Changes in Hematopoietic Stem Cells in Bone Marrow of Mice With Plasmodium Berghei Malaria

By Lillian Maggio-Price, Daniel Brookoff, and Leon Weiss

An impaired erythropoietic response to anemia has been noted in human patients with malaria and in rodents experimentally infected with Plasmodium berghei. We have attempted to characterize the erythropoietic response in mice with a fatal P berghei infection, with particular emphasis on changes in marrow hematopoietic stem cells. Mice infected with P berghei had dramatic decreases in bone marrow cellularity, erythroblasts, BFU-E, and CFU-E as early as 24 hours postinfection and before there was any change in hematocrit. With development of anemia, marrows became erythropoietic with some expansion of the CFU-E compartment, but the BFU-E pool remained depleted and reticulocyte response was inadequate. There was no significant change in CFU-S from marrows of malaria-infected mice one day after infection. The lethality of malaria infection may take three weeks to be revealed, but it may be determined within hours of the infection by the irreplaceable changes in marrow erythroid stem cells.

DECREASED ERYTHROCYTE SURVIVAL and depressed erythropoiesis characterize certain human and experimental malaria.1 Ineffective erythropoiesis2 decreased 59Fe incorporation into red cells, and decreased numbers of erythroblasts and circulating reticulocytes occur in Plasmodium vivax and Plasmodium falciparum infections.3 In this study we evaluated the hematologic response to a fatal murine malaria and noted significant changes in bone marrow cellularity and committed erythroid progenitors. These perturbations occur within 24 hours of infection and before the development of anemia. Changes in numbers or proliferation of erythroid stem cells may account for impaired erythropoietic capacity.

MATERIALS AND METHODS

Female C57Bl/6J mice between seven and 12 weeks of age were used in all malaria and bleeding experiments. Animals were housed under AAALAC-approved conditions and fed Purina Lab Chow and water ad lib.

Mice were infected with Plasmodium berghei by intraperitoneal injection of 106 parasitized donor syngeneic erythrocytes. Blood was obtained from the retro-orbital sinus for hematocrits (Hcts) and for leukocyte, reticulocyte, parasite, and differential counts. Animals were sacrificed and evaluated at one to 19 days following infection. Femora and spleen were immediately taken. Bone marrow and spleen were put in buffered formalin for light microscopy. The plasma clot culture technique was used to assay erythroid colony- and burst-forming cells (CFU-E and BFU-E) in marrows of experimental and control mice. Marrow was obtained by washing out femora with measured amount of alpha-MEM. Nucleated cell counts obtained with a Coulter counter (Coulter, Hialeah, Fla) gave a measure of marrow cellularity. The culture methods of McLeod and Axelrad were used with 10-4 mol/L betamercaptoethanol.4,5 Femoral marrows were cultured at concentrations of 5 × 104 nucleated cells per 0.1 mL clot for CFU-E and 1 × 105 nucleated cells per clot for BFU-E. The concentration of sheep erythropoietin (Step III, Lot Nos. 3029-1, 3031-1, 3034-1, 3035-1, 3070-1, Connaught Laboratories, Stillwater, Pa) routinely used was 0.2 U/mL clot for CFU-E and 2.0 U/mL clot for BFU-E. Cultures were maintained in a humidified atmosphere of 5% CO2 at 37 °C. After two days (colonies derived from CFU-E) or nine days (bursts derived from BFU-E) clots were removed, transferred to glass slides, fixed with glutaraldehyde, and stained with benzidine and Giemsa. Erythroid colonies of eight to 32 benzidine-positive cells were counted as CFU-E, and colonies of 50 to 1,000 benzidine-positive cells or three subcolonies totaling 50 or more cells were counted as BFU-E. Colonies were counted on three or four separate clots for the CFU-E assay, and bursts were counted on eight separate clots for the BFU-E assay.

The number of colony-forming units spleen (CFU-S) was assayed using the method of Till and McCulloch.6 Mice were given lethal whole body irradiation (950 rad), and spleens were excised from animals surviving at ten days posttransplantation and placed in Carnoy’s fixative for 24 hours. The number of surface macroscopic spleen colonies was determined using a dissecting microscope.

Erythropoietin levels were assayed by the polycythemic mouse bioassay.7 Serum from five to 15 experimental mice was obtained and pooled for determination of erythropoietin levels. Serum was obtained from malaria-infected mice at 1, 2, 3, 5, 10, 12, and 17 days after infection. Additional positive control mice were bled 0.5 mL daily for three days and serum assayed at each bleeding and one day after the final bleeding.

