**In Vitro Development of Bone Marrows from Patients with Neutropenia**

*By Peter Lau, Jerome I. Brody and Lawrence H. Beizer*

The purpose of this investigation was to determine whether a change from an in vivo to an in vitro environment could favorably influence the development of bone marrows, obtained from patients with neutropenias of unknown cause, which showed defective granulocytic maturation. The theory of leukoagglutination and quantitative experimental granulokinetics have never satisfactorily explained the association of low peripheral blood neutrophil counts with normocellular or hypercellular bone marrows containing a relative paucity of mature neutrophils in the presence of adequate numbers of other granulocytic forms. This combination might infer that certain inhibitory factors, inherent in the cellular components of the myeloid tissue or extrinsic to them, prevent the normal sequential growth of neutrophil precursors and ultimate release of mature granulocytes. Since, however, these bone marrows also resemble, morphologically, marrows of individuals undergoing leukapheresis, clinical neutropenia might also be explained on the basis of bone marrow exhaustion resulting from a drain on the circulating granulocytic pool. The study to be described, using the technic of in vitro tissue culture, further explores these problems.

**Methods and Materials**

I. Patient Groups

A. Patients with Neutropenia. This category included 7 patients, 3 male and 4 female, ranging in age from 16 to 71 years, with neutropenia of unknown etiology, all of whom had been observed clinically for a minimum of 6 months. They were referred to the Division of Hematology, either as outpatients or while in the hospital, because of the fortuitous discovery of neutropenia during studies completely unrelated to a blood disorder. None of the patients had knowingly been exposed to industrial toxins, household toxic agents or medications, and they had never received blood transfusions. Hepatosplenomegaly or significant lymphadenopathy were never found in any of their physical examinations. Serial leukocyte counts were obtained once or twice weekly for at least 6 weeks. Absolute neutrophil counts never exceeded 1500/mm³ except in two patients when the values rose, on a single occasion, to 1750/mm³ and 2500/mm³, respectively. Hematocrits and reticulocyte and platelet counts were always within normal limits on

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repeated examinations, as were the chest x-ray film, BUN, uric acid, complete liver function tests, tests for lupus erythematosus and rheumatoid arthritis, serologic tests for syphilis, leukocyte alkaline phosphatase, Coombs' test and serum immunoelectrophoresis. Their bone marrows were of normal or moderately increased cellularity and, according to standards established for differential cell counts, contained comparatively few band forms and mature neutrophils. The other granulocytic precursors were distributed in a quantitatively normal manner.

B. Control Patients. (1) Normal controls: Bone marrows and plasmas, from 12 patients admitted to the surgical and gynecology services because of injuries or incomplete abortions, who had no hematopoietic abnormalities, were used as normal control tissue culture components. These materials were obtained just prior to the patients' discharge, when they were almost completely recovered and no longer acutely ill. (2) Abnormal controls: Bone marrows and plasmas from 2 patients with chronic and 2 with acute granulocytic leukemia were included in the experimental protocol because these diseases have known inherent cellular abnormalities. The patients with chronic leukemia were in relapse and had received no busulfan for at least 10 days prior to performance of tissue culture. Those with acute leukemia were taking adrenocortical steroids and 6-mercaptopurine when their bone marrows and plasmas were used in the culture procedures.

II. Bone Marrow Culture

A. Preparation of Components. All bone marrows were withdrawn from the posterior iliac crest into a syringe containing 0.1 ml. heparin (1000 U./ml.). Approximately 8 to 10 ml. of aspirate were expressed into a sterile centrifuge tube and kept at room temperature for 30 to 60 minutes until the majority of the red blood cells had sedimented to the bottom of the tube. Occasionally, at the end of this period, if required, the marrow was centrifuged at 80 g for 5 minutes to complete the deposition of erythrocytes. The marrow-containing supernatant was then transferred to another centrifuge tube and centrifuged at 2500 g for 5 minutes. This supernatant was discarded and the bone marrow, appearing as a buffy coat, resuspended in 1 ml. of TC-199* culture media. A nucleated cell count was done by hemocytometer and preculture cover slip smears stained to be used later for comparative purposes. Plasma for tissue culture was heparinized in the proportion indicated earlier and was made cell-free by centrifugation at 2500 g for 30 minutes.

