

APPEARANCE OF ADHERENT CELLS SUPPRESSIVE TO ERYTHROPOIESIS DURING AN EARLY STAGE OF *PLASMODIUM BERGHEI* INFECTION IN MICE

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Abstract. We examined the effect of adherent cells from bone marrow or spleen of mice infected with *Plasmodium berghei* on dyserythropoiesis. Significant reduction in number of erythroid progenitors (erythroid colony-forming units : CFU-E and erythroid burst-forming units : BFU-E) was observed in bone marrow as early as 1 day after *P. berghei* infection. When adherent cells were removed from bone marrow or spleen cells of infected mice, the number of CFU-E and BFU-E was clearly increased. Furthermore, addition of adherent cells from infected mice to nonadherent cells from normal mice inhibited erythroid colony formation significantly in a dose-dependent manner. These results suggest that the adherent cells obtained from bone marrow or spleen of mice in the early stage of *P. berghei*-infection have a suppressive effect on erythropoiesis.

INTRODUCTION

Anemia is one of the most important symptoms of malaria infection in man and experimental animals (Weatherall *et al*, 1983). An impaired erythropoietic response to anemia has been noted in human patients with malaria (Dörmer *et al*, 1983; Perrin *et al*, 1982; Srichaikul *et al*, 1969, 1973) and in *Plasmodium berghei*-infected rodents (Rencricca *et al*, 1974; Silverman *et al*, 1987). Maggio-Price *et al* (1985) demonstrated that *P. berghei*-infected mice showed a dramatic decrease in numbers of bone marrow cells, erythroblasts, erythroid burst-forming units (BFU-E), and erythroid colony-forming units (CFU-E) as early as 24 hours post-infection without any changes in the number of peripheral erythrocytes. However, the mechanism of suppression of erythropoiesis in the early stage of malaria infection still remains unclear. In the present study, we demonstrate that the adherent cells in the bone marrow or spleen of mice at an early stage of *P. berghei*-infection confer a suppressive effect on erythroid colony formation *in vitro*.

MATERIALS AND METHODS

Animals and infection

Female C57BL/6 mice were raised in the animal center under clean conventional conditions and used at the age of 8-11 weeks. They were infected with *P. berghei* by intraperitoneal injection with 10^6 parasitized syngenic erythrocytes.

Preparation of cells

Bone marrow cells were obtained by flushing the marrow cavity of femurs from infected or normal mice with Hanks' balanced salt solution (HBSS) containing 10% fetal bovine serum (FBS; GIBCO, Gland Island, NY, USA). The spleen was gently squashed between two frost-ended slides in cold alpha-MEM (GIBCO). Erythrocytes were removed by isotonic lysis with a 0.83% solution of ammonium chloride. The cell suspension was washed three times and suspended in alpha-MEM containing 10% FBS. For the removal of adherent cells, the cell suspension was plated in plastic dishes (Falcon 3001, Becton Dickinson, Lincoln Park, NJ, USA) and incubated for 1 hour at 37°C in 5% CO₂-air environment. After gentle shaking, nonadherent cells were harvested, washed, and suspended in the fresh medium. The dishes were washed with warm HBSS containing 2.5 mM

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ERYTHROPOIESIS SUPPRESSION IN MURINE MALARIA

EDTA and allowed to stand on ice for 15 minutes. The adherent cells were removed by gentle scraping with a rubber policeman. Viability of nucleated cells isolated by the adherence technique was assessed by trypan blue exclusion and averaged over 90%.

***In vitro* erythroid colony assay**

The culture procedure was based on the viscous methylcellulose method described by Iscove and Sieber (1975). Briefly, 2×10^5 bone marrow cells or 2.5×10^5 spleen cells were plated in a 35 mm plastic dish (Falcon 1008, Becton Dickinson) in 1 ml of a mixture containing alpha-MEM without nucleosides (GIBCO), 0.8% methylcellulose (Nakalai Tesque, Kyoto, Japan), 30% FBS (Flow Laboratories Inc, Mclean, VA, USA), 10^{-4} M 2-mercaptoethanol and 2.0 Units of human urinary erythropoietin (kindly provided by Dr M Kawakita, Kumamoto University, Japan). The dishes were incubated at 37°C in a humidified atmosphere with 5% CO₂-air. Colonies were counted on day 2 for CFU-E and on day 9 for BFU-E. All assays were performed in quadruplicate and repeated at least twice.

Statistical analysis

The results were expressed as the mean \pm standard deviation (SD). P values were calculated by unpaired Student's *t*-test.

