The Effect of Neutropenia on Myeloid Growth and the Stem Cell in an In Vivo Culture System

By W. S. Tyler, Eero Niskanen, Frederick Stohlman, Jr., Jane Keane, and Donald Howard

An in vivo diffusion chamber (DC) culture technique was used to evaluate the role of circulating humoral factors in the control of myelopoiesis. Normal mouse bone marrow cell suspensions sealed in DC's were implanted intraperitoneally into mice or rats rendered neutropenic by pretreatment with cyclophosphamide, and the growth of cells in the DC's was evaluated at intervals thereafter. When marrow was cultured in hosts with graded neutropenia, a dose-related augmentation of myelopoiesis was demonstrated. The growth of macrophages and of pluripotent stem cells, measured by the spleen colony-forming technique, was also enhanced by culture in neutropenic hosts. These data provide further evidence that the rate of myelopoiesis is influenced by a circulating humoral factor. They further suggest that a humoral or hormonal factor is important in control of the pluripotent stem cell population.

The factors responsible for the regulation of myelopoiesis are of obvious clinical and biological significance, but evaluation of these control mechanisms has been hampered by certain unique features of myelopoiesis and the consequent lack of appropriate assay systems. The bone marrow myeloid compartment, unlike the erythroid, contains significant numbers of mature elements. This storage compartment provides a ready reserve for mobilization of granulocytes after an appropriate stimulus. It has been clearly demonstrated by several investigators that neutropenia1,2 or endotoxemia3 will generate a plasma factor, termed leukocytosis-inducing factor1 or neutrophilia-inducing activity (NIA),4 that mobilizes this reserve of granulocytes from the storage pool. The relationship, if any, of NIA to the differentiation of precursor cells into the myeloblast-promyelocyte compartments is unknown. It is entirely possible that a separate long-range humoral factor is responsible. Alternatively, it might be suggested that the initial depopulation...
EFFECT OF NEUTROPENIA

of the storage pool by NIA triggers an intramedullary feedback loop that is responsible for differentiation of myeloid precursor cells.

The recent studies on the growth of myeloid colonies in vitro\textsuperscript{4,5} suggest that a glycoprotein,\textsuperscript{6} termed colony-stimulating factor or CSF, might serve as a differentiating hormone. The culture of murine marrow in soft agar or methylcellulose requires the presence of CSF.\textsuperscript{7} CSF may be obtained from diverse sources, including newborn kidney tubules,\textsuperscript{4} embryo\textsuperscript{5} or fibroblast feeder layers,\textsuperscript{8} serum,\textsuperscript{9} urine,\textsuperscript{10} and pregnant uterus.\textsuperscript{11} Its concentration in serum has been shown to increase in neutropenic states\textsuperscript{12} or after the administration of endotoxin,\textsuperscript{13} suggesting a physiologic role. An increase in the plasma concentration of CSF has been shown to be temporally correlated with changes in the myeloid precursor pool and with a sequential wave of differentiation through the granulocytic compartment.\textsuperscript{12,13} However, it is unfortunately not possible to draw final conclusions as to the physiologic significance of CSF, its relationship to NIA, and its role in the control of myelopoiesis due to the above-mentioned considerations with respect to NIA, as well as the lack of a quantitative measure of myelopoiesis.

A recently described modification\textsuperscript{14} of the technique of Berman and Kaplan\textsuperscript{15} for culturing hemopoietic cells holds promise of circumventing the above dilemma. In this technique, hemopoietic cells are implanted intraperitoneally in Algire diffusion chambers. Growth of cells in these chambers is evaluated at various intervals thereafter. Since newly produced cells are retained in the chamber, NIA will not result in discharge of mature cells from the chamber; migration of stem cells will not occur as is the case with bone marrow,\textsuperscript{16} and theoretically the implanted marrow will be exposed to systemic humoral factors. This approach appears to provide an opportunity to observe more quantitatively the effects of humoral or hormonal regulation on in vivo myelopoiesis.

Results of experiments in which normal mouse bone marrow was cultured in diffusion chambers implanted intraperitoneally into animals that were rendered neutropenic by pretreatment with cyclophosphamide (CY) will be reported. Proliferation of granulocytic cells, macrophages, and hemopoietic stem cells was observed within the diffusion chamber, and their growth rates were influenced by the presence of neutropenia.

MATERIALS AND METHODS

Virgin female CF1 mice weighing 23–30 g, or virgin female Sprague-Dawley rats weighing 200–300 g, were used.

