Return of Alkaline Phosphatase in Chronic Myelocytic Leukemia Cells in Diffusion Chamber Cultures[1] (37288)

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Kinetics of proliferation of normal human bone marrow (BM) and peripheral blood cells have been studied in the diffusion chamber culture system (1–4). In this preliminary report the growth pattern, cell survival kinetics, leukocyte alkaline phosphatase (LAP) activity changes, and chromosomal analysis of two chronic myelocytic leukemia (CML) patients' BM cells cultured in the chamber system are presented.

Methods. Three studies were performed using two patients' (VN, AR1 and AR2) BM cells. Clinical data and hemograms of the patients found at the time of the studies are noted in Table I. At the time of obtaining their BM, both patients were receiving Allopurinol,[2] 600 mg/day. In addition, AR was receiving Busulphan 2 mg/day at the time of his first study. AR2 BM culture study was done 76 days after AR1 and 7 days after stopping Busulphan.

The techniques used were similar to those described (2–6). In brief, ~5 ml of BM was aspirated into a syringe containing 0.5 ml of 1% EDTA. The nucleated cells of the aspirate were isolated by a density gradient sedimentation method using isopaque–dextran solution (sp gr 1.0739). After washing the cells 3 times in Hanks' BSS (10X) medium,[3] they were resuspended in the medium. The final cell count per 0.1 ml was 1.35×10^6 from VN, 1.2×10^6 from AR1 and 0.9×10^6 from AR2 BM cells. The differential cellularity of the final suspensions are noted in Table I.

One-tenth milliliter of the suspension was placed in each of the diffusion chambers. The chambers (2 in each) were then implanted into the abdominal cavity of 6–8 wk old male mice of the Hale-Stoner-Brookhaven strain. Twenty-four hours previously each mouse had received a 700 rad whole body 250 kVp X-ray exposure. The chambers were reimplanted into newly irradiated hosts on Days 1, 8 or 9, 15 or 16 and 23. Commencing 24 hr after the original implantation in VN and AR1 and at 2 hr in AR2 and periodically thereafter in all three studies groups of 8–10 chambers were removed from the mice for 20–24 days.

The chambers were treated for 60–70 min in 0.5% pronase in isotonic buffered salt solution to suspend the cells. The cell suspension was removed and its cell concentration was determined by a hemocytometer method and total cells present calculated. Subsequently, all chambers' contents in each group were pooled, smears made and stained by May–Grünwald–Giemsa stain, and 1000–2000 cells were identified.

Leukocyte alkaline phosphatase (LAP) activity. LAP activity of the segmented neutrophils of the original BM (made from fresh aspirate not exposed to EDTA) and of the cells obtained from the diffusion chambers (DC) at intervals during the study were scored by the method of Kaplow (7). The method for the DC cells was as follows: for each point, four chambers were harvested, the chambers' contents were removed without treating them in pronase solution. The jelly-like material, always present in the chambers shortly after implantation, was broken up by repeated suction into and ejection from

[1] Research supported by the U.S. Atomic Energy Commission. Dr. G. Chikkappa is a Special Fellow of the Leukemia Society of America, Inc.,

[2] 4-hydroxypyrazolo[3,4-*d*]pyrimidine.

[3] Obtained from Grand Island Biological Co., Inc., Grand Island, NY.

TABLE I. Clinical Data of the Patients Noted at the Time of Study.

