Erythroid Cell Growth From Normal and W/W\textsuperscript{+} Murine Bone Marrow on Macrophage-coated Membranes

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Cellulose acetate membranes (CAM) placed in the peritoneal cavity of mice develop a macrophage layer capable of supporting in vivo hematopoietic colonies from intraperitoneally injected bone marrow cells. Modifications allowing for routine morphologic identification of colonies showed that both erythrocytic (E) and granulocytic (G) colonies occur with a consistent E:G ratio of 0.19 ± 0.037. Stimulating recipients by bleeding or phenylhydrazine injection did not produce a significant change in the total number of colonies, but led to an increase in erythroid colonies and a reduction in granulocytic colonies so that the E:G ratio significantly increased. Hypertransfusion of donor animals had no effect on the number of erythroid colonies that grew on CAM of anemic recipients. The total colony-forming ability of bone marrow cells from genetically anemic W/W\textsuperscript{+} mice was found not to differ from that of normal +/+ littermates; however, the E:G ratio of W/W\textsuperscript{+} marrow in bled recipients was significantly lower ($p < 0.01$) than that of +/+ marrow. These studies suggest that a CAM system supports an erythroid progenitor which is not affected by hypertransfusion of the donor animal, yet is dependent upon erythropoietin for colony formation, and that it is defective in the W/W\textsuperscript{+} mouse.

CELLULOSE ACETATE MEMBRANES (CAM) placed in the peritoneal cavity of mice develop a macrophage layer capable of supporting growth in vivo of hematopoietic cells from bone marrow cells injected intraperitoneally.\textsuperscript{1} These hematopoietic cells grow in a pattern of distinct large clusters and will be referred to as colonies. As described by Seki,\textsuperscript{1} there is a linear relationship between the number of cells injected and the number of colonies produced. Colonies are composed primarily of granulocytic cells and are markedly heterogeneous in size. Because most CAM colonies are granulocytic, other investigators have employed peroxidase staining for colony enumeration in the routine use of this assay.\textsuperscript{1,6} Counting CAM colonies with this specific staining technique, however, has at least two drawbacks. First, it eliminates the possible identification and quantitation of erythroid colonies; and second, it may not differentiate actual proliferating granulocytic colonies from clusters of mature neutrophils.

In the current study a modification of the Seki technique was used which allowed for routine morphologic identification of colonies. Erythroid as well as...
granulocytic colonies were found with a consistent erythrocytic to granulocytic (E:G) ratio which could be manipulated by stimulating membrane-bearing recipients. This study has investigated the nature of the CAM erythroid progenitor and its relationship to other known erythroid progenitors.

MATERIALS AND METHODS

Animals

Male and female specific pathogen-free (SPF) hybrid C57/A mice, 10-22 wk old, were used. The animals were bred in this laboratory, housed 5-7 per cage, maintained in a sterile environment with a 12-hr photoperiod, and fed Purina Lab Chow and acid water (pH 2.4) ad libitum. WBB6F1W/Wv, Wv/+, W/+ and +/+ mice were obtained from Jackson Laboratory, Bar Harbor, Maine.

Cellulose Acetate Membranes

The CAM for electrophoresis used by Seki1 were replaced in these experiments by 13-mm Millipore filters (HAWP, 0.45 μm; composed of mixed esters of cellulose acetate and nitrate). One side of the filter was covered by tape (Scotch Brand) to prevent macrophage accumulation and colony formation on that side. Before use taped membranes were immersed in Hanks' balanced salt solution (HBSS) containing penicillin and streptomycin (Lilly; 100 U and 50 μg/ml). Recipient mice were anesthetized with 0.01 cu cm/g body weight, and membranes were inserted into the peritoneal cavity. The peritoneum was sutured with 6-0 silk (Abbot), 0.01 cu cm/g body weight, and membranes were inserted into the peritoneal cavity. The peritoneum was sutured with 6-0 silk (Ethicon) and the skin was closed with 9-mm wound clips and flexible collodion (Mallinckrodt).