Diffusion chambers (DC) were made by gluing Millipore filters (0.22 μ average pore size and 13 mm diameter) on either side of a lucite ring, 14 mm in diameter. The interiors of the DC's were 2 mm deep and 10 mm in diameter. They were placed in a drying oven overnight at 80°C for sterilization. Cell suspensions were prepared by washing the tibial marrow from a minimum of three mice with ice-cold TC 199 or Hanks buffered salt solution (HBSS) containing 10% heparinized mouse plasma and 100 μg/ml of penicillin G and streptomycin. The concentration of nucleated cells was adjusted to 10⁶/ml; and each DC was filled with 0.1 ml of the suspension containing 10⁵ cells, sealed with a plastic plug and Millipore cement, and kept in culture medium on ice until implantation. CY was administered intravenously to rats or intraperitoneally to mice 24 hr before
implantation of DC's. Controls were injected with saline (SA). Two or four DC's were placed intraperitoneally into rats anesthetized with chloral hydrate or mice anesthetized with pentobarbital. DC's were implanted alternately into CY- and SA-pretreated hosts to normalize any changes in the cell suspension resulting from storage during surgery.

At intervals up to 7 days after implantation, groups of three to 14 treated and control hosts were anesthetized with ether, bled by cardiac puncture for white counts and differentials, and killed by cervical dislocation. Tibial cellularity of host animals was determined with a Coulter Model B as described by Fruhman. Bone marrow smears were made from the opposite tibia by the paintbrush technique and stained with Wright-Giemsa. The percentage of proliferative granulocyte precursors, including myeloblasts, promyelocytes, and myelocytes, was determined from 500 cell differential counts. The absolute number of proliferative granulocyte precursors per tibia was derived from the tibial cellularity and the per cent of proliferative precursors.

The chambers were removed after sacrifice; each was placed, unopened, in 2 cc of TC 199 or HBSS containing 5% Ficoll (Sigma Chemical) and 0.5% B grade Pronase (Calbiochem), a proteinase preparation used to dissolve the clots that form within DC's. They were agitated for 60-65 min in a water bath at 25°C. The chambers were then opened, and the contents were aspirated and placed into preweighed plastic cups. The interior of each chamber was washed with 3 vol of HBSS or TC 199, and the wash fluid was added to the cell suspension. Volume of the resultant cell suspension was determined by weight. Nucleated cells were counted in duplicate with a hemocytometer. The cells were then concentrated by centrifugation as described by Boyum and Borgstrom, smeared between cover slips, and stained with Wright-Giemsa for differential counts.

Cells harvested from diffusion chambers were classified morphologically as early proliferative cells (EPC's), myelocytes, nonproliferative granulocytes, and macrophages. The EPC's were mononuclear cells with a leptochromatic nucleus that in most instances contained a clearly defined nucleolus. The nuclear cytoplasmic ratio was greater than one, and the cytoplasm was basophilic. Their proliferative nature was confirmed by thymidine labeling. Myelocytes were identified by the characteristic murine, ring-shaped nucleus, and the nonproliferating metamyelocytes, bands, and mature neutrophils were identified by the usual criteria. The macrophages had eccentric nuclei and were occasionally binucleate. Their nuclei were more pachychromatic than those of the EPC's. The basophilic cytoplasm was heavily vacuolated.

When the numbers of splenic colony-forming units (CFU) per DC were measured, the contents of eight to 24 chambers were pooled and centrifuged at 400 g for 10 min. The cell pellet was resuspended in TC 199, the concentration of cells was measured, and the suspension was diluted for injection. Mice used to assay for CFU were given 950 R total-body irradiation from a cesium source within 6 hr before intravenous injection of the cell suspension. They were killed after 8 days, their spleens were fixed in Bouin's solution, and the macroscopic colonies were counted by two observers. CFU concentrations in the original bone marrow cell suspensions used for implantation were measured by injecting 35 or 50 X 10^6 cells into irradiated mice under similar conditions. To classify histologically the types of spleen colonies developing from normal marrow cells or from DC's, a series of sections from the center of each spleen was stained with hematoxylin and eosin and was examined.

To assess the effect of CY pretreatment and neutropenia on diffusion of protein and other nutrients into DC's 131I-labeled human serum albumin was injected intraperitoneally into host animals 4 days after implantation. The proportion of labeled albumin that had diffused into the chambers 4 hr thereafter was similar in both groups: 2.73 ± 0.25% for CY-pretreated hosts and 2.98 ± 0.33% for SA-pretreated hosts.