RESULTS

Hematologic parameters. There was little change in Hct or in parasite or reticulocyte count during the first five days after infection. By one week, the Hct had precipitously dropped to half normal levels, and the parasite and reticulocyte counts concurrently increased (Fig 1). The remainder of the infection was characterized by a steadily decreasing Hct and increasing parasite and reticulocyte counts. Mice died at 17 to 22 days with Hcts of 9% to 12% and both parasite and reticulocyte counts ranging from 40% to 70%. Reticulocyte counts were appreciably lower if corrected for the lowered Hcts. Mice were leukopenic with a lymphopenia for the first several days after infection (Table 1). As the infection progressed, mice exhibited a leukocytosis characterized by a marked monocytosis and neutrophilia. Myeloid-erythroid (M-E) ratios revealed a decrease in erythroblasts, with a

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STEM CELL CHANGES IN MICE WITH MALARIA

Knowledge of bone marrow and spleen. Marrows examined from mice one to three days after infection were primarily granulopoietic, with reduced or absent erythroblasts. Some marrows showed clusters of large immature cells, which appeared to be granulocyte or monocyte precursors, while other marrows had a predominance of mature granulocytes. By day 5, all marrows examined had immature erythroblasts and appeared like control marrows. Ten days after infection, marrows contained increased numbers of erythroblasts in all stages of maturation, with concomitant active granulopoiesis. There was little further change in the histologic appearance of marrow examined at 15 and 19 days. Similar to day 10, marrows were characterized by areas of intense erythropoiesis and granulopoiesis in a 1:1 ratio. There were minimal histologic changes in spleens from infected mice one to three days after infection. By five days, there were increased numbers of erythroblasts in red pulp and a slight expansion in white pulp with more germinal centers visible. The spleen was clearly different from controls at ten to 19 days. White pulp was markedly increased, consisting of large germinal centers with an expanded and intensely erythropoietic red pulp.

Bone marrow cellularity and stem cells. There were changes in bone marrow cellularity, BFU-E, and CFU-E in malaria-infected mice relative to control animals. These changes occurred as early as one day after infection and persisted throughout the course of the disease. Femoral marrow cellularity was significantly lower in malaria-infected mice. This decrease was noted early and persisted (Fig 2). There was an early, marked, and continuing depression of BFU-E from malaria marrows relative to control marrows (Fig 3). Numbers of marrow CFU-E changed over

| Table 1. Hematologic Parameters in Malaria-Infected Mice and Controls |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| No. of Mice    | WBC (1/mm³)     | Neutrophils (1/mm³) | Band (1/mm³) | Lymphocytes (1/mm³) | Monocytes (1/mm³) | Eosinophils (1/mm³) |
| Malaria        |                 |                  |                |                  |                  |                   |
| 4              | 3,220 (2,250–4,300) | 630 (450–860) |                | 2,030 (990–2,740) | 560              |                   |
| 2              | 2,660 (2,050–3,170) | 860 (440–2,170) | 10 (0–50) | 1,340 (1,230–1,400) | 410 (330–510) | 50 (0–150) |
| 3              | 6,480 (2,860–11,000) | 1,200 (520–1,330) |                | 3,230 (1,050–5,720) | 1,800 (1,060–2,640) | 50 (0–170) |
| 2              | 10,880 (5,450–16,300) | 820 (330–1,300) |                | 6,740 (3,050–10,430) | 3,320 (3,050–10,430) |                   |
| 6              | 37,660 (14,600–47,050) | 1,110 (4,090–17,250) | 330 (0–1,360) | 11,580 (6,420–22,740) | 14,560 (3,940–26,730) | 80 (0–470) |
| 10/12          | 26,790 (10,600–44,200) | 8,920 (1,910–21,220) | 1,060 (0–2,650) | 4,980 (1,910–10,030) | 12,600 (6,780–20,900) | 80 (0–420) |
| 4              | 43,830 (20,100–56,980) | 13,140 (6,320–20,200) | 620 (0–1,680) | 12,080 (3,820–21,080) | 17,990 (9,850–23,930) |                   |
| 4              | 20,680 (11,110–29,480) | 7,880 (3,220–14,740) | 200 (0–440) | 6,070 (3,780–8,640) | 6,670 (3,670–8,160) |                   |
| 4              | 15,440 (13,640–19,350) | 6,380 (4,360–9,680) | 160 (0–280) | 4,800 (3,800–6,000) | 4,040 (3,110–6,000) |                   |
| Controls       |                 |                  |                |                  |                  |                   |
| 18             | 8,000 (3,400–15,950) | 1,060 (210–1,910) |                | 6,180 (2,520–12,430) | 780 (200–1,750) | 40 (0–320) |

Values given are the means. The range is shown in parentheses.
the course of the malarial infection. CFU-E were significantly lower than controls before day 4 and increased above controls after day 4 (Fig 4). There was no significant change in numbers of spleen colonies from marrows of malaria-infected mice (one day after infection) relative to control marrows (Table 3).