RESULTS

As shown in Table 1, when whole bone marrow cells were used for the assays, a significant reduction

in the number of CFU-E or BFU-E in the bone marrow cells was observed as early as 1 day after *P. berghei*-infection. Bone marrow cells obtained from mice 3 days after infection showed a similar degree of reduction in the number of CFU-E or BFU-E.

To examine whether dyserythropoiesis is an actual reduction in number of erythroid progenitors or apparent reduction due to the presence of suppressor cells, the numbers of CFU-E (Table 2) and BFU-E (Table 3) in bone marrow or spleen cells were compared before and after the removal of adherent cells. As shown in Table 2, when adherent cells were removed from bone marrow cells of infected mice, the number of CFU-E in nonadherent cells significantly increased and became comparable to that of uninfected controls. Even before the separation of adherent cells, the number of CFU-E in spleen cells of infected mice was higher than that of controls. The removal of adherent cells from spleen cells of infected mice caused a further increase in the number of CFU-E. In contrast, the number of CFU-E in bone marrow or spleen cells of normal mice was not increased by the depletion of adherent cells. These results were fundamentally reproducible in BFU-E assays (Table 3).

To confirm further the suppressive effect of adherent cells on erythropoiesis, various numbers of adherent cells from bone marrow or spleen of *P. berghei*-infected or normal mice were added to the nonadherent cells from bone marrow or spleen cells of normal mice before measuring erythroid colony formation. As shown in Fig 1, a dose-dependent suppression in numbers of CFU-E and

Table 1

Number of CFU-E and BFU-E in bone marrow at an early stage of *P. berghei*-infection.

Experiment	Day ^a	CFU-E ^b	% control ^c	BFU-E ^b	% control
1	0	519 \pm 52	100	44.8 \pm 3.4	100
	1	296 \pm 54	57 ^d	27.8 \pm 4.1	62 ^d
2	0	500 \pm 38	100	46.0 \pm 4.2	100
	3	337 \pm 11	68 ^d	36.8 \pm 2.9	80 ^d

^a Days after *P. berghei*-infection

^b per 2×10^5 bone marrow cells plated

^c Percent of the normal control mice

^d Significant decrease ($p < 0.01$)

Table 2

Effect of adherent cells from *P. berghei*-infected mice on the formation of CFU-E.

Source ^a	Day ^b	Cells examined		% increase ^c
		Whole ^c	Nonadherent ^d	
BM	0	555 ± 22 (100) ^f	495 ± 28 (100)	-11
	1	324 ± 33 (58)	450 ± 33 (91)	30 ^g
	3	337 ± 11 (61)	460 ± 34 (93)	36 ^g
SPL	0	588 ± 11 (100)	430 ± 27 (100)	-27
	1	653 ± 10 (111)	1026 ± 44 (239)	57 ^g
	3	862 ± 21 (147)	1206 ± 49 (280)	40 ^g

^a Five mice were examined for each group. Bone marrow cells (BM, 2×10^5 cells/dish) or spleen cells (SPL, 2.5×10^5 cells/dish) were examined for CFU-E assay

^b Days after *P. berghei*-infection

^c Whole cell population was examined for CFU-E assay

^d Nonadherent cell population was examined for CFU-E assay

^e % increase = (number of CFU-E determined by using the nonadherent cell population / number of CFU-E determined by using the whole cell population - 1) \times 100

^f percent of CFU-E of uninfected mice

^g Significant increase ($p < 0.01$)

Table 3

Effect of adherent cells from *P. berghei*-infected mice on the formation of BFU-E.

Source ^a	Day ^b	Cells examined		% increase ^c
		Whole ^c	Nonadherent ^d	
BM	0	46 ± 4 (100) ^f	39 ± 5 (100)	-17
	1	30 ± 4 (66)	50 ± 7 (130)	64 ^g
SPL	0	23 ± 1 (100)	23 ± 3 (100)	0
	1	18 ± 0 (78)	29 ± 0 (126)	61 ^g

^a Five mice were examined for each group. Bone marrow cells (BM, 2×10^5 cells/dish) or spleen cells (SPL, 2.5×10^5 cells/dish) were examined for BFU-E assay

^b Day after *P. berghei*-infection

^c Whole cell population was examined for BFU-E assay

^d Nonadherent cell population was examined for BFU-E assay

^e % increase = (number of BFU-E determined by using the nonadherent cell population / number of BFU-E determined by using the whole cell population - 1) \times 100

^f percent of BFU-E of uninfected mice

^g Significant increase ($p < 0.01$)

BFU-E was observed when adherent cells from bone marrow of *P. berghei*-infected mice were added. Splenic adherent cells from infected mice showed similar inhibitory effect on the formation of erythroid colonies.