	Patients					
	VN		AR1		AR2	
	Blood	BM[a]	Blood	BM[a]	Blood	BM[a]
Total blood leukocyte count ($\times 10^3$/mm^3)	19.6	—[b]	39.5	—	19.5	—
Neutrophilic						
segments (%)	62.5	28.7	56.0	31.3	50.5	20.6
bands (%)	3.0	25.2	4.0	19.5	3.5	17.3
metamyelocytes (%)	7.5	13.8	9.0	14.3	2.0	13.4
myelocytes (%)	11.0	24.8	21.0	25.4	22.0	34.0
Promyelocytes + myeloblasts (%)	1.0	1.6	0.0	1.8	0.5	1.2
Hgb (g/100 ml)	12.6	—	13.0	—	14.0	—
Reticulocytes (%)	2.0	—	0.8	—	0.7	—
Platelets ($\times 10^5$/mm^3)	3.75	—	5.6	—	3.5	—
Spleen	Not felt		Tipped		Tipped	
Drugs						
Allopurinol (mg/day)	600		600		600	
Busulphan (mg/day)	0		2		0	

[a] Neutrophilic cells of the BM cells placed into the chambers.
[b] — = not applicable.

pasteur pipettes. The cell suspensions were pooled, smears were made and the LAP activity was evaluated.

Chromosome karyotyping. A direct hypotonic method of making preparations for karyotyping (8) was, with slight modification, used for the original BM. Colchicine was used instead of Velban.[4] The metaphase spreads were made on wet glass slides (distilled water at 4–5°) instead of dry glass slides. The smears were stained with Giemsa instead of orcein and the chamber cells were prepared as follows: each of two mice was injected ip with colchicine, (3 μg/g body wt) in 0.5 ml *N* saline. One hour later, chambers were removed and transferred into two vials each of which contained 62.4 μg of colchicine in 2 ml of 0.5% pronase solution. After 60–70 min of constant agitation, the chambers were removed and their contents were transferred into 3 ml of 1% sodium citrate solution at 37°. Twenty minutes later, a drop of fresh Carnoy's fixative (1 part of glacial acetic acid and 3 parts of absolute ethyl alcohol) was added, mixed and centrifuged at 85 g for 5 min. The supernatant was decanted, and 4 ml of Carnoy's solution was overlayed on the cell bottom. After refrigerating for 30 min at 5–7°, the cells were resuspended and centrifuged again. The supernatant was then removed and several drops of the Carnoy's solution was added to resuspend the cells. Two to 3 drops of the suspension were then placed on the center of a cold wet glass slide. The slide was dried by passing it over a flame or holding in a hot air stream. The slides were stained with Giemsa stain and evaluated.

Results. Changes in the total cellularity of the chambers with time are shown in Fig. 1. From the initial values the cellularity rose to reach a peak on Day 5 in VN, Day 4 in AR1 and between Days 3 and 4 in AR2. From the maximum, the cellularity decreased roughly as a single exponential except for a suggestive plateau around Day 6 in both studies of AR. These data indicate that the CML cells persisted and proliferated in the culture system.

Figure 2 contains the various neutrophilic

[4] Vinblastine.

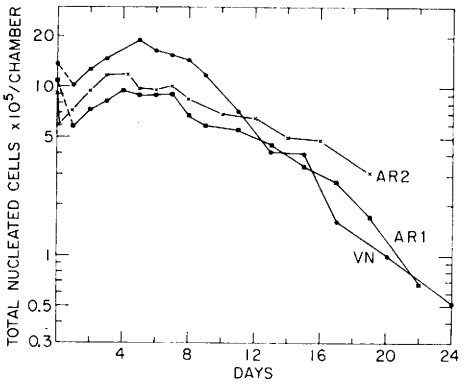

FIG. 1. Bone marrow cells of chronic myelocytic leukemia patients cultured in the diffusion chamber system. Total nucleated cells are shown. In order to evaluate the recovery of cells from the chambers, an earlier (2 hr) sampling was done in AR2. The drop in the cell count from time zero (cells placed into chambers) to the first determination appears to be related to our inability to recover the cells placed into the chambers.