X-Irradiation

Seven days after membrane insertion all recipient animals (except those in groups III and IVB, Table 1) received 900 R whole-body irradiation from a 250 kV Maxitron operating at 30 mA with 0.5 mm Cu and 1.0 mm Al filtration and 60 cm skin-target distance. Groups of 5-10 mice on a rotating platform were exposed to 94-98 R/min, as determined by a Victoreen condenser R-meter prior to each irradiation.

Bone Marrow Cell Suspension

Donors were killed by cervical dislocation and bone marrow cells were obtained by thoroughly flushing out femurs with HBSS (Microbiological Associates). The cell suspension was drawn through a 22-gauge needle and then passed through a 200-mesh/inch stainless steel screen. Total cell counts were made in 1% HCl diluting fluid in a hemocytometer. After appropriate dilution, marrow cells were injected intraperitoneally in 1.0 ml HBSS. Cells were injected within 2 hr of irradiation.

Staining

On day 7 post cell injection animals were killed and the CAM was removed and fixed for 2 min in 95% ethanol (EtOH). Membranes were then stained with routine Harris hematoxylin and eosin (H&E) followed by dehydration in successive baths of 70%, 80%, 95%, and 100% EtOH. Following dehydration the CAM were immersed for 3-5 min in a 1:1 mixture of xylene and 100% EtOH. The tape was then removed and membranes were flattened between two glass slides and immersed in xylene for clearing. Membranes may be left in xylene indefinitely; however, they should remain flattened between two slides to prevent curling. After clearing, the CAM were mounted (colony side up) with Permount (Fisher Scientific) and covered with coverslips.

In some experiments membranes were stained with benzidine prior to H & E to confirm erythroblast identification. In this staining procedure, described previously by McLeod et al.,7 the CAM were immersed for 5 min in a 1% solution of 3,3'-dimethoxybenzidine (Eastman Kodak Co., Rochester, N.Y.) and absolute methanol, followed by 2 min in 2.5% H₂O₂ (5 ml 30% H₂O₂ in 55 ml 70% EtOH).
Statistics

The mean ± SE of absolute colony count was obtained for each experimental group and compared for significant differences by Student’s t test. When experiments were pooled, values from each animal were entered. The mean E:G ratios were likewise obtained from individual ratios of all animals. A least squares fit was computed for E:G ratio versus hematocrit and for the number of erythroid versus granulocytic colonies per CAM in normal and bled recipients. The SE of the regression coefficient was computed and tested for significance by Student’s t test. The F test was used for comparison of the regression lines.

RESULTS

Granulocytic Colonies

Microscopic analysis of H&E stained colonies demonstrated the presence of numerous ringed-form precursor cells in many developmental stages as well as mitotic figures. Only colonies with 20 or more cells were counted. Figure 1 shows an example of a typical myeloid colony. Occasional clusters of uniformly mature neutrophils were observed; while these could represent an end-stage colony, it was assumed they had migrated to a membrane as mature cells and they were excluded from colony counts. No attempt was made to differentiate neutrophil precursors from monocytic precursors in colonies.

Fig. 1. Photomicrograph of 7-day myelocytic CAM colony. Harris hematoxylin and eosin. × 400.
Erythroid Colonies

Erythroid colonies were easily identified after H&E staining (Fig. 2), and some CAM were also stained with a benzidene reaction specific for hemoglobin to confirm erythroblast identification. Erythroid colonies were defined as aggregates of 10 to several hundred cells of different developmental stages which contained mitotic figures. Erythroid colonies occurred as single units or juxtaposed with myeloid colonies. In such instances the erythroid colony and myeloid colony were each counted as an individual unit.

