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Altered Erythropoiesis during the Course of Virulent Murine Malaria (40696)[1]

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Anemia is a major pathologic consequence of malaria in humans (1–3) and in rodents (4–7). There is abundant evidence which supports the thesis that the anemia associated with malarial infection is attributed to the destruction of parasitized and nonparasitized erythrocytes in concert with immune and phagocytic mechanisms (6–10). However, since the maintenance of normal circulating erythrocyte levels in mammals is governed by the precise homeostatic balance between erythropoiesis and erythrocyte destruction (11–13), it is conceivable that impaired erythropoiesis may also contribute to the anemic status of malaria-infected organisms. Supportive evidence has shown that during acute primary *P. vivax* and *P. falciparum* infections in humans, the proportions of marrow erythroid precursors (i.e., pronormoblasts through orthochromatophilic normoblasts) are depressed and impaired in their ability to incorporate radioiron *in vitro* (14, 15). In contrast, however, it has been suggested from semiquantitative data, that erythropoiesis in mice is enhanced at medullary and ectopic sites in response to the attendant anemia and hypoxemia of malarial infection (16). Nevertheless, it is clear that erythropoiesis does not keep pace with the enhanced rate of erythrocyte destruction and malarial mice quickly succumb, in part, to the deleterious consequences of severe uncompensated anemia (4–7). The present study was undertaken to elucidate the questionable relationship between the erythropoietic activity and anemia of mice during the course of virulent malarial infection.

Materials and methods. Virgin female CD-1 mice (Charles River Labs.), 14–16 weeks of age, were employed throughout. Malarial infection was transferred by a single intraperitoneal inoculum containing 15.0×10^6 erythrocytes parasitized with *Plasmodium berghei* (NK/65 strain). On Days 7, 14, and 21, control and infected mice were anesthetized with ether and sacrificed by cardiac puncture. Measurements on circulating blood, marrow, and spleen were performed as follows:

Blood measurements. Erythrocyte enumerations were obtained electronically with a Coulter Counter (Model Z_{B1}, Coulter Electronics, Inc.). The percentage hematocrits were determined by standard techniques. Hemoglobin was measured in accord with the cyanmethemoglobin procedure (17). The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) of blood samples were calculated by standard procedures (18). The percentage reticulocytes were determined from blood stained with new methylene blue (19) and % parasitemias were scored from methanol-fixed blood smears stained with Giemsa solution. Based on individual erythrocyte counts, the percentage reticulocytes and parasitemias were converted to absolute numbers/mm^3 of blood.

Medullary measurements. The numbers of nucleated cells/tibia were determined by flushing the bone with cold TC medium 199 (Difco Labs) and enumerating the monocellular suspension by hemocytometer subsequent to lysis of mature erythrocytes with 3% glacial acetic acid. The percentage of erythroid precursors/femur were scored from methanol-fixed paint-brush smears, stained with Wright's and Giemsa solutions (20). The numbers of erythroid precursors/tibia were calculated from the tibial nucleated cellularity and the proportion of femoral erythroid precursors.

Splenic measurements. The numbers of splenic nucleated cells were determined by hemocytometer, subsequent to splenic mincing in cold TC medium 199 and sequential

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passage through 19- to 23-gauge needles. The % erythroid precursors/spleen were scored from methanol-fixed smears stained with Wright's and Giemsa solutions (20). The numbers of splenic erythroid precursors were calculated from the splenic nucleated cellularity and the proportion of erythroid precursors.

All parameters were expressed as the daily group mean ± 1 standard error. Based on the nonparametric Mann–Whitney U test (21), P values < 0.05 were considered to be statistically significant.

Results. Table I demonstrates the erythroid status of CD-1 mice during the course of malarial (*P. berghei*) infection. It is clear that infected mice become progressively anemic, with circulating erythrocyte, hematocrit, and hemoglobin levels declining to approximately 20% of control values ($P < 0.05$) on Day 21. Specifically, the anemia was characterized by a 34% increase in mean corpuscular volume ($P < 0.05$), a 16% elevation in mean corpuscular hemoglobin ($P < 0.05$), and a 14% decrease in mean corpuscular hemoglobin concentration ($P < 0.05$) on Day 21. A slight 1.5-fold peak reticulocytosis was evidenced on Day 7, which declined thereafter. Tibial

TABLE I. ERYTHROID STATUS[a] OF CONTROL AND MALARIAL MICE[b]