It was not possible to produce a nonlethal malarial infection by decreasing the dose of parasitized red blood cells (prbc) used for infection. The malarial infection had a typical course in all mice developing a parasitemia. Several mice receiving a low-cell inocula (10² prbc per mouse) never developed a parasitemia and were normal 45 days postinfection.

Erythropoietin levels. Table 4 shows serum erythropoietin (epo) levels in malaria-infected mice and acutely bled mice. Epo levels did not increase in malaria-infected mice until there was a marked anemia (Hct, 15%) at ten days postinfection. Serum epo increased over the course of the infection and was highest at day 17 (1,600 mU/mL), the last point at which it was measured. Acutely bled mice had
erythroid progenitors (BFU-E and CFU-E) in mice with malaria. In order to clarify the erythropoietic response to infection, we have shown an early, marked, and persistent change in marrow CFU-E from malaria-infected mice (A) and matched controls (C). Before day 4, CFU-E were lower than controls (P < .001) and increased above controls (P < .01) after day 4. CFU-E for malaria mice increased at a greater rate (P < .001). Intercept for controls is 85.241 with slope 5.567; intercept for malaria mice is 58.391 with slope 11.830.

higher serum epo levels than did malaria-infected mice. Epo activity increased with developing anemia and was 16,900 mU/mL when the Hct was 9.5%.

**DISCUSSION**

The pathogenesis of the anemia of malaria is unclear. Erythrocyte survival is decreased, but the severity of anemia exceeds the degree of parasitemia. Therefore, anemia probably results from factors in addition to parasitization of bone marrow of mice with fatal P berghei infection. Despite changes in marrow BFU-E and CFU-E within hours of infection, there was no significant reduction in marrow CFU-S one day postinfection. Pluripotent stem cells, predominantly in Go, appear to be protected. Impaired in vitro and in vivo growth of progenitors may have accounted for our findings. Quantitative or qualitative changes in stem cells or in accessory cells may depress erythropoietin increased, marrow CFU-E expanded and splenic erythroblasts increased, suggesting that CFU-E also increased in the spleen. Therefore, marrow BFU-E may be decreased due to migration to spleen and differentiation to CFU-E in the marrow. Nonetheless, marrow CFU-E do not maintain an appreciable expansion, and proliferation and differentiation of erythroid progenitors in all locations fail to produce enough reticulocytes to provide a viable hematocrit. Singer showed that mice that have been splenectomized before infection with P berghei develop and succumb to anemia at the same rate as do nonsplenectomized mice. He noted a lower reticulocyte response and parasitemia, since P berghei preferentially parasitized reticulocytes. Dependent changes in marrow BFU-E and CFU-E within hours of infection, there was no significant reduction in marrow CFU-S one day postinfection. Pluripotent stem cells, predominantly in Go, appear to be protected. Impaired in vitro and in vivo growth of progenitors may have accounted for our findings. Quantitative or qualitative changes in stem cells or in accessory cells may depress erythropoietin proliferation in culture. Although malarial infec-

**Table 3. Spleen Colony Assay (CFU-S) at One Day after Malarial Infection**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>No. of BM Cells</th>
<th>Irradiation Controls</th>
<th>Control Bone Marrow</th>
<th>Malarial Bone Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Surviving/ irradiated</td>
<td>Mean Colonies (± SD)</td>
<td>Surviving/ irradiated</td>
</tr>
</tbody>
</table>

Donor marrow pooled from three mice in each group; recipient mice were sacrificed at ten days.

*All irradiated recipient mice were given chloroquine/acidified drinking water.

**Table 4. Serum Erythropoietin Levels**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Hct (%)</th>
<th>Epo (mU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malaria (day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>46.0</td>
<td>UD</td>
</tr>
<tr>
<td>2</td>
<td>44.0 ± 2.9</td>
<td>UD</td>
</tr>
<tr>
<td>3</td>
<td>42.0 ± 2.2</td>
<td>UD</td>
</tr>
<tr>
<td>5</td>
<td>41.5 ± 0.7</td>
<td>UD</td>
</tr>
<tr>
<td>10</td>
<td>15.0 ± 2.8</td>
<td>78</td>
</tr>
<tr>
<td>12</td>
<td>16.0 ± 3.6</td>
<td>1,375</td>
</tr>
<tr>
<td>17</td>
<td>12.0 ± 2.1</td>
<td>1,600</td>
</tr>
<tr>
<td>Bled (day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>44.1 ± 1.9</td>
<td>UD</td>
</tr>
<tr>
<td>2</td>
<td>25.5 ± 2.9</td>
<td>2,100</td>
</tr>
<tr>
<td>3</td>
<td>15.3 ± 1.8</td>
<td>6,500</td>
</tr>
<tr>
<td>4</td>
<td>9.5 ± 1.0</td>
<td>16,900</td>
</tr>
</tbody>
</table>

Values for hematocrit are mean ± SD for six to ten mice. The sera from six to ten mice were pooled for determination of epo levels. UD, undetectable.