DISCUSSION

In *P. berghei*-infection, a substantial decrease in number of erythroid progenitors was observed as early as 24 hours post-infection before any

ERYTHROPOIESIS SUPPRESSION IN MURINE MALARIA

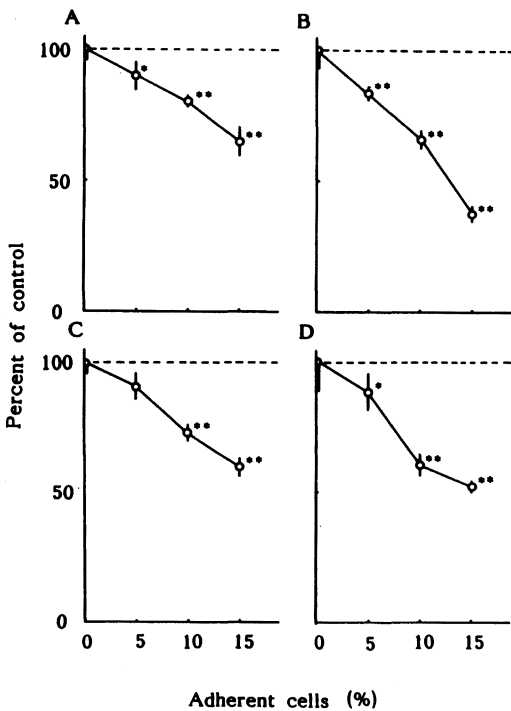


Fig 1—Effect of the adherent cells from *P. berghei*-infected mice on CFU-E (A, B) or BFU-E (C, D) formation. Various numbers of bone marrow (A, C) or spleen (B, D) adherent cells from *P. berghei*-infected mice were added to the 2×10^5 bone marrow (A, C) or 2.5×10^5 spleen (B, D) nonadherent cells from normal mice, and *in vitro* erythroid colony formation was examined. The results were expressed as the percent of the number of erythroid colonies (mean \pm SD) formed at day 2 (CFU-E) and at day 9 (BFU-E) in the presence of the same number of bone marrow (A, C) or spleen (B, D) adherent cells from normal mice. Statistical analysis by unpaired Student's *t*-test: * $p < 0.05$; ** $p < 0.01$

changes in the number of peripheral erythrocytes occurs (Maggio-Price *et al*, 1985). In the present study we could confirm and extend further these results by showing that the apparent decrease in number of erythroid progenitors was due to the presence of suppressive adherent cells, which was induced in the early stage of *P. berghei*-infection. Parasitized erythrocytes were readily observed in bone marrow from day 1 before the disease became patent in retroorbital plexus blood (Weiss, 1983).

Such an early appearance of parasitized erythrocytes may cause stimulation of suppressive adherent cells.

Our results show that the numbers of both CFU-E and BFU-E in bone marrow cells were reduced in the early stage of *P. berghei*-infection (Table 1). This fact suggests that erythropoiesis was depressed throughout the committed erythroid progenitor compartments. The suppressed number of CFU-E or BFU-E in bone marrow cells of *P. berghei*-infected mice was recovered to the normal level after the removal of the adherent cells (Tables 2, 3). Thus, the decrease in number of CFU-E or BFU-E is not a result of actual decrease in number of erythroid progenitor cells but is due to an apparent suppression by adherent cells.

The anemia characteristic in malaria is frequently much greater than would be expected from the degree of erythroid parasitism (Maggio-Price *et al*, 1985; Weatherall *et al*, 1983). Several hypotheses, such as an increased erythrophagia (Facer and Brown, 1981) or the existence of autoimmune reactions (Abdalla *et al*, 1980; Facer, 1980) have been offered to explain the pathway of genesis of the anemia, leading to accelerated destruction of erythrocytes. In fact, histopathological observations have demonstrated accelerated phagocytosis of parasitized and normal red cells in malarial subjects (Weiss, 1983). This phenomenon may be the result of nonspecific stimulation of the phagocytic system by the parasites. Although the relationship between the accelerated phagocytosis and the adherent cells suppressive of erythropoiesis remains unclear, macrophages may regulate the number of peripheral erythrocytes by two ways: the control of erythropoiesis and of erythrophagia.