Parameters	Days of infection		
	7	14	21
Parasitized erythrocytes ($\times 10^5/mm^3$)			
Controls	0 (20)	0 (20)	0 (12)
Experimentals	3.5 ± 0.3 (20)[c]	6.6 ± 0.6 (20)[c]	5.2 ± 0.5 (17)[c]
Erythrocytes ($\times 10^6/mm^3$)			
Controls	8.5 ± 0.2 (20)	8.3 ± 0.3 (20)	8.2 ± 0.2 (12)
Experimentals	5.6 ± 0.3 (20)[c]	2.6 ± 0.2 (20)[c]	1.4 ± 0.1 (17)[c]
Hematocrit (%)			
Controls	44.4 ± 0.9 (20)	43.9 ± 1.0 (20)	43.3 ± 0.6 (12)
Experimentals	29.8 ± 1.2 (20)[c]	16.8 ± 1.0 (20)[c]	9.9 ± 0.8 (17)[c]
Hemoglobin (g/dl)			
Controls	14.4 ± 0.4 (20)	15.2 ± 0.5 (20)	14.7 ± 0.3 (12)
Experiments	9.6 ± 0.4 (20)[c]	4.8 ± 0.4 (20)[c]	2.9 ± 0.1 (17)[c]
Mean corp. volume (μm^3)			
Controls	52.2 ± 0.6 (20)	52.9 ± 1.4 (20)	52.8 ± 2.3 (12)
Experimentals	53.2 ± 1.3 (20)	64.6 ± 4.5 (20)[c]	70.7 ± 4.3 (17)[c]
Mean corp. hemoglobin (pg)			
Controls	16.9 ± 0.3 (20)	18.3 ± 0.5 (20)	17.9 ± 0.8 (12)
Experimentals	17.1 ± 0.6 (20)	18.5 ± 1.0 (20)	20.7 ± 1.3 (17)[c]
Mean corp. hemoglobin conc. (%)			
Controls	32.4 ± 0.4 (20)	34.6 ± 0.5 (20)	33.9 ± 0.4 (12)
Experimentals	32.2 ± 0.6 (20)	28.6 ± 0.7 (20)[c]	29.3 ± 1.2 (17)[c]
Reticulocytes ($\times 10^5/mm^3$)			
Controls	1.7 ± 0.2 (19)	2.0 ± 0.2 (20)	1.6 ± 0.3 (11)
Experimentals	2.6 ± 0.3 (20)	2.2 ± 0.3 (20)	2.0 ± 0.3 (16)
Nucleated cells/tibia ($\times 10^6$)			
Controls	12.8 ± 0.9 (20)	13.0 ± 0.9 (20)	14.9 ± 1.0 (12)
Experimentals	10.9 ± 0.9 (20)[c]	10.5 ± 0.6 (20)[c]	10.7 ± 0.8 (17)[c]
Erythroid precursors/tibia ($\times 10^6$)			
Controls	2.3 ± 0.2 (19)	2.3 ± 0.2 (20)	2.4 ± 0.2 (12)
Experimentals	1.7 ± 0.3 (19)[c]	1.8 ± 0.2 (18)[c]	1.8 ± 0.2 (17)[c]
Nucleated cells/spleen ($\times 10^8$)			
Controls	2.2 ± 0.2 (20)	2.5 ± 0.2 (20)	2.6 ± 0.2 (12)
Experimentals	4.3 ± 0.8 (18)[c]	10.4 ± 1.0 (20)[c]	9.5 ± 0.9 (15)[c]
Erythroid precursors/spleen ($\times 10^7$)			
Controls	1.7 ± 0.2 (19)	2.2 ± 0.3 (19)	1.9 ± 0.4 (12)
Experimentals	8.7 ± 1.1 (17)[c]	40.7 ± 5.6 (20)[c]	37.2 ± 6.4 (15)[c]

[a] Values represent the daily group mean ± 1 standard error and reflect the combined data from three separate experiments. Values in parentheses refer to the total numbers of mice per group. Statistical significance between daily control and experimental groups was assessed by the nonoparametric Mann–Whitney U test.

[b] Mice were infected on Day 0 with an intraperitoneal inoculum containing 15.0×10^6 parasitized (*P. berghei*) erythrocytes.

[c] Significantly different from daily control values ($P < 0.05$).

nucleated cellularity was depressed to approximately 85% ($P < 0.05$) of control levels on Day 7 and further declined to 72% ($P < 0.05$) on Day 21. The numbers of tibial erythroid precursors remained at approximately 74% ($P < 0.05$) of control values throughout the course of infection. In contrast, the numbers of splenic nucleated cells were elevated 2-fold ($P < 0.05$) on Day 7 and increased approximately 4-fold ($P < 0.05$) on Days 14 and 21; at which time nearly 20-fold increases in erythroid precursor levels ($P < 0.05$) were apparent.

Discussion. In consideration of the severe anemia associated with virulent rodent malaria, much attention has been directed toward an understanding of processes associated with the destruction of parasitized and nonparasitized erythrocytes (6–10), but alternative possibilities remain ill defined. The present study was undertaken to further characterize the erythropoietic status of mice during the course of *P. berghei* infection in an attempt to determine if erythropoietic impairment contributed to their anemic status.

Coincident with the intense parasitemia which peaked on Day 14, infected mice became progressively anemic, with circulating erythrocyte, hematocrit, and hemoglobin values declining to approximately 20% of control levels on Day 21. These data agree with previous reports (4–7). Specifically, however, we noted that the anemia of malarial mice was characterized by a 34% increase in mean corpuscular volume, a 16% elevation in mean corpuscular hemoglobin, and a 14% decrease in mean corpuscular hemoglobin concentration on Day 21 of infection.

