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Macrophage Migration Inhibitory Factor Release by Macrophages after Ingestion of *Plasmodium chabaudi*-Infected Erythrocytes: Possible Role in the Pathogenesis of Malarial Anemia

JAMES A. MARTINEY,[1]* BARBARA SHERRY,[1] CHRISTINE N. METZ,[2] MARISOL ESPINOZA,[1] ANGEL S. FERRER,[1] THIERRY CALANDRA,[2]† HAL E. BROXMEYER,[3] AND RICHARD BUCALA[2]

Laboratory of Cytokine Biology[1] and Laboratory of Medical Biochemistry,[2] The Picower Institute for Medical Research, Manhasset, New York 11030, and Department of Microbiology and Immunology and The Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202[3]

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Human falciparum malaria, caused by *Plasmodium falciparum* infection, results in 1 to 2 million deaths per year, mostly children under the age of 5 years. The two main causes of death are severe anemia and cerebral malaria. Malarial anemia is characterized by parasite red blood cell (RBC) destruction and suppression of erythropoiesis (the mechanism of which is unknown) in the presence of a robust host erythropoietin response. The production of a host-derived erythropoiesis inhibitor in response to parasite products has been implicated in the pathogenesis of malarial anemia. The identity of this putative host factor is unknown, but antibody neutralization studies have ruled out interleukin-1β, tumor necrosis factor alpha, and gamma interferon while injection of interleukin-12 protects susceptible mice against lethal *P. chabaudi* infection. In this study, we report that ingestion of *P. chabaudi*-infected erythrocytes or malarial pigment (hemozoin) induces the release of macrophage migration inhibitory factor (MIF) from macrophages. MIF, a proinflammatory mediator and counter-regulator of glucocorticoid action, inhibits erythroid (BFU-E), multipotential (CFU-GEMM), and granulocyte-macrophage (CFU-GM) progenitor-derived colony formation. MIF was detected in the sera of *P. chabaudi*-infected BALB/c mice, and circulating levels correlated with disease severity. Liver MIF immunoreactivity increased concomitant with extensive pigment and parasitized RBC deposition. Finally, MIF was elevated three- to fourfold in the spleen and bone marrow of *P. chabaudi*-infected mice with active disease, as compared to early disease, or of uninfected controls. In summary, the present results suggest that MIF may be a host-derived factor involved in the pathophysiology of malaria anemia.

Malaria is a disease caused by an intracellular parasitic protozoa of the genus *Plasmodium* and is transmitted by the infected female *Anopheles* mosquito during blood meals. Malaria is still a major cause of death and severe illness in most of the world, with 300 to 500 million new infections per year resulting in approximately 1 to 2 million deaths, mostly in children under the age of 5 years (28). The complications of severe anemia and cerebral malaria are the major causes of morbidity and mortality due to malaria. Of the four strains which infect humans, *Plasmodium falciparum* is the most prevalent and accounts for most malaria-related deaths.

The *P. falciparum* life cycle includes a nonpathogenic, asymptomatic hepatic stage (extraerythrocytic), which is followed by the invasion of mature erythrocytes by infective forms (merozoites) and the initiation of the pathogenic intraerythrocytic stages. The intraerythrocytic parasite derives most of its amino acid requirements from host hemoglobin catabolism within a specialized acidic organelle, the food vacuole (19). Heme is released during hemoglobin digestion and rendered nontoxic by cross-linking into an insoluble polymer, hemozoin, through a parasite-specific biochemical activity (44). The fate of hemozoin is connected to many of the sequelae of malaria infection. After the release of merozoites (invading forms) from host erythrocytes during schizogony, hemozoin is left behind as a residual body and accumulates to a significant degree as "malaria pigment." The intraerythrocytic stages encompassing hemoglobin catabolism (pigmented trophozoites) and erythrocyte lysis (schizogony and hemozoin release) are responsible for many of the pathologic sequelae of malaria.

The pathogenesis of *P. falciparum* malarial anemia is complex and multifactorial and remains poorly understood, despite being a major cause of death in regions of high endemicity (reviewed in references 32 and 34). Severe anemia can be observed at low levels of parasitemia, during chronic infection, and even after the complete chemotherapeutic elimination of organisms (2, 33). Several mechanisms that have been implicated in the pathogenesis of severe anemia (erythrocyte lysis and phagocytosis, increased sequestration of parasitized red blood cells [PRBC], and autoimmune erythrocyte destruction) do not adequately explain the severity and extent of malarial anemia. Hematologic studies of patients with severe malarial anemia have demonstrated ineffective erythropoiesis (17), bone marrow dyserythropoiesis, and lower erythroblast proliferative rates and numbers (17). Similar observations have been made in murine malaria models (13, 24, 36, 37, 43). The suppression of erythropoiesis in cases of severe malaria occurs despite an adequate production by the host of functional erythropoietin (the growth factor necessary for erythrocyte progenitor development) (8, 21). A vigorous host erythropoietin response also was observed in *P. berghei*, *P. vinckei*, and *P. chabaudi* infection of mice (24, 37, 43). The mechanistic basis

* Corresponding author. Mailing address: Forchheimer 520 Department of Pathology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-2048. Fax: (718) 430-2140. E-mail: martiney@freewweb.com.