RESULTS

Growth of Mouse Marrow Cells Implanted into Rats

Table 1 summarizes the effects of CY on the peripheral blood neutrophils
Table 1. Effect of Cyclophosphamide on Host Rats

<table>
<thead>
<tr>
<th>Day*</th>
<th>Treatment</th>
<th>Number of Rats</th>
<th>Blood Neutrophils/ cu mm</th>
<th>Proliferative Granulocyte Precursors/ Tibia x 10^-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>SA</td>
<td>6</td>
<td>1449 ± 144†</td>
<td>15.3 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>CY 50 mg/kg</td>
<td>6</td>
<td>79 ± 17</td>
<td>7.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>CY 100 mg/kg</td>
<td>3</td>
<td>136 ± 125</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>SA</td>
<td>7</td>
<td>783 ± 207</td>
<td>11.8 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>CY 50 mg/kg</td>
<td>8</td>
<td>1193 ± 212</td>
<td>19.2 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>CY 100 mg/kg</td>
<td>3</td>
<td>1063 ± 380</td>
<td>18.6 ± 6.2</td>
</tr>
<tr>
<td>7</td>
<td>SA</td>
<td>6</td>
<td>1053 ± 225</td>
<td>15.1 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>CY 50 mg/kg</td>
<td>6</td>
<td>1086 ± 360</td>
<td>13.5 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>CY 100 mg/kg</td>
<td>3</td>
<td>3266 ± 1027</td>
<td>21.3 ± 0.8</td>
</tr>
</tbody>
</table>

* After implantation of chambers. CY or SA was injected 1 day before implantation. † SE of the mean.

and the numbers of proliferative granulocytic precursors in the bone marrow of host rats. The degree of neutropenia 3 days after CY was not significantly different at the two dose levels, but the extent of marrow depopulation and the subsequent recovery rate were greater in animals given 100 mg/kg. By the sixth day after CY, complete recovery with some overshoot was evident in both groups.

The number of cells in DC’s after 2–7 days of culture is shown in Fig. 1. By the second day, the number of cells/DC had decreased by about 40% in CY-treated hosts and 60% in SA-treated hosts. This difference was not statistically significant, however. After the second day the number of cells/DC increased strikingly in all groups, and a dose-related increase in cell growth was observed in CY-pretreated hosts. Thus, by the fifth day a highly significant difference was seen between chambers from each neutropenic group and controls, as well as between the two neutropenic groups (p < 0.001). Between the fifth and seventh days the rate of cell growth in DC’s declined.

Most of the cells harvested from DC’s clearly belonged to the granulocytic series or were macrophages. Normoblasts were present in smears of DC cells on day two but had disappeared after 5 days of culture. Small number of typical lymphocytes were seen in all smears, and there were occasional megakaryocytes.

Changes in early proliferative cells, myelocytes, and nonproliferative granulocytes are shown in Fig. 2. The values on day zero were derived from differential counts of the marrow of 20 normal CF1 mice. The numbers of myeloblasts plus promyelocytes in the original bone marrow suspension were used as the value for EPCs at zero time (Fig. 2A). When plotted in this fashion, the number of EPCs increased exponentially from day 0 to day 5 in all groups. There was no significant difference between groups on day 2, but by the fifth day, chambers from rats pretreated with 100 mg/kg CY contained significantly
more EPCs than did those from rats given 50 mg/kg CY or SA ($p < 0.01$). The differences between rats given 50 mg/kg and those given saline were not statistically significant. Between the fifth and seventh days EPCs decreased in all groups. In contrast to the rapid initial growth of EPCs, myelocytes (Fig. 2B) increased only slightly, and nonproliferative granulocytes (Fig. 2C) decreased in number during the first two days of culture. Between the second and the fifth days, however, a dose-related increase in both myelocytes and nonproliferative granulocytes was seen. Between the fifth and seventh days the numbers of myelocytes did not change, but the more mature granulocytes continued to accumulate.

As there were no macrophages in normal bone marrow, a zero point is not shown in Fig. 3. By the second day of culture there were 16,000/DC from hosts pretreated with 100 mg/kg CY, 7700/DC from hosts given 50 mg/kg CY, and 6600/DC from control hosts. The difference in macrophages in DC's from rats given 100 mg/kg CY and those from each of the other two groups was significant ($p < 0.01$). After the second day, macrophages accumulated at the same rate in all three groups. Although the number of macrophages/DC was slightly higher in rats pretreated with 50 mg/kg CY than in controls at each point shown, the two groups, over-all, were not significantly different from each other.