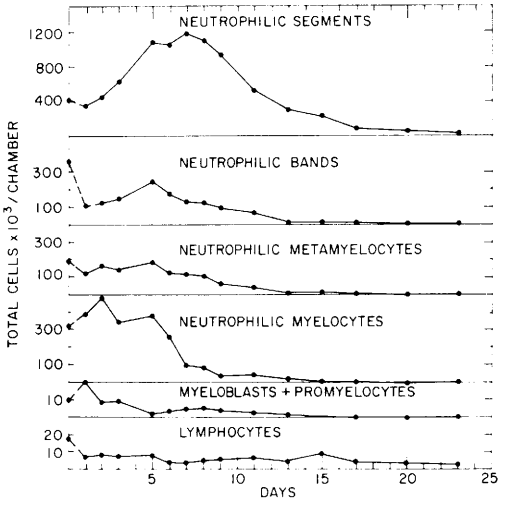

FIG. 2. Absolute neutrophilic cells and lymphocytes counted in the diffusion chambers of the VN marrow study are shown.

and lymphocytic cells of VN marrow study. There is an orderly proliferation of neutrophilic cells as shown by the sequential rise in the most immature cells, myeloblasts plus promyelocytes, followed by a rise in the myelocytes and later in the nondividing neutrophilic cells. The results of AR1 and AR2 marrow culture studies (not shown) were similar to that of VN marrow, except that the myeloblast plus promyelocyte compartment peaked at 48 hr in the AR1 study.

Rate of change in the total cellularity and the various fractions of the granulocytic cells, except for the metamyelocytes and bands, which did not show well formed smooth patterns in any of the studies, are noted in Table II. The rate of changes in the total cells and the various fractions are similar in all the studies with minor differences noted in Table II.

LAP studies. The pattern of the LAP activity noted in segmented neutrophils of the original BM cells and that obtained in the culture studies are shown in Fig. 3. Distribution of the proportion of the cells with 0–4+ enzyme activity of cultured VN cells

TABLE II. Fractional Changes[a] in Total Cells, Various Types of Neutrophilic Granulocytes and LAP[b] Activity of the Segmented Neutrophils Seen in the Chambers.

	Increase in %/day[c]			Decrease in %/day[c]		
	VN	AR1	AR2	VN	AR1	AR2
Total cells	11.8	11.8	18.3	4.7	5.5	4.8
Neutrophilic segments	11.7	14.2	11.8	12.4	8.4	6.1
Neutrophilic myelocytes	17.0	17.3	17.9	13.7	12.2	20.0
Myeloblasts + promyelocytes	55.0	55.0	55.9	22.5	29.6	22.8
LAP	14.3	12.5	10.2	9.5	5.2	5.6

[a] Fractional changes in various cells and LAP are calculated keeping the peak values as 100% and assuming linearity.

[b] Leukocyte (segmented neutrophil) alkaline phosphatase activity.

[c] Rates of increases and decreases estimated from the ascending and descending limbs of the various cells and LAP activity.

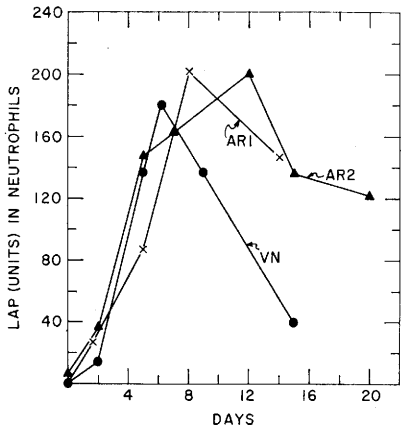

FIG. 3. Leukocyte alkaline phosphatase (LAP) activity measured in the segmented neutrophils from the bone marrow cells placed into the chambers is shown in zero day point. Subsequent measures of LAP activity in the chamber cells are shown against time.

is shown in Fig. 4. Except for fewer (<10%) cells with 4+ activity at the peak, LAP results for AR1 and AR2 were similar to those for VN.

These results indicate that the LAP-positive cells increased in the cultures along with the increase in neutrophils, and the peaks of LAP activity roughly coincided with the segmented neutrophil peaks. The lower section of Table II shows the rate of rise and fall in the LAP activity. These results approximate those noted for segmented neutrophils.