† Present address: Division of Infectious Diseases, Department of Internal Medicine, BH-19.111, CHUV, CH-1011 Lausanne, Switzerland.

for the suppression of erythropoiesis in the presence of erythropoietin is unknown.

Clark et al. proposed that certain pathogenic manifestations of malaria, such as severe anemia and cerebral malaria, may be due to proinflammatory cytokine release by host macrophages in response to malaria parasites or their products (11, 12, 15). A soluble mediator released from the bone marrow and spleen cells of *P. berghei*-, *P. chabaudi*-, or *P. vivax*-infected (but not uninfected or chemically anemic) mice was shown to depress in vitro erythropoietin-induced proliferation of erythroid precursors and to be partly responsible for anemia (26, 49). Stevenson and colleagues have ruled out tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), or gamma interferon (IFN-γ) as the host-derived, soluble inhibitor of erythropoiesis (50). On the other hand, they have shown that IL-12 levels in resistant B6 mice and susceptible A/J mice correlate with the extent of anemia, with the A/J mice having defective IL-12 production (39). The identity of additional host-derived mediators contributing to malarial anemia remain unknown.

We now report a macrophage product released upon ingestion of *Plasmodium*-infected erythrocytes or malaria pigment (hemozoin): macrophage migration inhibitory factor (MIF). MIF is a macrophage and T-cell mediator that counter-regulates the anti-inflammatory effects of glucocorticoids and is required for T-cell activation, antibody production by B cells, and delayed-type hypersensitivity reactions (reviewed in reference 25). In this study, we show that MIF inhibits erythropoiesis in vitro in the presence of erythropoietin. We demonstrate MIF production during *P. chabaudi* infection of BALB/c mice and find that serum MIF levels correlate with disease severity. Finally, we show MIF production within the bone marrow and liver and by spleen cells isolated from *P. chabaudi*-infected mice with active disease. Taken together, our results suggest that MIF is a likely candidate for a host-derived factor contributing to malarial anemia.

MATERIALS AND METHODS

Mice and experimental infection. Female BALB/c, BALB/c *nu*/*nu*, C3H/HeN (Harlan Bioproducts for Science, Indianapolis, Ind.), and C3H/HeJ mice (Jackson Laboratories, Bar Harbor, Maine) between 8 and 10 weeks of age were housed in groups of five mice per cage with free access to food and water and were acclimated for 10 days before experimentation. The animals were housed in an American Association for Accreditation of Laboratory Animal Care-approved facility. Normal age-matched mice were infected by a single intraperitoneal inoculum of 10^6 *P. chabaudi*-infected erythrocytes collected from a syngeneic donor animal. The course of infection was monitored daily from tail blood smears stained with DiffQuik (Baxter Scientific Products, West Chester, Pa.). Parasitemia (percent PRBC) was determined by microscopic examination of 300 to 500 red blood cells (RBCs). Multiple infections of RBCs were often observed during acute disease but were recorded as a single infection. Each animal was monitored every other day, and at least half of the experimental group was monitored every day. The percent hematocrit was determined from 100 μl of tail blood collected in a heparinized capillary tube using an Adams Micro-HCT microcentrifuge. Parasites were maintained by serial passage in BALB/c mice and passaged at least twice before experimental use. After 8 to 10 passages, the parasite preparation was discarded and a fresh inoculum was prepared from stock kept in liquid nitrogen was used.

Tissue collection. At various times postinfection, three to five animals per group were killed by CO_2 asphyxiation, and the blood was collected by cardiac puncture. Blood was allowed to clot, and serum was obtained by centrifugation and then stored at −20°C until assay. Bone marrow samples were collected by flushing 1 ml of lysis buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate, 2 mM EDTA, and 50 mM Tris [pH 7.5]) through the lumenal space of two femurs with a 21-gauge syringe. The bone marrow plug was subsequently homogenized, the cellular debris was pelleted, and the organ lysate supernatant was concentrated using an Amicon Centricon 10 (Amicon, Beverly, Mass.). Protein concentration was determined by using the Micro BCA Protein Kit (Pierce, Rockford, Ill.).