Normal Recipients

In each of four separate experiments 10 membrane-bearing mice (female) were exposed to 900 R and then injected intraperitoneally with $1 \times 10^6$ cells. The animals were killed 7 days later. Three of the 40 CAM contained only giant cells and they were excluded from the study. The remaining CAM had, on a per membrane basis, $5.6 \pm 1.04$ erythroid colonies and $37.9 \pm 4.66$ granulocytic colonies, a total colony yield of $43.0 \pm 5.33$ with an E:G ratio of $0.19 \pm 0.037$ (group I, Table 1) (See Materials and Methods for calculation of mean E:G ratio.)
Table 1. Effect of Bleeding and Phenylhydrazine on Erythroid Colonies

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Donor</th>
<th>Recipient (No.)</th>
<th>Total CAM Colonies/1 × 10^6 Cells</th>
<th>E:G</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal</td>
<td>Normal + 900 R (37)</td>
<td>43.0 ± 5.33</td>
<td>0.19 ± 0.037</td>
</tr>
<tr>
<td>II</td>
<td>Normal</td>
<td>Bled + 900 R (26)</td>
<td>39.0 ± 6.79</td>
<td>0.48 ± 0.058*</td>
</tr>
<tr>
<td>III</td>
<td>Normal</td>
<td>400R + phenylhydrazine (9)</td>
<td>35.2 ± 8.12</td>
<td>0.64 ± 0.178†</td>
</tr>
<tr>
<td>IV A</td>
<td>Plethoric</td>
<td>Bled + 900 R (8)</td>
<td>54.6 ± 6.24</td>
<td>0.22 ± 0.032</td>
</tr>
<tr>
<td>IV B</td>
<td>Plethoric</td>
<td>400R + phenylhydrazine (8)</td>
<td>33.6 ± 6.10</td>
<td>0.34 ± 0.032</td>
</tr>
<tr>
<td>V A</td>
<td>W/W*</td>
<td>Bled + 900 R (9)</td>
<td>60.2 ± 11.45</td>
<td>0.10 ± 0.022*</td>
</tr>
<tr>
<td>V B</td>
<td>W/W*</td>
<td>Bled + 900 R (10)</td>
<td>33.4 ± 6.10</td>
<td>0.17 ± 0.011†</td>
</tr>
<tr>
<td>C</td>
<td>W/W*</td>
<td>Normal + 900 R (10)</td>
<td>37.8 ± 3.58</td>
<td>0.01 ± 0.003†</td>
</tr>
</tbody>
</table>

*Significantly different from paired control, p < 0.001.
†p < 0.01.
‡p < 0.02.

Bled Recipients

In three separate experiments 10 membrane-bearing recipients (female) were bled approximately 0.4 ml from the orbital sinus 1 and 2 days prior to irradiation and cell injection, and a like number were not bled. At the time of cell injection the mean hematocrit of the bled group was 36.4% ± 0.45% compared to 45.6% ± 1.02% for nonbled controls. As shown in Table 1 (group II), the mean number of total colonies did not differ significantly between the bled and nonbled groups. However, when colony yield was expressed qualitatively as E:G ratios, the bled group had a mean ratio significantly greater (p < 0.01) than that of the nonbled group and that of group I.

Figure 3 illustrates the linear relationship between granulocytic and erythroid colonies in normal recipients (n = 25, correlation coefficient 0.76, p < 0.01, slope 2.43, and y intercept 20.16) and in bled recipients (n = 26, correlation coefficient 0.83, p < 0.01, slope 2.23, and y intercept 3.34). As determined by the F test for the comparison of regression lines, the residual variance and the slopes of the two lines do not differ; however, the y intercepts are significantly different at α < 0.001.

Phenylhydrazine-treated Recipients

In two separate experiments 10 membrane-bearing female recipients were given 400 R total-body irradiation plus 60 mg/kg phenylhydrazine 5 days before cell injection. A like number of recipients received 400 R and cells without phenylhydrazine, and a third group received 400 R only. At the time of cell injection the mean hematocrit of the phenylhydrazine-treated group was
21.9% ± 0.53% compared to 44.8% ± 1.01% for the 400-R controls. The mean number of total colonies in the phenylhydrazine-treated group did not differ from that of controls, but the E:G ratio of the phenylhydrazine-treated group was significantly higher ($p < 0.01$) than those of both the nontreated controls and group I (group III, Table 1). No colonies were found on membranes from mice receiving 400 R without bone marrow injection.

