Marrow Culture in Diffusion Chambers in Rabbits.
I. Effect of Mature Granulocytes on Cell Production

By Roel Willemze, Richard I. Walker, John C. Herion, and Jeffress G. Palmer

Factors influencing granulopoiesis have been evaluated using diffusion chambers implanted in the peritoneal cavity of rabbits. An increase in granulopoiesis in chambers implanted in hosts made neutropenic by nitrogen mustard occurs in mice made neutropenic by x-ray or drug. The intraperitoneal injection of leukocytes inhibited the growth of cells in chambers implanted in rabbits. Removal of mature granulocytes from marrow prior to chamber inoculation produced a marked increase in cell growth, especially of granulocytes. Mature granulocytes clearly inhibited cell replication and this inhibition involved both myeloid and erythroid elements, although the data suggest a greater effect on myelopoiesis. In contrast to the mouse, erythropoiesis in chambers in rabbits remained prominent for over 1 wk.

In recent years factors influencing granulopoiesis have been studied in vitro in systems that support the growth and differentiation of granulocyte colonies and in diffusion chambers (DC) that isolate implanted cells from host cells while allowing exchange of nutrients, wastes, and presumed humoral stimulators or inhibitors. Analysis of granulocyte kinetics suggests that granulopoiesis may be subject to feedback inhibition by factors derived from late differentiated granulocytes. Mature granulocytes have been shown to inhibit lymphocyte proliferation in vitro, and more recently there has been reported a soluble small molecular weight polypeptide substance derived from mature granulocytes that inhibits granulocyte proliferation while having no effect on other replicating cells. It is considered to be a "chalone" analogous to the inhibitor described for skin cells. Intraperitoneal injection of a granulocyte chalone or granulocyte conditioned medium into mice bearing implanted DC has been reported to cause inhibition of granulocyte production in those chambers. The physiologic role of this inhibitory activity associated with or derived from granulocytes has not been established.

A granulopoietic stimulator analogous to erythropoietin has yet to be demonstrated. A factor or factors derived from mononuclear cells, macrophages, and other sources have been shown to be required for growth of granulocytes in vitro. Although the physiologic role of this factor (called colony-stimulating activity) is uncertain, the concentration of this activity in urine and serum fluctuates inversely with the blood neutrophil count.

The increased production of cells in diffusion chambers implanted into ani-
mals rendered neutropenic by drug27,28 or x-ray28,29 has been interpreted as evidence for an increase in the amount of some stimulator of granulopoiesis. Since these animals are also neutropenic, the increased cell production in the chambers could arise due to a reduction in inhibitor carried by mature neutrophils to the locus of the chambers. The studies reported here tested the effect of granulocytes on cell production in DC in neutropenic animals.

The rabbit was chosen as the experimental animal because its size makes it possible not only to implant multiple chambers in a single host, but also to use autologous marrow with technical ease.

**MATERIALS AND METHODS**

Male white New Zealand rabbits weighing 2.4 - 3.0 kg were used. Marrow cells were aspirated from the ilium, suspended in culture medium, and inoculated into DC. Chambers were then placed in the peritoneal cavity of the same rabbit for varying periods of time.

Culture medium was made of 85% McCoy's 5A medium and 15% fetal calf serum (Grand Island Biological). Penicillin and streptomycin were added to a final concentration of 100 U/ml and 100 μg/ml, respectively.

Total cell counts were made in duplicate with a Coulter model B Cell Counter. Smears of bone marrow and blood were made with a Shandon-Elliot cytocentrifuge using bovine albumin to preserve cell morphology during smear preparation. Smears were stained with May-Grünwald-Giemsa stain for differential counts. Cells in the myeloid series were categorized as proliferating (blasts, promyelocytes, and myelocytes) or postmitotic (metamyelocytes and segmented forms). Normoblasts were classed as early (pronormoblasts and basophilic normoblasts) or late (polychromatophilic and orthochromatic normoblasts). Large mononuclear cells were classified as macrophages/mononuclear cells.