Erythroid (burst-forming unit–erythroid [BFU-E]), multipotential (CFU-granulocyte, erythroid, macrophage, megakaryocyte [CFU-GEMM]), and CFU-granulocyte-macrophage (CFU-GM) progenitor cell assays. Human bone marrow cells were obtained by aspiration from the posterior iliac crest of healthy volunteers who had given informed consent. Low-density marrow cells (<1.077 g/cm^3) were isolated by density-cut separation on Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) and enriched for progenitors as previously described (6, 7). Enriched low-density human bone marrow cells were plated at 10^5 cells/ml in 1% methylcellulose with recombinant human (rhu) erythropoietin (Epo; 1 U/ml) (Amgen Corp., Thousand Oaks, Calif.) plus rhu IL-3 (100 U/ml) plus rhu steel factor (50 ng/ml) (both from Immunex Corp., Seattle, Wash.). Cultures were maintained in a humidified atmosphere of 5% CO_2 in lowered (5%) oxygen at 37°C and scored for colonies after 14 days of incubation. The effects of MIF or anti-MIF immunoglobulin G (IgG) on colony formation were assessed as follows: rhu MIF or control medium were incubated for 90 min at room temperature with either control medium or neutralizing anti-MIF monoclonal antibody (MAb) (50 μg of antibody per 10 ng of MIF) and then added to the plated human bone marrow cells.

Stimulation of macrophage cultures with parasite products. Thioglycolate-elicited peritoneal macrophages were isolated by using standard methods. Briefly, 8- to 12-week-old mice were inoculated intraperitoneally with 2.0 ml of sterile Brewer's thioglycolate broth. Macrophages were harvested under aseptic conditions 3 days later by peritoneal lavage with 5 ml of ice-chilled 11.6% sucrose solution. The cells were washed twice, and the numbers were determined by hemocytometer counting. Viability was determined by trypan blue exclusion. The cells were suspended in RPMI 1640–5% fetal bovine serum (FBS) to a concentration of 2×10^6 cells/ml, and 1-ml aliquots were dispensed into wells of a 24-well plate. Parasitized (~50% parasitemia) or uninfected syngeneic RBCs at various RBC/macrophage ratios (1:1, 10:1, and 50:1) were added in an equal volume of medium and incubated overnight under a humidified atmosphere of 5% CO_2 in air at 37°C. The culture supernatants were collected after 24 h, clarified by centrifugation, and stored frozen at −20°C until assayed for MIF content by enzyme-linked immunosorbent assay (ELISA).

MIF ELISA. MIF levels in serum and bone marrow lysates were measured by an MIF-specific sandwich ELISA method with purified mouse recombinant MIF (rMIF) as standard. Briefly, 96-well ELISA plates (Immunolon II; Dynatech, Chantilly, Va.) were coated with 10 to 15 μg of anti-MIF MAb (XIV.14.3) per ml in phosphate-buffered saline (PBS) overnight at room temperature. The plates then were washed and blocked with Superblock (Pierce) containing 2% goat serum. After an additional wash in Tris-buffered saline (TBS)–0.05% Tween 20, the samples were plated in triplicate and incubated overnight at 4°C. The plates then were washed, and detector rabbit anti-MIF polyclonal antibody (diluted 1:250) was added for 2 h at room temperature. After a wash in TBS–0.05% Tween 20, alkaline phosphatase-conjugated goat anti-rabbit IgG was added at a 1:4,000 dilution for 35 min at room temperature. Captured antibody complexes were detected by the addition of *p*-nitrophenyl phosphate (pNPP)-ethanolamine substrate, and the positive signals were read at 405 nm against a standard curve obtained for purified rMIF (114 to 83,000 pg/ml). The limit of detection of the assay is 250 pg/ml, and the intra- and interassay coefficients of variation are 5 and 11%, respectively. Data are normalized as values per nanogram per milligram of total protein, measured by using the Micro BCA Protein Kit (Pierce) with a bovine serum albumin (BSA) standard curve.

Preparation of spleen cell cultures. Uninfected and infected mice were killed by CO_2 asphyxiation at various days postinfection, and the spleens were removed aseptically. Cell suspensions were prepared by grinding spleens between two sterile frosted-end microscope slides. Debris was removed by filtering through a sterile fine wire mesh. The resulting suspension was washed three times in RPMI medium (GIBCO, Gaithersburg, Md.) supplemented with 5% FBS, 2 mM L-glutamine, and gentamicin. Viable cells were enumerated by a hemocytometer by the trypan blue exclusion method. Cells were cultured at a density of 10^6 per ml in 24-well plates under a humidified atmosphere of 5% CO_2 in air at 37°C. After 24 h, the supernatants were collected, clarified by centrifugation, and stored at −20°C until assayed for MIF content by ELISA.