**E:G Ratio**

The E:G ratio was found to have an inverse linear relationship with the packed red cell volume of female recipient mice (correlation coefficient 0.91, slope $-43.85$, $y$ intercept 53.15, regression coefficient significant at the level of $p < 0.05$; Fig. 4).

**Plethoric Versus Normal Donors**

In one experiment 20 male membrane-bearing recipients were rendered anemic by bleeding. Half of the bled recipients received $2 \times 10^6$ cells from plethoric donors; the other half received $2 \times 10^6$ cells from normal donors. Donor mice were rendered plethoric by intraperitoneal injection of 1.0 ml packed red blood cells (washed three times) on each of three consecutive days. Control donors were injected at the same time with 1.0 ml HBSS. The results (group IV A, Table 1) indicated that bone marrow cells from plethoric donors had the same colony-forming ability and E:G ratio as those of the normal donors. However, the E:G ratio of the control group was not significantly greater than that obtained from group I, nor was it comparable to that obtained from bled female recipients. This difference might be due in part to the sex difference of the recipients.5

The experiment was repeated in male recipients made anemic by sublethal irradiation and phenylhydrazine treatment 5 days before injection of $1 \times 10^6$ cells (group IV B, Table 1). As in the first experiment, bone marrow from plethoric donors did not differ in total colony-forming ability or E:G ratio from marrow of normal donors. The E:G ratios, although not comparable to the
ratio obtained from normal marrow in phenylhydrazine-treated female recipients, were significantly greater \((p < 0.02)\) than the group I E:G ratio.

**W/W' Versus +/+ Donors**

In two experiments 20 membrane-bearing +/+, W/+ and W'/+ mice (male and female) were made anemic by bleeding. In the first experiment half of the recipients received 2 \times 10^6 marrow cells from W/W' donors; the other half received 2 \times 10^6 cells from +/+ littermate donors. In the second experiment the cell dose was 5 \times 10^5 cells. As summarized in Table 1 (group V A, B), in both experiments the total colony-forming ability of W/W' bone marrow was found not to differ significantly from that of +/+ bone marrow. In contrast, the E:G ratios obtained from W/W' mice were significantly lower than those obtained from littermate controls. In a third experiment 20 nonanemic membrane-bearing littermates received 5 \times 10^5 cells from either W/W' or +/+ donors (Group V C, Table 1). The total number of colonies formed by W/W' bone marrow in normal recipients was slightly but not significantly smaller than that formed by +/+ marrow. However, the E:G ratio obtained from the W/W' marrow was found to be statistically significantly smaller than that of the +/+ marrow.

**DISCUSSION**

Seki\(^1\) described a technique for morphologic identification of colonies on the macrophage layers which involved peeling the layer off the membrane, mounting it on a glass slide, and staining with H&E. He reported that using this technique approximately 95% of the colonies appeared to be granulocytic and 5%
erythrocytic. In subsequent studies, granulocytic colonies were routinely enumerated by staining for a peroxidase reaction. We found it not only very tedious and time consuming, but also difficult to separate intact macrophage layers from the membranes; consequently, we developed a routine H&E staining procedure for the entire CAM.

The staining procedure used in the present study allowed for routine microscopic evaluation of colony number and type and showed that both erythrocytic and granulocytic colonies occurred with a consistent E:G ratio of $0.19 \pm 0.037$. As shown in Fig. 3, the number of erythrocytic colonies per CAM had a linear relationship with the number of granulocytic colonies. However, linearity of erythroid colonies with the number of donor cells injected has not been determined. The predominance of granulocytic colonies might be explained in part by the fact that CAM have been shown to produce colony-stimulating activity in amounts sufficient to stimulate growth of granulocytic colonies in semisolid media in this strain of mouse.