Erythropoiesis of bone marrow matrix occurs in erythroblastic islands. These islands consist of one or two central reticular cells or macrophages surrounded by developing erythroblasts (Bessis, 1972). Gordon *et al* (1980) demonstrated that normal human bone marrow macrophages could enhance red cell production *in vitro*. Although monocytosis occurs in *P. berghei*-infected mice, macrophage functions appear abnormal (Gross *et al*, 1988; Loose, 1984). In the present study, we showed the adherent cells in both bone marrow and spleen have a suppressive effect on erythropoiesis in the early stage of *P. berghei*-infection. Similarly,

the presence of adherent cells suppressive of erythropoiesis has been reported in the bone marrow cells of the patients with the anemia of chronic diseases other than malaria (Roodman *et al*, 1983) or fungal infection (Zanjani *et al*, 1982). The mechanisms of dyserythropoiesis caused by the suppressive adherent cells needs to be clarified in future studies.

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REFERENCES

- Abdalla S, Weatherall DJ, Wickramasinghe SN, Hughes M. The anaemia of *P. falciparum* malaria. *Br J Haematol* 1980; 46 : 171-83.
- Bessis M. *Cellules du Sang Normal et Pathologique*. Paris: Masson 1972.
- Dörmer P, Dietrich M, Kern P, Horstmann RD. Ineffective erythropoiesis in acute human *P. falciparum* malaria. *Blut* 1983; 46 : 279-88.
- Facer CA. Direct coombs antiglobulin reactions in Gambian children with *Plasmodium falciparum* malaria. *Clin Exp Immunol* 1980; 39 : 279-88.
- Facer CA, Brown J. Monocyte erythrophagocytosis in falciparum malaria. *Lancet* 1981; 1 : 897-8.
- Gordon LI, Miller WL, Branda RF, Zanjani ED, Jacob HS. Regulation of erythroid colony formation by bone marrow macrophages. *Blood* 1980; 55 : 1047-50.
- Gross A, Geva S, Frankenburg S. *Plasmodium berghei*: lymphocyte and macrophage dynamics in the spleen of Balb/c mice in the course of infection and after rechallenge of cured mice. *Exp Parasitol* 1988; 65 : 50-60.
- Iscove NN, Sieber F. Erythroid progenitors in mouse bone marrow detected by macroscopic colony formation in culture. *Exp Hematol* 1975; 3 : 32-43.
- Loose LD. Characterization of macrophage dysfunction in rodent malaria. *J Leukoc Biol* 1984; 36:703-18.
- Maggio-Price L, Brookoff D, Weiss L. Changes in hematopoietic stem cells in bone marrow of mice with *Plasmodium berghei* malaria. *Blood* 1985; 66 : 1080-5.
- Perrin LH, Mackey LJ, Miescher PA. The hematology of malaria in man. *Semin Hematol* 1982; 19:70-82.
- Rencricca NJ, Stout JP, Coleman RM. Erythropoietin production in virulent malaria. *Infect Immun* 1974; 10 : 831-3.
- Roodman GD, Horadam VW, Wright TL. Inhibition of erythroid colony formation by autologous bone marrow adherent cells from patients with the anemia of chronic disease. *Blood* 1983; 62: 406-12.
- Silverman PH, Schooley JC, Mahlmann LJ. Murine malaria decreases hemopoietic stem cells. *Blood* 1987; 69 : 408-13.
- Srichaikul T, Wasanasomithi M, Poshyachinda V, Panikbutr N, Rabieb T. Ferrokinetic studies and erythropoiesis in malaria. *Arch Intern Med* 1969; 124 : 623-8.
- Srichaikul T, Sirisawakul T, Poshyachinda M, Poshyachinda V. Ferrokinetics in patients with malaria : normoblasts and iron incorporation *in vitro*. *Am J Clin Path* 1973; 60 : 166-74.
- Weatherall DJ, Abdalla S, Pippard MJ. The anaemia of *Plasmodium falciparum* malaria. In: Evered D, Whelan J, eds. *Malaria and the red cell. Ciba Found Symp* 1983; 94 : 74-97.
- Weiss L. Hematopoietic tissue in malaria: facilitation of erythrocytic recycling by bone marrow in *Plasmodium berghei*-infected mice. *J Parasitol* 1983; 69 : 307-18.
- Zanjani ED, McGlave PB, Danies SF, Banisadre M, Kaplan ME, Sarosi GA. *In vitro* suppression of erythropoiesis by bone marrow adherent cells from some patients with fungal infection. *Br J Haematol* 1982; 50 : 479-90.