MIF immunohistochemistry. At the time of sacrifice, animals were anesthetized with metophane and perfused by cardiac puncture with 20 to 30 ml of cold PBS. The liver was dissected and immersion fixed in Parafix (depolymerized 1.3 M formaldehyde solution; Molecular Histology) for 12 to 15 h at room temperature, extensively washed, and paraffin embedded. Paraffin sections (40 μm) were cut onto gelatin-coated slides. For immunohistochemistry, fixed tissue was dehydrated through a graded series of ethanol and cleared with xylene. Endogenous peroxidase activity was quenched with 3% H_2O_2 in PBS for 30 min. The sections were washed in 1× PBS–0.05% Tween 20, and nonspecific immunoreactivity was blocked with 10% BSA. Anti-MIF antiserum was added at a 1:1,000 dilution in 1% BSA-PBS overnight at 4°C. Immunoreactive MIF was visualized with the use of anti-rabbit IgG-horseradish peroxidase (Dako, Carpinteria, Calif.) at 1:200 in 1% BSA-PBS for 30 min at room temperature, followed by incubation with the horseradish peroxidase substrate diaminobenzidine.

Statistics. Within these experiments, statistical significance was analyzed by using the Student's two-tailed *t* distribution test ($P < 0.05$). When data from similar experiments were combined, the one-way-analysis-of-variance ranks test was used to determine significance. Two-tailed *P* values of <0.05 were considered significant differences.

RESULTS

***P. chabaudi*-infected erythrocytes induce MIF secretion by syngeneic elicited peritoneal macrophages.** We have previously observed that the addition of endotoxin-free, synthetic hemozoin (chemically identical to natural pigment [45]) to the murine monocyte cell line RAW264.7 or to thioglycolate-elicited macrophages resulted in the induction of MIF, as determined by Western blotting (unpublished observations). We cocultured elicited peritoneal macrophages with syngeneic uninfected or *P. chabaudi*-infected erythrocytes at RBC/macrophage cell ratios of 1:1 to 50:1. Elicited macrophages constitutively secrete MIF (2 ng/ml) over a 24-h period in the absence of any stimulus (Fig. 1A, solid bar). Coculture with syngeneic uninfected erythrocytes induced a modest twofold increase in MIF release that was independent of the RBC/macrophage ratio (Fig. 1A, open bars). *P. chabaudi*-infected erythrocytes, in contrast, induced a dose-dependent increase in macrophage MIF secretion, which was up to 10-fold higher at a 50:1 ratio (Fig. 1C, hatched bars).

We next determined whether T cells were either a contaminating source of MIF or required for macrophage MIF production. Elicited peritoneal macrophages obtained from BALB/c *nu/nu* (T-cell-deficient) mice secrete MIF in response to syngeneic *P. chabaudi*-infected erythrocytes to the same extent as BALB/c mice (Fig. 1B, open and closed bars). These data demonstrate that MIF is produced by macrophages independent of T-lymphocyte contamination or help. To ensure that the release of MIF from macrophages was not a result of lipopolysaccharide (LPS) contamination, we compared the MIF response of elicited macrophages from LPS-responsive (C3H/HeN) and hyporesponsive (C3H/HeJ) mice (Fig. 1B, hatched bars). There was no significant difference in the amount of MIF secreted by these two strains of macrophages upon coculture of macrophages with syngeneic infected erythrocytes, ruling out contaminating LPS as a source of MIF stimuli.

Effect of MIF on myelopoiesis. To determine whether MIF was able to modulate the production of RBCs (erythropoiesis), rhu MIF or neutralizing anti-MIF antibody was added to human bone marrow cultures under erythropoietic induction. Erythropoiesis was quantified in vitro by counting the Epo-responsive erythroid (BFU-E) and multipotential (CFU-GEMM) progenitors which develop into colonies from bone marrow cells. The addition of either anti-MIF IgG or an irrelevant antibody had no effect on the normal bone marrow development of BFU-E or CFU-GEMM (Fig. 2A and C, solid bars). On the other hand, the addition of 0.1 to 100 ng of MIF (within the range observed after ingestion of PRBC) per ml dose-dependently inhibited BFU-E and CFU-GEMM development (Fig. 2A and C, open bars). For example, a dose of 10 ng of MIF per ml (equivalent to that released by macrophages at a 10:1 PRBC/macrophage ratio) inhibited BFU-E and CFU-GEMM development by approximately 50%. Anti-MIF IgG, but not control IgG, restored full erythropoietic potential, demonstrating the specificity of the inhibition (Fig. 2A and C, hatched bars). Additionally, MIF also inhibited colony formation by CFU-GM (Fig. 2B). These results demonstrate that MIF suppresses the development of erythroid and other myeloid progenitors in the presence of functional Epo and other growth factors. No colonies formed from BFU-E or CFU-GEMM in the absence of Epo (data not shown).