Membrane-bearing recipients made anemic before injection of bone marrow cells had significantly higher E:G ratios than control mice. The ratios had an inverse linear relationship with the hematocrit of recipient mice (Fig. 4), suggesting that the CAM erythroid progenitor is dependent upon erythropoietin for colony formation.

The significantly greater E:G ratios of anemic recipients were not caused simply by an increment of erythroid colonies with a corresponding increase in total colony number. The mean number of total colonies did not differ between the bled and nonbled groups (group II, Table I). However, for any given number of total colonies the bled group had more erythroid colonies and fewer granulocytic colonies than the nonbled group. This conclusion was further supported by the regression analysis shown in Fig. 3. The only significant difference between the two lines is the $y$ intercept. If bleeding simply added erythroid colonies to the total colony number, the lines would have different slopes but the same $y$ intercept. These observations suggest that a certain proportion of the CAM colony progenitors might be capable of either granulocytic or erythroid expression depending upon the stimuli present.

Transfusion-induced plethora has been reported to reduce the number of cells which form small erythrocytic colonies (CFU-E) in vitro, but not the number of cells which produce the very large erythrocytic colonies (BFU-E); nor does inducing plethora in the donor influence the number of macroscopic spleen colonies which are formed in lethally irradiated mice (CFU-S). Making the donors plethoric did not influence the number of total colonies nor the number of erythroid colonies formed on CAM of normal or anemic recipients. This observation, plus the fact that some of the erythroid CAM colonies contained several hundred cells, suggests that the cell forming erythroid colonies on the CAM is more closely related to the BFU-E or the CFU-S than to the CFU-E.

Bone marrow from the W/W$^v$ mouse shows a defective proliferative responsiveness to stimuli and does not form normal numbers of macroscopic spleen colonies, nor normal numbers of BFU-E, but it does form normal numbers of CFU-F per cell dose. Therefore, it was postulated that if CAM
erythroid progenitors were more comparable to BFU-E or CFU-S than to CFU-E, bone marrow from W/W<sup>+</sup> donors would form fewer erythrocytic colonies in anemic recipients than bone marrow from +/+ littermates. The total colony yield obtained from W/W<sup>+</sup> mice agreed with previous work of Kitamura et al.<sup>6</sup> that showed that the number of peroxidase-positive colonies obtained from W/W<sup>+</sup> marrow is consistently, but not significantly, less than that obtained from +/+ marrow. However, the erythrocytic colony-forming ability of W/W<sup>+</sup> marrow was significantly less than that of +/+ littermates, suggesting that the CAM erythroid progenitor, like the BFU-E and the CFU-S, is defective in the W/W<sup>+</sup> mouse.

Thus, it would appear that the progenitor cell responsible for erythroid colony formation on the CAM differs considerably from the committed CFU-E, and seems to resemble more closely earlier precursors such as the BFU-E or CFU-S in that it is not affected by plethora and it is defective in the W/W<sup>+</sup> mouse. The possibility that some CAM colony precursors are capable of either granulopoiesis or erythropoiesis, as suggested by increased erythrocytic colonies and decreased granulocytic colonies in bled recipients compared to nonbled recipients, is also suggestive of an early precursor such as the CFU-S. However, other studies have shown that irradiation and cytosine arabinoside sensitivities of granulocytic colony-forming cells on the CAM differ appreciably from those of the CFU-S.<sup>16</sup> In those respects CAM granulocytic precursors share certain characteristics with cells forming endogenous spleen colonies and cells forming granulocytic colonies in vitro (CFU-C). These observations suggest that most CAM colonies may be derived from early committed precursors such as the BFU-E or the CFU-C.

ACKNOWLEDGMENT

The authors are indebted to Susan Brown for her excellent technical assistance.

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