Marrow cells were obtained from the ilium of lightly anesthetized rabbits by aspiration into a syringe containing 0.5 ml 2% EDTA in saline. The aspirate was forced gently through a 22-gauge needle into a tube containing sterile culture medium. Differential counts of 25 freshly aspirated normal rabbit marrows revealed the following distribution (mean ± SD): proliferating granulocytes, 9% ± 0.3%; postmitotic granulocytes, 34% ± 1.3%; lymphocytes, 11% ± 0.9%; monocytes, 1% - 2%; early normoblasts, 12% ± 0.9%; and late normoblasts, 34% ± 1.3%. In some studies, marrow cell suspensions were made poor in mature granulocytes by passing them through a column of sterile absorbent cotton.12 On elution from the cotton column the differential counts of 25 cell suspensions revealed the following distribution (mean ± SD): proliferating granulocytes, 13% ± 1.6%; postmitotic granulocytes, 16% ± 0.7%; lymphocytes, 13% ± 1.6%; monocytes, 1% - 2%; early normoblasts, 16% ± 1%; and late normoblasts, 42% ± 2.7%. Cell concentration was adjusted by dilution as required to deliver in 0.1 ml the desired number of cells to each DC.

DC were made by gluing 0.45-μm pore size, 13-mm Millipore filters to plastic rings with MF-1 glue (No. 70.000.00). At least 3 days elapsed after construction before chambers were used. After sterilization in ethylene oxide, the DC were allowed to ventilate for at least 12 hr. Chambers were loaded using a Hamilton repeating syringe that delivered 0.1 ml. The loading hole was sealed by a plastic peg rendered semifluid by heating in a flame. Filled chambers were kept in ice-cold medium while awaiting implantation. Under pentobarbital anesthesia, usually 16-30 chambers were placed in the peritoneal cavity of each of these animals. At intervals after implantation, chambers were harvested from animals killed with intravenous pentobarbital. Cells were recovered from the chambers by the technique described by Benestad5 utilizing incubation in a solution of Ficoll and Pronase to dissolve any clot within the DC.

Neutropenia was produced in rabbits by the intravenous injection of 2.5 mg of nitrogen mustard (mechlorethamine HCl, HN2) per kilogram of body weight. This dose of HN2 predictably produces virtual agranulocytosis 96 hr after injection.30 In experiments on animals given HN2, marrow was obtained immediately prior to the administration of the drug and DC were implanted 1-1½ hr after the drug was given. Large quantities of granulocytes were obtained from peritoneal exudates induced by intraperitoneal injection of 200 ml 0.5% glycogen in sterile saline.31 The exudates were harvested about 16 hr after the glycogen injection by aspiration and
Table 1. Total and Differential Counts of Cells Harvested on Day 9

<table>
<thead>
<tr>
<th>Culture Condition</th>
<th>No. of Animals</th>
<th>Total Cells</th>
<th>Proliferating Granulocytes</th>
<th>Postmitotic Granulocytes</th>
<th>Macrophages/</th>
<th>Mononuclear Early Late</th>
<th>Normoblasts</th>
<th>Early Normoblasts</th>
<th>Late Normoblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-BM +</td>
<td>4</td>
<td>2267 ± 174</td>
<td>113 ± 10.2</td>
<td>403 ± 37</td>
<td>820 ± 85</td>
<td>519 ± 36</td>
<td>771 ± 97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE-cells</td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.005</td>
<td></td>
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</tr>
<tr>
<td>N-BM</td>
<td>5</td>
<td>3343 ± 141</td>
<td>162 ± 16.2</td>
<td>609 ± 48.3</td>
<td>581 ± 30.9</td>
<td>285 ± 19</td>
<td>1579 ± 112</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-BM in HN2-host</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.005</td>
<td>NS</td>
<td>NS</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-BM in HN2-host+</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.005</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>p &lt; 0.025</td>
<td>p &lt; 0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP-BM</td>
<td>4</td>
<td>4658 ± 128</td>
<td>356 ± 1.9</td>
<td>1250 ± 80</td>
<td>720 ± 18.4</td>
<td>487 ± 18.8</td>
<td>1732 ± 14.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP-BM in HN2-host</td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.005</td>
<td>NS</td>
<td>NS</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.005</td>
<td></td>
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</tr>
<tr>
<td>GP-BM in HN2-host+</td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.005</td>
<td>NS</td>
<td>NS</td>
<td>p &lt; 0.025</td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.005</td>
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</table>

Each value is the mean ± SE of mean × 10^-2 from number of animals indicated. The value from each animal is the mean from 8-16 chambers.