**Growth of Mouse Marrow Cells Implanted Into Mice**

The effect of CY, 350 mg/kg, on the blood neutrophil counts of mice is shown in Table 2. There was profound neutropenia 2 days after CY. Recovery was nearly complete at 8 days. The pattern of changes in total cellularity and numbers of nonproliferating granulocytes seen when mouse marrow was

![Fig. 1. Total numbers of nucleated cells per DC after culture of normal mouse marrow in rats. Pretreatment of host is indicated. Values are mean (± SE) of 14–20 DC's for the saline and 50 mg/kg groups, and 5–6 DC's for the 100 mg/kg group.](image-url)
implanted into control or neutropenic mice were nearly identical to that seen when rats were used as hosts (Fig. 4). By day 5, the total numbers of cells and of nonproliferative granulocytes were again significantly greater in DC’s from neutropenic hosts.

Macrophages (Fig. 5) also proliferated rapidly. Analysis of the combined results from days 1 to 7 showed that macrophage numbers were significantly greater in DC’s from CY-pretreated hosts ($p < 0.05$, f test).

The numbers of splenic colony-forming units per diffusion chamber were measured in three experiments. The combined results are shown in Fig. 6. An average of 21 CFU were implanted in each chamber. The number of CFU/DC did not change significantly during the first two days of culture, but by the fifth day CFU/DC had increased to 37 in control mice and to 59 in neutropenic mice ($p < 0.01$). The histologic classification of splenic colonies from one experiment is shown in Table 3. It was clear that cells in the diffusion chambers retained their pluripotent characteristics and produced erythroid and megakaryocytic colonies, as well as granulocytic colonies. However, it is of interest that a significantly greater proportion of colonies derived from chamber cells than from normal marrow cells was not sufficiently differentiated to be classified.

DISCUSSION

When bone marrow from normal mice was implanted intraperitoneally into normal mice or rats, growth of myeloid elements, pluripotent stem cells, and macrophages, but not of erythroid and megakaryocytic cells, was observed. These differential growth patterns presumably reflect environmental conditions that favored myeloid differentiation. The growth of macrophages under these conditions...
conditions suggests that the microenvironmental characteristics required for growth of these cells is similar to that for myeloid elements. Alternatively, it might be interpreted as suggesting a common cell of origin for both granulocytic cells and macrophages. Macrophage growth has also been characteristic of in vitro systems in which myeloid elements may be cultured in semisolid media. In these in vitro systems, granulocytic elements are seen to develop prior to the evolution of macrophages. Metcalf, from observations on single cell transfers from colonies developing in soft agar, has suggested a common precursor for macrophage and granulocytic elements. Our studies, however, do not provide an answer to the question of whether granulocytes and macrophages derive from a single precursor cell or whether the environmental conditions favor the growth of both but from different precursor elements.

Table 2. Effect of Cyclophosphamide on Blood Neutrophils of Host Mice

<table>
<thead>
<tr>
<th>Day*</th>
<th>Cyclophosphamide (300 mg/kg)</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of Mice</td>
<td>Neutrophils/ cu mm</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>72 ± 31†</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>25 ± 10</td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td>170 ± 48</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>588 ± 143</td>
</tr>
</tbody>
</table>

* After implantation of DCs. CY or SA was injected 1 day before implantation.
† SE of the mean.

![Fig. 3. Total numbers of macrophages per DC after culture of mouse marrow in rats. Numbers of DC's as in Fig. 1.](image-url)
During the initial phase of culture there was a decrease in the numbers of cells within the chambers. This was due to a loss of erythroid elements and presumably some of the preexisting granulocytes and lymphocytes. Thereafter, there was an increase in total cellularity predominately due to an increase in myeloid cells and to a lesser extent an increase in macrophages. The pattern of increase suggests differentiation from more immature precursor cells, such as the committed myeloid stem cells (CFC). The contribution of multiplication within the differentiated, but still proliferating, compartments cannot be assessed from the present data. Further studies with isotopic labeling and the evaluation of CFC using the in vitro culture technique should provide additional insight into this question.

The initial growth rate of myeloid cells in the diffusion chambers was enhanced when the chambers were implanted to neutropenic animals. In our studies, neutropenia was produced by a single injection of cyclophosphamide 24 hr before transplantation. In the experiments in which chambers were implanted in rats, the neutrophil counts had returned to control levels by the sixth postcyclophosphamide day (fifth postimplantation day); in mice the neutrophils were within control range by the eighth postcyclophosphamide day (seventh postimplantation day). The period of differential growth observed in these animals was during the phase of neutropenia; as the peripheral white cell count of host animals returned to normal levels, differential growth between cyclophosphamide-treated and saline-treated animals was no longer apparent. Thus, it would seem that the enhancement of growth is related to a circulating humoral factor present in the blood stream of neutropenic animals. It is of interest that Boyum et al.21 and Rothstein et al.22 have observed a similar enhancement of myeloid growth in animals neutropenic in consequence of exposure to whole-body irradiation. Rothstein et al.22 observed the implants