B. Culture Technic. Suspension cultures were made in disposable 16 × 22 mm., snap-cap glass vials containing 5 ml. of TC-199 culture media, 3 ml. of human plasma, and 100 μg. each of crystalline penicillin G and streptomycin sulfate. Sufficient suspending marrow was added to the tissue culture components so that each vial finally contained 8 to 10 million nucleated cells per culture. The various bone marrow and plasma combinations used were: (a) normal bone marrow with autologous plasma; (b) normal bone marrow with normal homologous plasma; (c) normal bone marrow with leukemic plasma; (d) leukemic bone marrow with autologous plasma; (e) leukemic bone marrow with normal plasma; (f) neutropenic bone marrow with autologous plasma; (g) neutropenic bone marrow with normal plasma; (h) normal bone marrow with neutropenic plasma. They were purposely arranged to consider the effect of other factors, in addition to environment, on bone marrow growth. It is conceivable, for example, that homologous normal plasma, plasmas from patients with leukemia, leukopenic plasmas, or the ingredients of the culture media might, by themselves, either promote or inhibit cellular development. All cultures were incubated in air, in the vertical position at

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Table 1.—Mature/Immature Mean Ratios Obtained with Different Bone Marrow and Plasma Combinations

<table>
<thead>
<tr>
<th>Category</th>
<th>Combinations*</th>
<th>24 Hours</th>
<th>48 Hours</th>
<th>72 Hours</th>
<th>96 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Marrow</td>
<td>Plasma</td>
<td>No.</td>
<td>Ratio</td>
<td>SE</td>
</tr>
<tr>
<td>A</td>
<td>Nor.</td>
<td>Ant.</td>
<td>12</td>
<td>1.2</td>
<td>.08</td>
</tr>
<tr>
<td>B</td>
<td>Nor.</td>
<td>Nor. Hom.</td>
<td>4</td>
<td>1.4</td>
<td>.17</td>
</tr>
<tr>
<td>C</td>
<td>Nor.</td>
<td>Leuk.</td>
<td>2</td>
<td>1.2</td>
<td>—-</td>
</tr>
<tr>
<td>D</td>
<td>Leuk.</td>
<td>Ant.</td>
<td>4</td>
<td>1.1</td>
<td>—-</td>
</tr>
<tr>
<td>E</td>
<td>Leuk.</td>
<td>Nor.</td>
<td>5</td>
<td>1.0</td>
<td>.06</td>
</tr>
<tr>
<td>F</td>
<td>Neut.</td>
<td>Ant.</td>
<td>8</td>
<td>1.5</td>
<td>.17</td>
</tr>
<tr>
<td>G</td>
<td>Neut.</td>
<td>Nor.</td>
<td>8</td>
<td>1.4</td>
<td>.16</td>
</tr>
<tr>
<td>H</td>
<td>Nor.</td>
<td>Neut.</td>
<td>7</td>
<td>1.7</td>
<td>.21</td>
</tr>
</tbody>
</table>

*Nor. = normal; Ant. = autologous; Hom. = homologous; Leuk. = leukemic; Neut. = neutropenic.

1Standard error.
2Not applicable.
3Based on 6 determinations; see Table 2 at 96 hours.

Their pH was adjusted as frequently as necessary and maintained between 7.2 and 7.6.

C. Culture Sampling and Interpretation. Serial samples of each culture were taken at 24-hour intervals up to 96 hours since considerable macrophage transformation occurs after this time. A sterile capillary pipet aspirated aliquots from the center as well as from the peripheral quadrants of each vial floor. Approximately 0.3 to 0.5 ml. of material so obtained was expressed into a test tube and centrifuged to sediment the cells. Coverslip smears of the sediments, 2 for each vial, were stained with Wright’s-Giemsa. They were scanned as for conventional bone marrow interpretation and representative fields chosen for differential cell counts. For each coverslip, at least 400 identifiable, separate granulocytic elements were counted. The cells sequentially preceding and including the metamyelocyte were considered immature. Band forms and segmented neutrophils, since both are seen normally in the peripheral blood, were called mature cells. The mature/immature ratio then was computed.

In order, however, to indicate the changing cellular proportions in a meaningful and comparable manner, the preculture mature/immature ratio, after its absolute calculation, was then given a hypothetical value of 1 and the subsequent or corrected ratios determined as follows:

\[
\text{Corrected mature/immature ratio} = \frac{\text{Actual mature/immature ratio at given hr.} \times 1}{\text{Actual mature/immature ratio at 0 hr.}}
\]

RESULTS

Table 1 summarizes the results of bone marrow cultures. The values shown are the numerical means and their standard errors obtained for each experimental group. Normally, as indicated by categories A and B, bone marrow maturation begins in vitro during the first 24 hours. At this time the mature/immature ratio is at or exceeds unity. This development appears progressive, attains a peak at 72 hours and plateaus or regresses thereafter. A similar sequence was observed with category C in which leukemic plasma did not prevent the development of normal neutrophils. On the other hand, the inherently abnormal leukemic bone marrows, whether in their own or normal homologous plasmas, demonstrated a singular inability to fully mature
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Fig. 1.—Preculture appearance of bone marrow from a patient with neutropenia. An occasional band form is seen but mature neutrophils are virtually absent ($\times$ 450).

(categories D and E). In these instances the corrected ratios were mainly at or just below 1. The ratio of 1.1 at 24 hours observed in category D, similar to the normal 1.2 ratio, may reflect the presence of a minor, ineffective, short-lived nonleukemic bone marrow population. Particularly significant, and in direct contrast to the leukemic marrows, was the in vitro developmental patterns (Figs. 1 and 2) of the neutropenic marrows (categories F, G) which paralleled those for normal bone marrows grown with autologous, homologous normal, and leukemic plasma (categories A, B, and C).