*N-BM, normal bone marrow in chambers in normal hosts; HN2-host, host received nitrogen mustard; PE-cells, host received peritoneal exudate cells; GP-BM, granulocyte-poor bone marrow in chambers.

Lavage with sterile saline. Exudate cells, consisting of about 90% granulocytes and 10% mononuclear cells, were washed once in sterile isotonic saline and resuspended in 25 ml McCoy's 5A medium.

Between 1.25 and 2 × 10^8 such cells were obtained for each injection in experiments in which they were injected intraperitoneally into DC hosts. Such cell injections were limited to the period of severe leukopenia (usually between days 2 and 6 post-HN2 injection^30). In control animals peritoneal exudate cells were given on the same days. They produced no detectable effect on the recipients' blood neutrophil counts.

The growth curves of freshly aspirated unmodified and granulocyte-depleted marrow in DC were established in normal hosts and in hosts rendered neutropenic by treatment with HN2. The effect of intraperitoneal injections of isologous peritoneal exudate granulocytes on cell growth in DC implanted in normal and neutropenic animals was then studied.

Studies were done in 4-9 rabbits at each time point and in each rabbit a minimum of 8 and a maximum of 16 DC containing a given sample of marrow were used. Thus, each point for each rabbit represented the mean value of from 8-16 chambers and the value for a given day represented the mean of the means for all the rabbits from which chambers were harvested on that day. After individual total counts were done, the contents of the chambers containing the same beginning marrow cell preparation were pooled to prepare the slides for differential counting.

Significance of difference between mean cell counts was determined by application of the non-paired, two-tailed Student's t test; p values were calculated on the raw data shown in Table 1, but were not determined on derived data expressed as ratios of cells harvested/cells inoculated, as plotted in the graphs.

To test the accuracy of cell delivery into the chambers, experiments with four different cell concentrations were done. After measuring the cell concentration in a suspension, replicates were delivered into each of five small centrifuge tubes and the number of cells in each tube was promptly enumerated. When 0.1 ml of a suspension containing 3300 cells/μl was inoculated, 3455 ± 370 (SD) cells/μl were recovered; when 2460 cells/μl were inoculated, 2523 ± 115 cells/μl were recovered; when 3950 cells/μl were inoculated, 3784 ± 110 cells/μl were recovered; and when 2900 cells/μl were inoculated, 2830 ± 198 cells/μl were recovered.

Efficiency of harvest was tested by loading DC, sealing them, allowing them to stand for 1 hr in a beaker of culture medium, and then harvesting them. Using this routine, 80%-90% of the
Fig. 1. Effect of number of normal marrow cells implanted on cell growth in chambers in control animals.

Fig. 2. Distribution of cell types in chambers inoculated with $3 \times 10^5$ normal marrow cells and implanted in control animals.
cells computed to be within the chamber could be recovered. Incubation of chambers in the Ficoll-Pronase solution did not alter this yield.

RESULTS

Cell Proliferation in DC in Normal Rabbits

Figure 1 shows that optimum growth occurred in chambers containing $3 \times 10^5$ cells. In each instance cell numbers fell for 4 or 5 days and rose to a maximum on the ninth or tenth day after implantation. After this time cell numbers declined gradually for at least the next 8 or 9 days. Not shown is the observation that cell growth with an inoculum of $2 \times 10^5$ cells did not differ significantly from that found with $3 \times 10^5$ cells, but the use of smaller numbers of cells created problems with quantitation of cell counts. Thus $3 \times 10^5$ was selected as the number of cells for routine use.

Figure 2 shows the distribution of cell types harvested when $3 \times 10^5$ cells were implanted in each chamber. Proliferating and postmitotic granulocytes plateaued from day 7 at about one-half the number inoculated, while macrophages and unidentified mononuclear cells, initially scarce, increased to about 40% of the cells harvested on day 13. Cells identified as lymphocytes diminished gradually during the first week of culture. The number of early normoblasts remained essentially constant throughout the period of culture, while late red cell precursors increased by day 9 to about 1.5 times the number implanted.

