1279-1289, 1960.
- Friend, C., Patuleia, M. C., and de Harven, E. Erythrocytic Maturation *In Vitro* of Murine (Friend) Virus-induced Leukemic Cells. *Natl. Cancer Inst. Monograph*, 22: 505-522, 1966.
- Friend, C., Scher, W., Holland, J. G., and Sato, T. Hemoglobin Synthesis in Murine Virus-induced Leukemic Cells *In Vitro*: Stimulation of Erythroid Differentiation by Dimethyl Sulfoxide. *Proc. Natl. Acad. Sci. U. S.*, 68: 378-382, 1971.
- Golde, D. W., Byers, L. A., and Cline, M. J. Chronic Myelogenous Leukemia Cell Growth and Maturation in Liquid Culture. *Cancer Res.*, 34: 419-423, 1974.
- Golde, D. W., and Cline, M. J. Identification of the Colony-stimulating Cell in Human Peripheral Blood. *J. Clin. Invest.*, 51: 2981-2983, 1972.
- Golde, D. W., and Cline, M. J. Growth of Human Bone Marrow in Liquid Culture. *Blood*, 41: 45-57, 1973.
- Golde, D. W., and Cline, M. J. Regulation of Granulopoiesis. *New Engl. J. Med.*, 291: 1388-1395, 1974.
- Horoszewicz, J. S., Leong, S. S., and Carter, W. A. Friend Leukemia: Rapid Development of Erythropoietin-independent Hematopoietic Precursors. *J. Natl. Cancer Inst.*, 54: 265-267, 1975.
- Ikawa, Y., Furusawa, M., and Sugano, H. Erythrocyte Membrane-specific Antigen in Friend Virus-induced Leukemic Cells. *Bibliotheca Haematol.*, 39: 955-967, 1973.
- Liao, S-K., and Axelrad, A. A. Erythropoietin-independent Erythroid Colony Formation *In Vitro* by Hemopoietic Cells of Mice Infected With Friend Virus. *Intern. J. Cancer*, 15: 467-482, 1975.
- LoBue, J., Dornfest, B. S., Gordon, A. S., Hurst, J., and Quastler, H. Marrow Distribution in Rat Femurs Determined by Cell Enumeration and  $^{59}\text{Fe}$  Labeling. *Proc. Soc. Exptl. Biol. Med.*, 112: 1058-1062, 1963.
- Messner, H. A., Till, J. E., and McCulloch, E. A. Interacting Cell Populations Affecting Granulopoietic Colony Formation by Normal and Leukemic Human Marrow Cells. *Blood*, 42: 701-710, 1973.
- Metcalf, D., Furth, J., and Buffett, R. F. Pathogenesis of Mouse Leukemia Caused by Friend Virus. *Cancer Res.*, 19: 52-58, 1959.
- OKunewick, J. P., and Phillips, E. L. Change in Marrow and Spleen CFU Compartments following Leukemia Virus Infection: Comparison of Friend and Rauscher Virus. *Blood*, 42: 885-892, 1973.
- OKunewick, J. P., Phillips, E. L., and Brozovich, B. Inactivation of *In Vivo* Colony Forming Units from Rauscher Viral Leukemic Mice by *In Vitro* Treatment with Antiserum (Abstract). *Proceedings of the American Society of Hematology*, 1974, p. 166.
- Ostertag, W., Melderis, H., Steinheider, G., Kluge, N., and Dube, S. Synthesis of Mouse Haemoglobin and Globin mRNA in Leukaemic Cell Cultures. *Nature New Biol.* 239: 231-234, 1972.
- Pike, B. L., and Robinson, W. A. Human Bone Marrow Colony Growth in Agar-gel. *J. Cellular Physiol.*, 76: 77-84, 1970.
- Rossi, G. B., and Friend, C. Further Studies on the Biological Properties of Friend Virus-induced Leukemic Cells Differentiating along the Erythrocytic Pathway. *J. Cellular Physiol.*, 76: 159-166, 1970.
- Sumner, M. A., Bradley, T. R., Hodgson, G. S., Cline, M. J., Fry, P. A., and Sutherland, L. The Growth of Bone Marrow Cells in Liquid Culture. *Brit. J. Haematol.*, 23: 221-234, 1972.
- Tambourin, P., and Wendling, F. Malignant Transformation and Erythroid Differentiation by Polycythaemia-inducing Friend Virus. *Nature New Biol.*, 234: 230-233, 1971.
- Wendling, F., Tambourin, P., Gallien-Lartigue, O., and Charon, M. Comparative Differentiation and Numeration of CFUs from Mice Infected Either by the Anemia- or Polycythemia-inducing Strains of Friend Viruses. *Intern. J. Cancer*, 13: 454-462, 1974.
- Zajdela, F. Contribution à l'étude de la Cellule de Friend. *Bull. Cancer Paris*, 49: 351-373, 1962.