High circulating levels of MIF during peak parasitemia. Having demonstrated the production of macrophage MIF after exposure to parasite products in vitro, we next tested whether MIF was expressed during malaria infection in vivo. *P. chabaudi* is a murine malarial parasite whose infection of genetically susceptible BALB/c mice results in a dose- and passage-dependent course of infection (46). The inverse relationship between parasitemia and hematocrit at the infective inoculum of our experiments (10^6 PRBC) is illustrated in Fig. 3. BALB/c mice developed severe anemia and high levels of parasitemia, with extremely low hematocrit levels at days 7 to 9 postinfection, and then succumbed to infection between 9 and 10 days postinfection as previously described (46).

We measured the serum concentration of MIF at various days postinfection by both ELISA and Western blotting. Serum MIF increased dramatically during the course of infection,

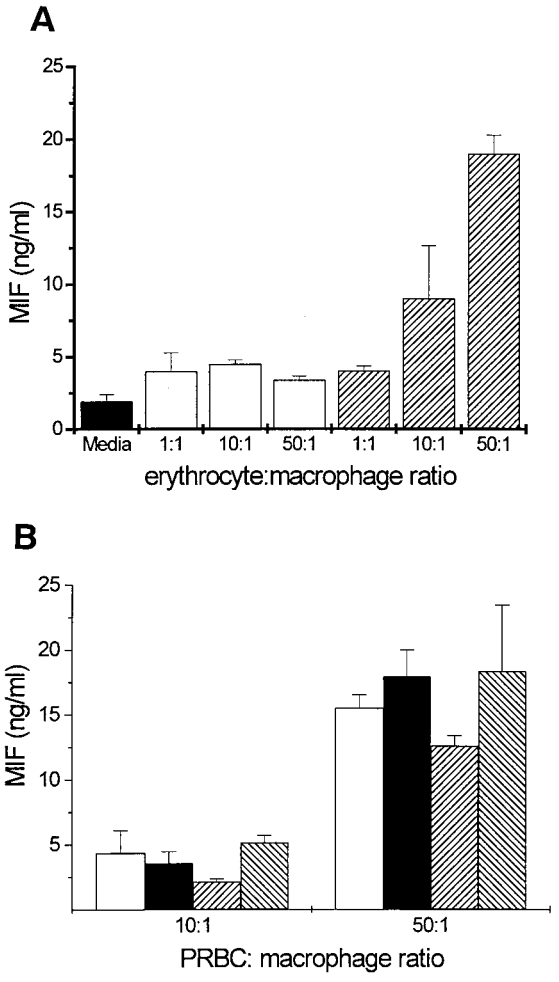

FIG. 1. MIF secretion by murine macrophages cocultured with PRBC. (A) Uninfected (open bars) or *P. chabaudi*-infected (hatched bars) erythrocytes were cocultured with 10^6 syngeneic (BALB/c) thioglycolate-elicited macrophages at 1:1, 10:1, and 50:1 RBC/macrophage ratios. Closed bars represent elicited macrophage MIF produced in the absence of any stimulus. The means at the 10:1 and 50:1 ratios for infected erythrocytes are statistically different ($P < 0.05$) from that of the uninfected erythrocytes. (B) Uninfected or *P. chabaudi*-infected erythrocytes were cocultured with syngeneic macrophages as described above (BALB/c, open bars; BALB/c *nu/nu*, solid bars; C3H/HeN, left-hatch bars; C3H/HeJ, right-hatch bars). Data are plotted as "net" MIF production: MIF induced by uninfected RBCs subtracted from that induced by PRBC. The supernatants were collected after 24 h, and MIF concentrations were quantified by sandwich ELISA. For details, see Materials and Methods. The means at each specific ratio are not statistically different from each other according to the two-population *t* test (two-tailed). The means at the 50:1 ratio are more statistically significant ($P < 0.05$) than the means at 10:1 ratio. Each column represents the mean ± standard deviation (SD) values of three replicas of one typical experiment.