Effect of HN2 Treatment of the Hosts on the Number and Composition of Cells Harvested From Implanted DC

Figure 3 shows the effect of 2.5 mg of HN2/kg on the circulating granulocytes of rabbits. Blood granulocytes were maintained for about 2 days and then fell rapidly to reach a minimum on day 4. Circulating granulocytes rose rapidly thereafter to slightly above normal.

![Fig. 3. Effect of HN2 injected intravenously in rabbits on circulating leukocytes and granulocytes. Each point represents the mean of the leukocyte or granulocyte counts of at least 20 rabbits.](image-url)
Chambers harvested from animals treated with HN₂ contained more cells after the fifth day than those harvested from control animals. By day 9 chambers from HN₂-treated hosts contained 1.5 times the number inoculated, while chambers from control animals contained the same number as in the inoculum (Table 1). The means of absolute values (Table 1) obtained on day 9 in five control and four HN₂-treated animals were significantly different ($p < 0.01$). The distribution of cells found in the chambers in these animals is presented in Fig. 4. Proliferating and postmitotic granulocytes in diffusion chambers from HN₂-treated animals were significantly higher in comparison with those of controls after day 5, while there was no difference in the number of macrophages in the two kinds of hosts. Early normoblasts were significantly higher at day 9 in the HN₂-treated hosts and late normoblasts were higher in this group at day 11.

**Effect of Granulocyte Removal on Subsequent Growth in DC**

Marrow from which postmitotic granulocytes had been removed by passage through a cotton column had a greater proportion of cells capable of replication than did the unmodified marrow. To adjust for this increase in potentially
proliferative cells, 20% fewer cells of granulocyte-poor marrow \((2.4 \times 10^3)\) were inoculated per chamber than the \(3 \times 10^3\) unmodified marrow cells used for the control. Preliminary studies showed no difference between cell yield when \(2 \times 10^3\) and \(3 \times 10^3\) unmodified marrow cells were inoculated in the DC.

The removal of mature granulocytes from marrow greatly enhanced subsequent growth over that of the unmodified marrow, both kinds of marrow samples being obtained from the same animal prior to \(HN_2\) treatment and then implanted in that animal after \(HN_2\) administration (Table 1). The distribution of cells in these chambers is shown in Figs. 5 and 6. On day 9, marrow from which mature granulocytes had been removed prior to implantation had an increased number of proliferating granulocytes, postmitotic granulocytes, and late normoblasts. There was no significant difference between the number of macrophages or early normoblasts. An increase in proliferating granulocytes was apparent in the first few days of culture, while postmitotic granulocytes increased dramatically from day 7 (Fig. 6).

**Effect of Addition of Peritoneal Exudate Cells**

Four experiments were performed in which \(HN_2\)-pretreated rabbits bearing 16 chambers with normal and 16 chambers with granulocyte-poor marrow re-
ceived daily intraperitoneal injections of 1–2 × 10⁹ heterologous peritoneal exudate cells on days 2–6, the period when they were leukopenic. Normal control rabbits bearing chambers containing normal marrow were also given granulocytes on the same days after chamber implantation. Blood granulocytes were determined daily and were not altered by the peritoneal exudate granulo-

Fig. 7. Effect of intraperitoneal injections of peritoneal exudate (PE) cells on the number and distribution of cells in chambers implanted in HN₂-treated hosts; 3 × 10⁵ normal marrow cell and 2.4 × 10⁵ granulocyte-poor marrow cells were inoculated per chamber. Normal controls are included for comparison. Macrophage values: macrophages harvested, divided by total cells inoculated. Normoblast and granulocyte values: number of the specific cell type harvested, divided by the number of the specific cell type inoculated. All results obtained on day 9 after DC implantation.
cyte injections. DC were harvested on day 9. Results of these studies are shown in Table 1 and Fig. 7. In these leukopenic hosts and the normal control hosts, peritoneal exudate cells significantly reduced the total cell number, the proliferating granulocytes, and both early and late normoblasts in unmodified normal marrow. The numbers of macrophages and postmitotic granulocytes were not significantly different from the numbers of these cells in HN2 leukopenic hosts not injected with peritoneal exudate cells. There was a slight increase of macrophages in chambers in the control hosts given peritoneal exudate cells.

Using marrow modified by removal of mature granulocytes, it was observed that injection of peritoneal exudate cells significantly reduced all cell types except macrophages. Intraperitoneal injection of culture medium containing no cells had no effect on total and differential counts of chambers harvested from HN2-treated recipients at day 9.