Some leukopenic plasmas, when supporting growth of normal bone marrow (category II), produced ratios different from and higher than those obtained with other marrow-plasma combinations. For this reason, Table 2 separately indicates these individual experimental values. With leukopenic plasmas 1 and 4 the change is apparent at 24 hours since these ratios exceed the ones observed with the other nonleukemic marrows at this time as is the case with plasmas 1 and 2 at 48 hours. At 72 hours, plasmas 1, 2, 5, and 7 have produced ratios which are greater than the mean values recorded at the same period for cultures not containing leukopenic plasma. The ratios at 96 hours have begun to diminish, as with normal plasma, but leukopenic plasmas 1, 2, and 5 continue to maintain an apparently stimulating effect.

DISCUSSION

The first major observation of this study was that neutropenic bone marrow, once removed from the body and grown in either autologous or homologous normal plasma, underwent normal maturation as shown by progressive altera-
Fig. 2.—Same marrow after 72 hours of in vitro growth. Mature granulocytes now are scattered throughout this representative microscopic field (× 450).

Discussion of bone marrow cellular ratios (Table 1). The results strongly suggest that these cells are potentially capable of normal sequential development. The identical behavior of normal marrows suspended both in normal autologous and homologous plasma makes it unlikely that environmental change alone or that constituents of the culture media influence maturation in a nonspecific manner. The inability of bone marrows from patients with leukemia to undergo normal development, regardless of the plasmas in which they were suspended, is additional evidence that external factors cannot correct disorders which are primarily cellular in origin. The validity of the latter comment is supported by the failure of plasmas from patients with these same blood dyscrasias to adversely affect normal bone marrows.

The second important observation, complementing the first, is that certain leukopenic plasmas appeared to accelerate cellular maturation of normal bone marrows. Although the differences between the means of categories A and H were statistically significant at the 5 per cent level only after 24 hours, the tabulated individual ratios themselves still must be considered in the overall evaluation of the data. It is not possible to disregard the persistently elevated ratios of plasmas 1, 2, and 5 (Table 2) which set these plasmas apart from other members of category H and reflect the comparatively high standard errors of this group (Table 1). The contrasting low standard errors shown in category A (Table 1) supports the reliability and reproducibility of the system when normal components are used in the assay. The latter values also suggest that the results obtained with neutropenic plasmas 1, 2, and 5 are not fortuitous and infer that these plasmas contained a component which influenced,
in some way, the kinetics of the complex granulocytic maturation cycle. Since, in the isolated cultured bone marrow particles, there would be no further input of myeloblasts from a stem cell pool, this effect may be related to a shortening of the cellular transit times through the theoretical granulocytic compartments, or to the failure of a greater portion of myelocytes to revert to a more primitive cell type before completing maturation. These elevated ratios also imply that sustained peripheral neutropenia, whatever its cause, distorts the normal feedback mechanism which regulates leukopoiesis and results in the presence of excess circulating bone marrow stimulants analogous to factors controlling the normal release of red cells and platelets. The ability of plasmas, obtained from animals and man made neutropenic by leukopheresis and nitrogen mustard, to produce experimental leukocytosis is in accord with this hypothesis.

The current observations do not indicate whether the normal in vitro maturation of leukopenic bone marrow is related to the elimination of an in vivo inhibitor or reflects the absence of peripheral leukocyte removal and decreased bone marrow stress. The in vitro behavior of leukopenic plasma, as used in culture with autologous and normal bone marrows, makes the inhibitor theory somewhat untenable. The results do provide evidence, however, that the myeloid elements in certain benign forms of neutropenia are innately capable of normal sequential growth.

**SUMMARY**

Bone marrows from patients with neutropenia, characterized morphologically by a paucity of mature neutrophils, underwent normal maturation when removed from the body and grown in tissue culture. In addition, certain leukopenic plasmas appeared to stimulate development of normal bone marrows under similar circumstances. These observations suggest that the granulocytic elements in benign forms of neutropenia are innately capable of normal sequential growth and that sustained peripheral neutropenia may distort the normal feedback mechanism which regulates leukopoiesis.

**SUMMARIO IN INTERLINGUA**

Medullas ossee ab patientes con neutropenia, characterisate morphologicamente per un paucitate de neutrophilos matur, se provava capace de matura-
tion normal, post lor extraction ab le corpore, in lor subsequente crescentia in histoculturation. In plus, certe plasmas leucopenic pare stimular le desenvolpamento de normal medullas ossee sub simile circumstancias. Iste observationes suggestiona que le elementos granulocytic in formas benigne de neutropenia es intrinsecamente capace de un normal crescentia sequential e que persistente neutropenia peripheric distorque possibilemente le normal mechanismo retro-agential que effectua le regulation del leucopoiese.

REFERENCES


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