BFU-E migration to spleen. The decline in marrow BFU-E that we noted was more marked, was associated with a decline in marrow cellularity, occurred before a fall in circulating hemoglobin, and persisted until death. It is likely that BFU-E did migrate from marrow to spleen, since, histologically, the spleen red pulp becomes erythropoietic at the same time that marrow erythropoiesis returns (day 5 postinfection). This migration may have been mediated through endotoxin released during infection. As serum erythropoietin increased, marrow CFU-E expanded and splenic erythroblasts increased, suggesting that CFU-E also increased in the spleen. Therefore, marrow BFU-E may be decreased due to migration to spleen and differentiation to CFU-E in the marrow. Nonetheless, marrow CFU-E do not maintain an appreciable expansion, and proliferation and differentiation of erythroid progenitors in all locations fail to produce enough reticulocytes to provide a viable hematocrit. Singer showed that mice that have been splenectomized before infection with P berghei develop and succumb to anemia at the same rate as do nonsplenectomized mice. He noted a lower reticulocyte response and parasitemia, since P berghei preferentially parasitized reticulocytes.
tion of the cultured cells might interfere with colony development, we considered such infection unlikely. Colonies from malarial animals were morphologically normal and infected erythroblasts or erythrocytes were absent. Indeed, late in infection, marrow cultures of heavily infected mice showed a relative increase in erythroid colony number. It is also unlikely that parasites directly infect stem cells in the bone marrow. Electron microscopic studies have shown parasitized reticuloocytes in vascular sinuses one day post-malarial infection, but parasites were rarely seen within erythroblasts in hematopoietic cords.

Because proliferation of several hematopoietic cell lines occurs in malaria, completion among stem cell compartments may have accounted for a reduction in the marrow erythroid pool. For example, marrow CFU-C fall in mice with heightened erythropoiesis. We observed an early wave of granulopoiesis/macrophagy in marrows of malarial mice, suggesting an earlier expansion of CFU-C.

Decreased epo levels are associated with the anemia of chronic infection and inflammation. However, we and others found increased epo levels in mice with P. berghei anemia. CFU-E pool kinetics are largely controlled by epo levels, and the increase in marrow CFU-E in malarial mice may well be due to increased epo production. A normal response to epo, however, depends on other factors, such as continuous protein intake, and it is possible that the nutrition of mice with severe malaria is compromised.

Although the iron status of these animals was not monitored, the hemolytic component of this anemia would suggest readily available iron. Corrected reticulocyte counts that range from 3.8% to 21.1% also indicate that iron was being delivered to the developing erythrocytes. With progression of infection, however, impaired release of iron from reticuloendothelial stores may have contributed to an inadequate erythropoietic response.

The changes in erythropoiesis we report may be related to the immunologic responses that occur in malaria. BFU-E reside in the null cell fraction and require T lymphocytes or their soluble products for optimal growth. Alterations in B, T, and null cell populations occur in human and rodent malaria. T cells and macrophages appear to collaborate to produce inducer substances as burst-promoting activity (BPA) that act on erythroid progenitor cells. This cellular collaboration, necessary for adequate erythropoiesis, may be impaired in fatal malaria. For example, although monocyteosis occurs in P. berghei-infected mice, macrophage functions appear abnormal; impaired processing of antigen, phagocytosis, and detoxification occurs. In addition, thymocytes (Thy 1.2+) increase markedly in the spleen in nonfatal Plasmodium yoelli infections, but their proliferation is impaired in fatal P. berghei infections. In normal mice, different fractions of thymocytes have been shown to modulate erythropoiesis through their enhancing or suppressing effect on CFU-E. Thymocytes enhancing CFU-E were restricted to the high theta density fraction while suppressing thymocytes concentrated in the low theta density fraction.

There are dramatic changes in bone marrow of mice infected with fatal P. berghei malaria. These changes occur 24 hours postinfection and include a decline in bone marrow cellularity, erythroblasts, BFU-E, and CFU-E. These perturbations occur early, before the development of anemia, and may offer clues to the impaired erythropoietic response in murine malaria. Examination of self-limiting malarial infections should help to resolve this question.

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