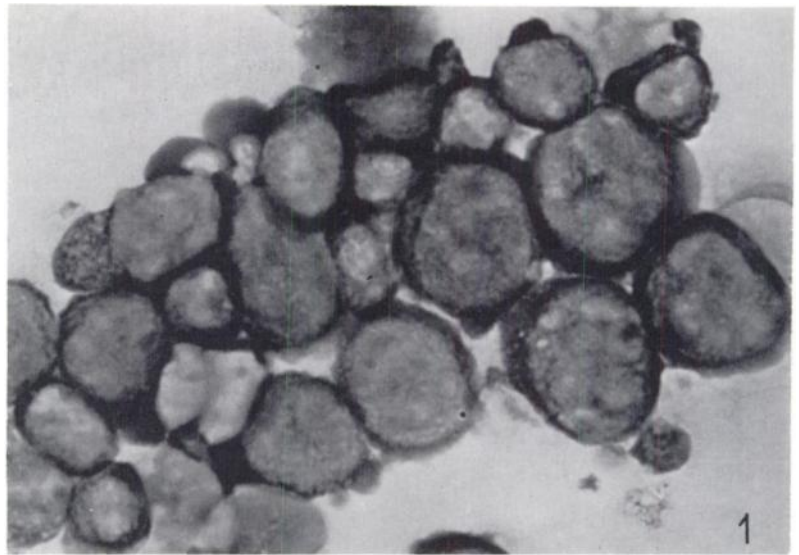


Fig. 1. Leukemic spleen cells before culture stained for peroxidase and counterstained with Giemsa. More than 97% of the cells were undifferentiated blasts.

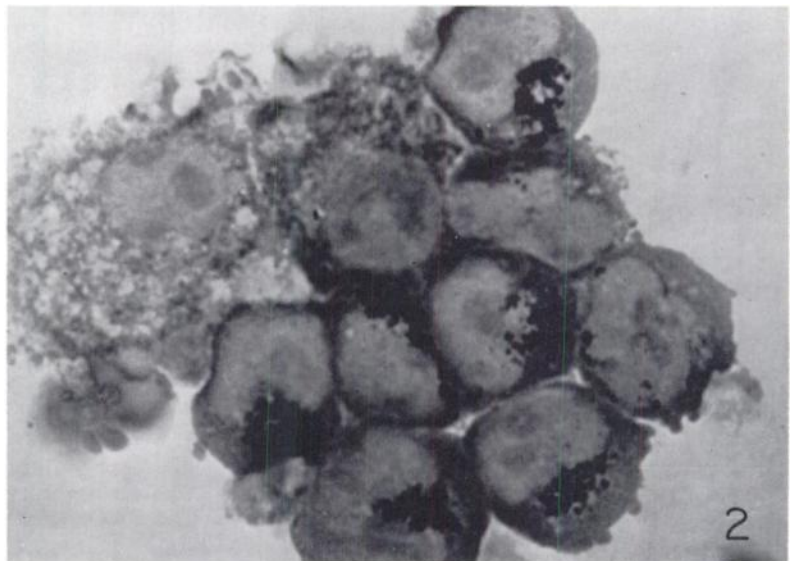


Fig. 2. Leukemic spleen cells after 5 days in liquid culture with PMU. Note the development of peroxidase-positive granules in the Golgi zone of the immature cells. A young macrophage is seen in the upper left.

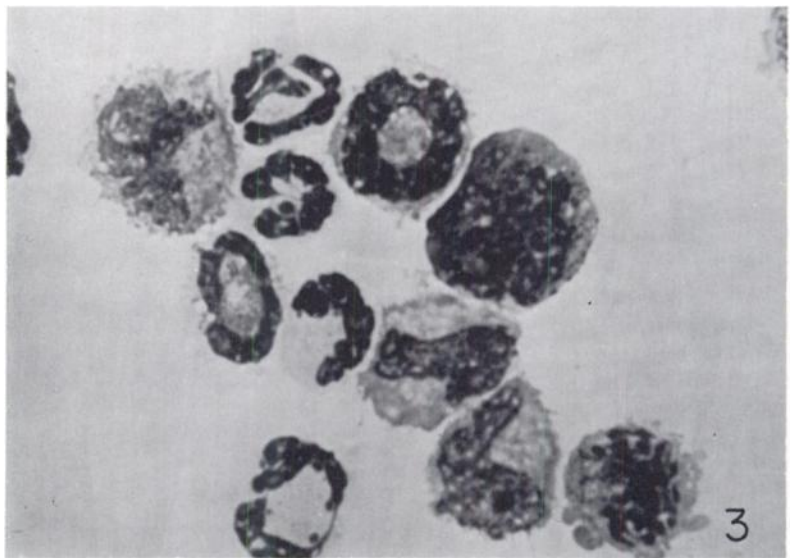


Fig. 3. Mature granulocytes and mononuclear phagocytes in liquid culture of leukemic spleen cells at 8 days.