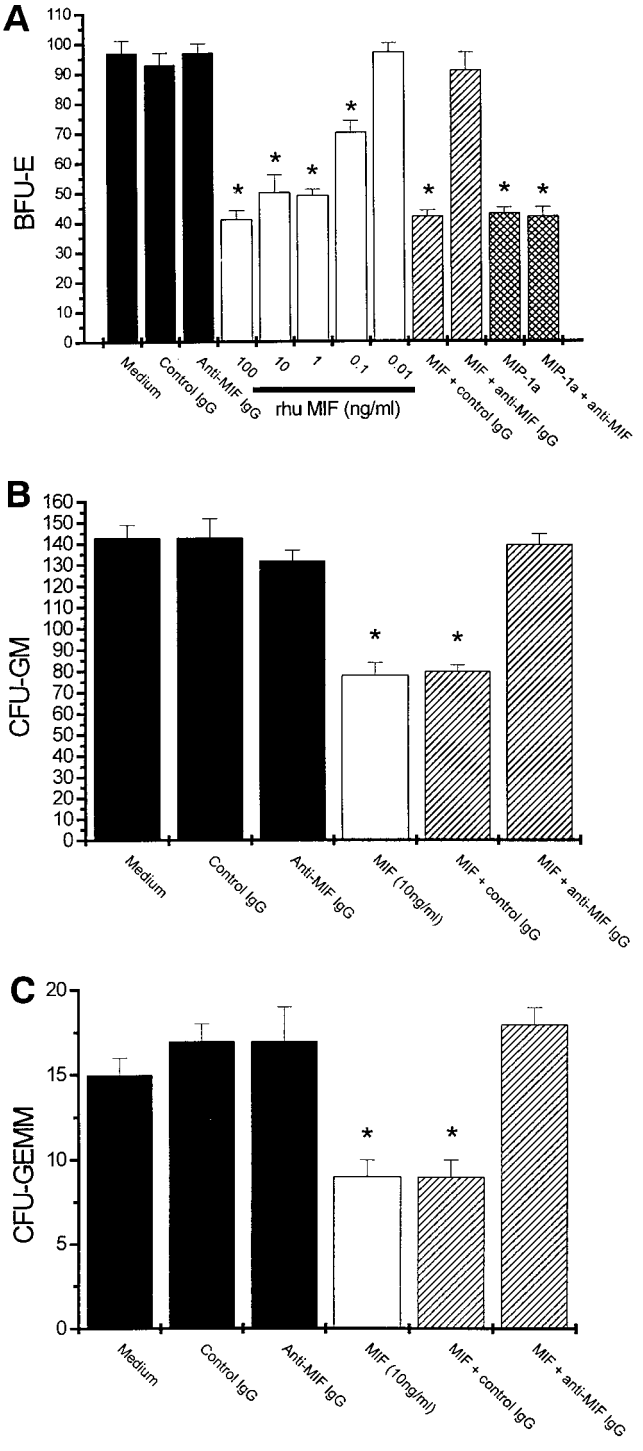

FIG. 2. Inhibition of myelopoiesis by MIF. Human bone marrow cells were plated and stimulated according to standard protocols. The numbers of progenitor colonies were counted after 14 days of stimulation with the appropriate growth factors (solid bars): A, BFU-E; B, CFU-GM; and C, CFU-GEMM. See Materials and Methods for more details. Antibody (control rabbit IgG or anti-MIF IgG) was preincubated with medium prior to addition to the culture (solid bars). rhu MIF protein (0.01 to 100 ng/ml) was added in the same volume at the beginning of the experiment (open bars). A total of 10 ng of MIF per ml was preincubated with antibody (control IgG or anti-MIF) prior to the addition to the culture (cross-hatched bars). Values that are statistically different ($P < 0.05$) from the control values are denoted by an asterisk. There is no statistically significant difference between medium alone, control IgG, and anti-MIF IgG. For details, see Materials and Methods. Each column represents the mean ± standard error of the mean (SEM) values of two different experiments.