Chromosome analysis. Philadelphia (Phl) chromosome was present in the original BM cells in 8 out of 11 metaphases in AR1 and in 11 out of 20 in VN. Metaphase spreads of AR1 cultures showed the chromosome in 4 out of 7 on Day 5, 2 out of 6 on Day 14, and 1 out of 1 on Day 21. The chromosome was noted in 3 out of 4 spreads on culture Day 10 of VN marrow. It was also noted in 2 out of 2 metaphases on Day 21 in the cultures of AR2. Presence of the Phl chromosome in the cultures as late as 21 days after the initiation of the study strongly suggests that the chromosome was probably derived from a cell that was not in the recognizable granulocytic series on Day 0 since the recognizable neutrophilic cells capable of mitosis would not be expected to survive more than 10 days in the cultures (9). This is true provided the rate of maturation of neutrophilic precursors in the culture system is either normal or faster than normal (9).

Discussion. These data indicate that leukemic cells can be cultured in the chamber system for at least 24 days. The sequential rise in cellularity starting from myeloblast plus promyelocyte compartment, passing through division and maturation in the myelocyte and finally terminating in the mature cells indicates that there is an orderly proliferation and maturation. The cell loss from the chambers seems to be taking place by senescence since vacuolization and disintegration in the cytoplasm and pyknosis in the nuclei of the segmented neutrophils can be observed early, reach a maximum between Days 5 and 9 of the study.

Continued persistence of the myeloblasts and promyelocytes in the chambers as long as 21 days (Fig. 2), suggests that there is a constant formation of new cells from a stem cell pool. This assumes that the myeloblasts are not self-perpetuating in this system and/or the life span of cells

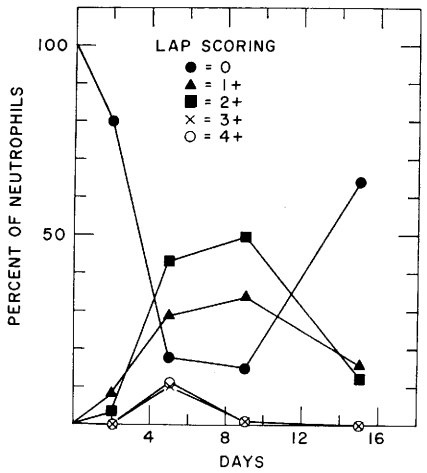

FIG. 4. Percent changes in the segmented neutrophils with variable intensity of leukocyte alkaline phosphatase (LAP) in the culture system are shown. Zero hour points were obtained from the cells of the original marrow placed in the chambers.

is not longer in culture than *in vivo* (9).

A definite rate of transition and survival of the cells in the cultures cannot be estimated with the available data. However, the rate of increase of the granulocytes in the cultures can be computed by using the model for normal granulocytopoiesis suggested by Cronkite and Vincent (9) are: that the myeloblast divides once to give rise to two promyelocytes, which then divide to produce four myelocytes. Transition period for each of these two cells is ~20 hr. Each myelocyte divides twice in an average period of 104 hr to produce 4 metamyelocytes. The average amplification from a stem cell through the differentiated multiplicative pool is 16 (9). The myelocytes are subdivided into two groups: one that has not yet divided; and a second which has divided once. The relative ratios of these groups are 1:2. In Fig. 5, the expected growth rate for neutrophilic cells is compared with the total cells noted in the chambers. This comparison of the obtained total cells with the expected neutrophilic cells does not alter the meaning since more than 90% of the cells in the chambers are in the neutrophilic series during the period evaluated. It is apparent from Fig. 5 that the cells in the cultures seem to proliferate somewhat faster than they would have in the bone marrow under normal circumstances.