Malarial mice developed a peak 1.5-fold reticulocytosis on Day 7, which declined thereafter. Accordingly, the reticulocyte response reported herein was meager in comparison to what one might expect in other situations characterized by similar degrees of anemia (22). While these data lend support to the suggestion that impaired erythropoiesis contributes to the anemic status of malarial mice, based on the reticulocyte response alone, this conclusion must be viewed with caution since *P. berghei* parasites have a definite predilection for reticulocytes, as opposed to the more mature erythrocytic element (6, 10). Furthermore, the reticulocytosis may, in fact, be an underestimation of the true erythropoietic response by virtue of a shortened cell lifespan, as reported in malaria (6–10).

In view of the problems associated with assessing the dynamics of erythropoiesis in malaria from circulating parameters, we chose to monitor the erythroid responses at bone marrow and splenic sites. On Day 7 the numbers of tibial nucleated cells and erythroid precursors in infected mice were depressed to 85 and 74% of control values, respectively. Tibial nucleated cellularity declined further to 72% of control levels on Day 21, while the numbers of erythroid precursors remained approximately 26% depressed throughout the course of infection. These data are in agreement with previous studies which have shown that in humans, during acute primary *P. vivax* and *P. falciparum* infections, marrow erythroid precursors are likewise decreased (14, 15). However, it was not feasible in their human study to monitor the total marrow erythroid content/bone. Hence, by comparison, our data perhaps affords more conviction in view of the significant depression in absolute numbers of erythroid precursors/tibia throughout the course of murine malaria. Marrow depression/inhibition has been suggested in the past during acute malarial infections (18, 23, 24).

The depressed medullary erythroid findings we observed are not in agreement with a previous study (16) which reported that erythropoiesis in malarial mice is enhanced at medullary sites. In that study, Singer employed histologic sections of bone marrow and clearly stated the lack of strict quantitation thereof. In our investigation, however, we circumvented this problem by preparing a monocellular suspension from which the absolute numbers of erythroid precursors were determined. Hence, our manipulation may have permitted a better representation of the medullary erythroid capacity of *P. berghei*-infected mice, and negates the possibility that erythropoiesis might be expanded into normally nonerythropoietic portions of the marrow, since the lower one-third of the tibia is such an area. In any case, the depressed medullary erythroid response which we report during murine malaria, may perhaps be likened to that seen in situations involving other infections (25).

In contrast to the depressed medullary response noted in *P. berghei*-infected mice, the

numbers of splenic nucleated cells were elevated 2-fold on Day 7 and rose to approximately 4-fold on Days 14 and 21, at which time nearly 20-fold increases in erythroid precursor levels were apparent. These data are in agreement with the qualitative observations made by Singer (16). Apparently normal mice, subjected to intense erythropoietic challenge, have the capacity to undergo erythropoietic expansion at both medullary and splenic sites. In the past, we (22) have, for example, shown that subsequent to intense phenylhydrazine-induced hemolytic anemia, erythropoiesis is increased approximately 2-fold in medullary sites and 50-fold in the spleen. Conversely, in *P. berghei*-infected mice, medullary erythropoiesis was significantly depressed and splenic erythropoiesis, although expanded, did not increase to the extent expected. Furthermore, since the murine spleen during normalcy usually accounts for approximately 20% of total red cell production (26), the contrasting increased splenic and decreased medullary erythropoiesis during malaria, could perhaps account for the meager reticulocytosis, as reported herein.

Although the importance of chemical and cellular modulators of erythropoiesis is well recognized (27–29), the specific mechanisms responsible for impaired erythropoiesis in malarial mice are presently unknown, but may perhaps relate to: (a) hemopoietic stem cell (i.e., CFU-s, BFU-e, and/or CFU-e) deficits, and/or (b) committed erythroid stem cell (i.e., BFU-e and/or CFU-e) resistance to erythropoietin, and/or (c) the existence of ineffective erythropoiesis. Plans to elucidate these possibilities are in progress.

Summary. Peripheral, medullary, and splenic erythroid parameters were quantitatively assessed in mice during the course of virulent malarial (*P. berghei*) infection. Mice became progressively anemic, with circulating erythrocytes, hematocrit, and hemoglobin levels declining to approximately 20% of control by Day 21. The anemia was further characterized by a 34% increase in mean corpuscular volume, a 16% elevation in mean corpuscular hemoglobin, and a 14% decrease in mean corpuscular hemoglobin concentration by Day 21. A meager 1.5-fold reticulocytosis was noted on Day 7, which declined thereafter. Tibial nucleated cellularity was depressed to 85 and 72% of control values on Days 7 and 21, respectively, while erythroid precursor numbers remained at 74% of control levels throughout the course of infection. In contrast, splenic nucleated cells were elevated 2-fold on Day 7 and increased approximately 4-fold on Days 14 and 21. Although nearly 20-fold elevations in erythroid precursor levels were apparent, these increases were below what one might expect in noninfected mice with a similar degree of anemia.

These data support the contention that the erythropoietic capability of mice infected with malaria is impaired. Accordingly, it is suggested that this situation contributes to the severe anemic status of malarial mice, in addition to the well-documented mechanisms leading to the destruction of parasitized and nonparasitized erythrocytes.

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