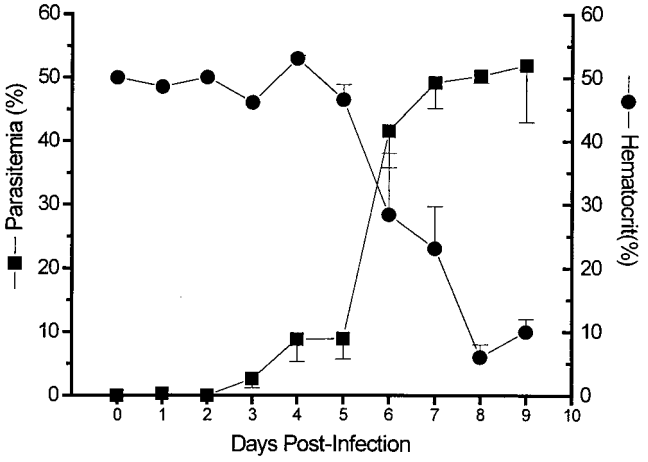

FIG. 3. Course of *P. chabaudi* infection in BALB/c mice. Inoculation of mice and measurement of the percent parasitemia and hematocrit was performed as indicated in Materials and Methods. Data are of the means ± the SEM of five different experiments, with 5 to 12 animals per time point.

reaching a maximum (7 to 11 ng/ml) between 7 and 9 days postinfection coinciding with high levels of parasitemia (>40%) and low hematocrit (<25%) (Fig. 4, open bars). Western analyses confirmed the increase in serum MIF with progressing disease course (data not shown). Interestingly, animals which received a lower parasite inoculum (10^4 PRBC) and cleared the infection by 10 days postinfection had MIF levels comparable to day 4 postinfection (2 to 3 ng/ml) (Fig. 4, solid bar), suggesting that MIF is produced during the course of infection.

MIF production by spleen cells. Spleen cell-conditioned medium prepared from *P. chabaudi*-infected C57BL/6 mice has been shown previously to inhibit erythropoiesis (50). We next sought to determine MIF levels in the supernatants of cell suspensions obtained from spleens taken from *P. chabaudi*-infected mice. ELISA results demonstrated a biphasic two- to threefold increase in MIF production (25 to 40 ng/ml) by

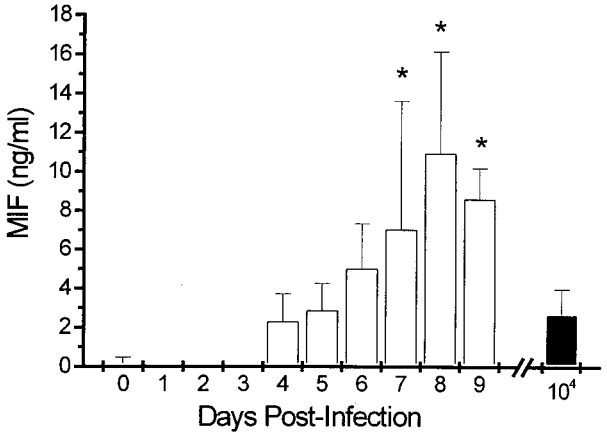

FIG. 4. Serum concentration of MIF during *P. chabaudi* infection. Quantification of MIF was performed by using an MIF-specific sandwich ELISA as described in Materials and Methods. Each point represents the mean ± the SEM of three different experiments, each experiment performed in duplicate wells. The last column denotes a group of animals that were injected with 10^4 infected erythrocytes, a 100-fold-lower inoculum (solid bar). Values that are statistically different ($P < 0.05$) from day 4 postinfection are denoted by an asterisk.

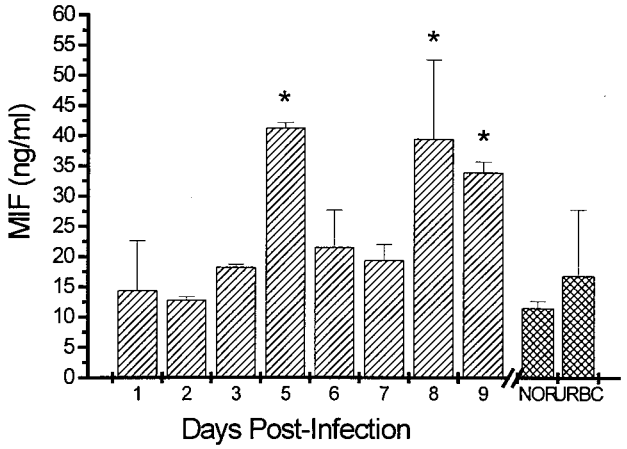

FIG. 5. MIF levels in supernatants of spleen cell cultures derived from *P. chabaudi*-infected mice. Spleen cell cultures were prepared from aseptically dissected spleens of *P. chabaudi*-infected mice at various days postinfection as described in Materials and Methods. Spleen supernatants from normal, uninfected mice are shown in the column labeled NOR. Spleen supernatants from animals injected with uninfected erythrocytes as an additional control is shown in the column labeled URBC. Quantification of MIF was performed with an MIF-specific sandwich ELISA. Each point represents the mean ± the SD of triplicate wells from a representative experiment. Values that are statistically different ($P < 0.05$) from day 0 (uninfected) are denoted by an asterisk.

spleen cells derived from animals with significant parasitemia (>20% at 5, 8, and 9 days postinfection) compared to the presymptomatic stage (<15 ng/ml at 1 to 3 days postinfection) (Fig. 5, hatched bars). As controls, we plated spleen cells derived from normal (uninfected) animals and from animals injected with a 10^6 inoculum of syngeneic uninfected erythrocytes (mock infection). These cultures produced MIF levels (10 to 15 ng/ml) that were equivalent to spleen cell cultures from mice at 1 to 3 days postinfection (Fig. 5, cross-hatched bars).