Fig. 4. Total numbers of nucleated cells and of nonproliferative granulocytes after culture of normal mouse marrow in mice pretreated as indicated. Values are for pooled cell suspensions (8-24 DC's) from four replicate experiments on days 2 and 5, two experiments on day 1, and a single experiment on day 7.
over a 24-hr period. More recently, Boyum et al.\textsuperscript{51} reported differential growth of implanted marrow in irradiated animals with a more sustained neutropenia. These data, together with ours, indicate a sustained stimulus to growth of myeloid elements in neutropenic animals that is thought to reflect the presence of a circulating humoral factor perhaps related to CSF.

The growth characteristics of the pluripotent stem cell in diffusion chambers implanted into neutropenic hosts have not been previously reported. It is clear that in both the saline-treated controls and the neutropenic hosts the pluripotent stem cells continued to maintain their pluripotent characteristics and increased in numbers during the period of study. Moreover, the growth rate of pluripotent stem cells in the neutropenic animals was significantly greater than that in the controls. Investigation of the mechanisms controlling turnover in the pluripotent compartment has engaged the interest of a number of investigators in the recent past. It has been suggested that this regulation of the stem cell compartment is short ranged and of a stimulatory nature. Rencricca et al.\textsuperscript{16} observed in animals with phenylhydrazine-induced anemia that as the numbers of CFU decreased in the bone marrow the remaining CFU entered cycle. In the spleen there was an increase in CFU due to migration from the marrow, and the CFU did not enter cycle. This suggested to them that a short-range interaction based on cell concentration was operating and that the cellular interaction was of an inhibitory nature, perhaps by a mechanism analogous to contact inhibition. Gregory et al.\textsuperscript{25} observed that pretreatment of irradiated donors with cyclophosphamide accelerated recovery of CFU and

![Diagram](image-url)

Fig. 5. Total number of macrophages after culture of mouse marrow in mice. Number of replications as in Fig. 4.
suggested that perhaps this resulted from release of a humoral factor from
cells damaged or killed by cyclophosphamide. They further suggested that
this factor might be an α2 macroglobulin, which has been shown to affect the
recovery of animals from irradiation injury.26 Although the present study does
not exclude the existence of a short-range type of regulation of the CFU com-
partment size, it appears to favor a long-range control involving a diffusible
humoral factor, perhaps, as has been suggested, α2 macroglobulin. Examination
of the effect of varying the concentrations of cells and of CFU in the diffusion
chamber and the effect of the introduction of thymocytes into the chamber

Table 3. Histologic Classification of Spleen Colonies Derived From Bone Marrow Cells
After Culture in DC’s

<table>
<thead>
<tr>
<th>Source of Cells</th>
<th>Number of Spleens Examined</th>
<th>Total Colonies</th>
<th>Erythroid</th>
<th>Granulocytic</th>
<th>Megakaryocytic</th>
<th>Mixed</th>
<th>Undifferentiated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal marrow</td>
<td>6</td>
<td>27</td>
<td>15</td>
<td>4</td>
<td>0</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>CY day 1*</td>
<td>6</td>
<td>40</td>
<td>16</td>
<td>3</td>
<td>1</td>
<td>7</td>
<td>13†</td>
</tr>
<tr>
<td>SA day 1</td>
<td>6</td>
<td>59</td>
<td>32</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>19†</td>
</tr>
<tr>
<td>CY day 2</td>
<td>6</td>
<td>61</td>
<td>13</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>22‡</td>
</tr>
<tr>
<td>SA day 2</td>
<td>6</td>
<td>80</td>
<td>28</td>
<td>7</td>
<td>5</td>
<td>16</td>
<td>28†</td>
</tr>
<tr>
<td>CY day 5</td>
<td>6</td>
<td>20</td>
<td>8</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>SA day 5</td>
<td>7</td>
<td>28</td>
<td>7</td>
<td>5</td>
<td>0</td>
<td>3</td>
<td>13‡</td>
</tr>
</tbody>
</table>

* Cells injected into irradiated mice were cultured for 1 day in CY-pretreated hosts.
† Proportion of undifferentiated colonies significantly different from normal bone marrow group (p <0.05).
‡ Proportion of undifferentiated colonies significantly different from normal bone marrow group (p <0.01).
on the growth of CFU should provide further insight into the factors responsible for control of the CFU compartment and the importance of short- as compared with long-range control.

REFERENCES

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