DISCUSSION

Previous studies of hematopoiesis in DC have been performed largely in mice and rats. The experience with rabbits reported here indicates that this animal is suitable for such studies and offers the advantage of larger size, the ability to use more chambers per animal, and the ability to utilize marrow from the same animals in which the DC are placed. In DC containing either autologous or isologous rabbit marrow, erythropoietic activity is much greater than in the studies with mouse marrow, and thus the technique is suitable for studies of factors affecting erythropoiesis. Erythropoiesis derived from circulating cells has been reported in chambers implanted in severely bled rabbits. Cells in DC may die and disintegrate or remain intact, or live unchanged. Cells may also divide and/or undergo morphologic change, presumably to a more differentiated form of the same cell line. A net decrease in total cells, granulocytes, and late normoblasts occurred during the first week after DC implantation, a phenomenon that has been noted by others. While this effect may in part be due to disintegration from cell damage caused by manipulation, the apparent selective loss of granulocytes and late normoblasts suggests the possibility of maturation with death of polymorphonuclear neutrophils and loss of red cell nuclei. Reticulocytes and erythrocytes have not been enumerated in these studies.

After 6 days an increase in the number of granulocytes, macrophages, and late normoblasts regularly occurred. Although the total cell number rose slightly and later declined, the proportion of different cells changed. Both cell division and maturation appeared to contribute to the changes observed.

As reported by others, neutropenia in the host animal led to increased numbers of granulocytes in DC. The increased growth of the granulocytes in DC implanted into the peritoneal cavity of neutropenic animals has been ascribed to an increase in a humoral stimulator of granulopoiesis in these animals.

Mature granulocytes have long been known to inhibit cell replication, and during the past 10 yr evidence has accumulated indicating inhibition of granulopoiesis by substances derived from or associated with granulocytes. Whether these substances represent specific inhibitors of granulopoiesis and what their
physiologic role might be remain to be determined. Material derived from medium in which peritoneal exudate granulocytes and monocytes had been incubated, when injected intraperitoneally into neutropenic mice bearing DC containing normal marrow, reduces granulocyte growth within the chambers.\textsuperscript{19,20} Boyum et al.\textsuperscript{33} recently reported a significant depression of granulopoiesis and stimulation of macrophage formation in 7-day cultures when mature granulocytes from human blood or syngeneic mouse peritoneal cells were added to the chambers containing mouse bone marrow cells.

In these studies, designed to determine if granulocytes given intraperitoneally to neutropenic animals would decrease cell production in DC implanted in those animals, the intraperitoneal injection of $1-2 \times 10^9$ granulocytes during the time of neutropenia (usually days 2–6) substantially inhibited cell growth as measured on the ninth day after chamber implantation. Blood neutrophil counts were not detectably altered by these injections. This finding does not exclude the presence in neutropenic animals of increased amounts of a circulating humoral stimulator of granulopoiesis, but it raises the possibility that at least some of the increased growth of marrow cells implanted in DC could occur due to the inability of the neutropenic animal to deposit granulocytes with their inhibitory factor(s) in the locus of the chambers. Red cell production was also inhibited by intraperitoneal injection of exudate granulocytes.

A marked increase in cell production occurred in marrows modified by the removal of mature granulocytes prior to the inoculation of the DC. Increased cell production in these experiments involved not only granulocytes, but also erythroid cells. The removal of mature granulocytes from inoculated marrow had its most striking effect in increasing the number of postmitotic granulocytes produced. Stimulation of macrophage production was not observed in any of the studies. Decreased growth in DC caused by intraperitoneally injected granulocytes and the striking increase in growth resulting when mature granulocytes were removed from the inoculum suggest that mature granulocytes contain inhibitors of cell replication. This inhibitory effect involved both the myeloid and erythroid cells. The existence of a specific inhibitor of granulopoiesis associated with mature granulocytes could not be established from this study, although the striking increase in granulopoiesis produced by removal of mature granulocytes suggests that the inhibitory effect of the granulocyte involves granulopoiesis more than erythropoiesis.

ACKNOWLEDGMENT

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RABBIT MARROW CULTURE IN DC


Marrow culture in diffusion chambers in rabbits. I. Effect of mature granulocytes on cell production

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