The characteristic finding of low or absent LAP in the neutrophils of CML patients is noted in the original BM cells of our patients. The appearance of LAP-positive cells with greater than normal activity in all three studies indicates that the CML cells can under appropriate conditions synthesize LAP. As postulated by others (10), enzyme synthesis is controlled by a derepressor–repressor system. The derepressor is either absent, deficient or ineffective; alternatively, the repressor system is continuously active in classical CML patients. This abnormal enzyme regulating mechanism(s) is corrected in the culture system allowing the cells to recover the synthesizing capacity.

The rise in LAP activity in these studies with the neutrophil count suggests that a common factor may be involved in control of LAP synthesis and proliferation of neutrophilic precursors. It is tempting to suggest that a diffusible factor(s) is present in mice that stimulates both neutropoiesis and LAP synthesis. Stress of radiation and/or surgery is known to increase LAP in normal neutrophils (11, 12). However, this is an unlikely explanation since periodic reimplantation of the chambers into new irradiated hosts did not change the smooth rise and fall in the enzyme activity. It has been suggested that there are two cell lines in CML—one LAP-negative and another LAP-positive (13, 14). One could hypothesize that only the LAP-positive cells proliferated. This, however, is not likely because the Ph1 chromosome, a feature of most cases of

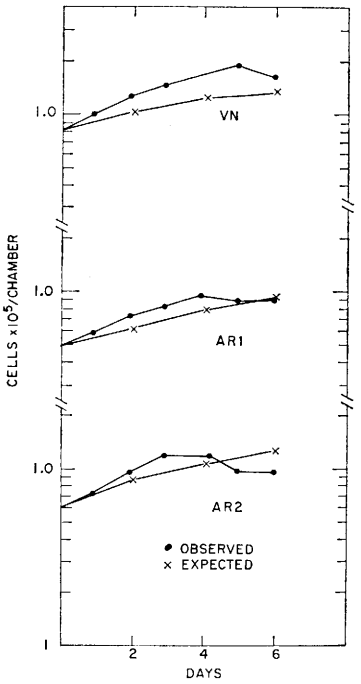

FIG. 5. Total nucleated cells obtained from the culture system and that expected from the cells placed into the chambers are shown. For calculations of the expected rise of cells in chambers, the zero hour points in VN and AR1 are derived by extrapolating the lines joining the observed points on Days 1 to 5 in VN and 1 to 4 in AR1 to ordinate. In AR2, zero hour point was obtained by extrapolating a line through the points of 2 hr to Day 3. See text for details of computation of the expected cell changes in the chambers.

classical CML, persisted in the cultures for 21 days.

If the rate of proliferation and maturation of the neutrophilic precursors in the cultures is similar to that observed in normal human BM, the recognizable immature granulocytic cells placed originally into the chambers should have been exhausted by about 10 days after the commencement of the study (9). As noted above, the Ph1 chromosome was recognized in the cells of the chambers for 21 days. This strongly implies that the chromosome abnormality is maintained in stem cells (15, 16) and transmitted to discernible neutrophilic precursors by differentiation into recognizable granulocytic cells.

Summary. Bone marrow cells of chronic myelocytic leukemic patients were cultured in a diffusion chamber system. The proliferation of neutrophilic granulocytes was orderly, starting in the myeloblasts and promyelocytes, passing through successive phases of maturation to become segmented neutrophils.

Leukocyte alkaline phosphatase activity of the neutrophils introduced into the chambers was negative. The neutrophils in the chambers were positive when first tested on Day 2. This suggests that the factor(s) controlling the synthesis of LAP is activated in the culture system environment.

The Ph1 chromosome, a hallmark of most CML, was present in the cultures for 21 days, indicating that the chromosome aberration persists and is not influenced by the milieu of the dividing cells in culture, unlike the LAP activity. The results of LAP and chromosome studies together suggest that the defect in CML involves a chromosomal aberration and a defective mechanism for transfer of the genetic message.

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Received Jan. 19, 1973. P.S.E.B.M., 1973, Vol. 143.