Liver MIF immunohistochemistry. Extensive deposition of hemozoin and PRBC occurs in the liver during malarial infection. We next evaluated by immunohistochemistry the MIF protein levels in the liver during the course of *P. chabaudi* infection. We detected a small amount of immunoreactivity in normal animals, as previously described (4), and early in disease (1 to 3 days postinfection [data not shown]). Figure 6A and B depict paraffin-embedded liver serial sections from an animal with acute disease (43% parasitemia). Figure 6A shows the background obtained with preimmune serum, while Fig. 6B demonstrates the immunoreactivity seen with anti-MIF antiserum. The sections were not counterstained in order to appreciate the amount of hemozoin deposition on the tissue (black precipitates). MIF immunoreactivity localized to inflammatory cells within the lumen of liver vessels (Fig. 6C), hepatocytes, endothelium, and Küpffer cell lining the liver sinusoids (Fig. 6D). In summary, (i) there is a marked increase in MIF immunoreactivity in the liver during active disease and (ii) several cell types (inflammatory cells, Küpffer cells, endothelial cells, and hepatocytes) are potential sources of MIF during acute disease.

Bone marrow MIF production. Localized MIF production within the bone marrow of *P. chabaudi*-infected animals could result in the suppression of erythropoiesis, contributing to the severity of malarial anemia. Ultrastructural studies of bone marrow from anemic children with severe malaria have shown the presence of bone marrow macrophages with ingested, *Plasmodium*-infected erythrocytes (2) and hemozoin (33). We observed a similar histopathology in our murine model (data not shown). Therefore, we quantified bone marrow MIF from *P. chabaudi*-infected mice at various times postinfection. Our data showed significant MIF levels (12 to 15 ng/ml) during active disease (6 to 7 days postinfection) compared to early infection (<7 ng/ml at 1 to 5 days postinfection) (Fig. 7).

DISCUSSION

The pathogenesis of malaria remains unclear. It is characterized by increased RBC destruction, decreased RBC production, and bone marrow dyserythropoiesis typified by incomplete mitosis, multinucleation, chromatin disintegration, intercytoplasmic bridges, karyorrhexis, and distorted nuclei (reviewed in reference 34). In addition, there is marked suppression of erythropoiesis, even in the presence of adequate functional erythropoietin production. Several alternative models have been proposed for the mechanism of anemia: sequestration of PRBC (2), rupture of PRBC during schizogony, macrophage-mediated ingestion of PRBC, bone marrow hypoxia due to blockage of microvasculature by PRBC, low iron availability, immune-mediated hemolysis mediated by RBC surface IgG and complement receptor C3 (1, 18, 38), disseminated intravascular coagulation (16, 20; H. A. Reid, letter, Lancet **i:**167–168, 1975), and decreased survivability of uninfected RBCs (23, 48). However, none of the above models adequately account for the severity of anemia nor the active suppression of erythropoiesis in the presence of erythropoietin. Maggio-Price et al. proposed that erythropoietic changes were related to the immunologic responses to malarial infection by host white blood cells (24).

The interaction between host leukocytes and pigmented trophozoites and/or hemozoin plays a central role in both the protective and pathogenic sequelae of malarial infection. Host macrophages avidly phagocytize several parasite-specific products during the symptomatic stages of infection (summarized in reference 41). Ingestion of these products has a profound effect on macrophage function (41) and cytokine production (40). The first and best-characterized parasite-induced cytokine was TNF-α, induced in macrophages by *Plasmodium*-infected erythrocytes, hemozoin or malarial pigment, and certain glycolipids. Mononuclear cells from the spleen and bone marrow of *Plasmodium*-infected mice produce a soluble factor that inhibited the response of erythroid progenitors to erythropoietin (26). Since one of the biological activities of TNF-α is the suppression of erythropoiesis, it was suggested that host TNF-α production in response to parasite products was the basis of the severe anemia in malaria (13, 15). However, antibody-neutralizing studies demonstrated that the host-derived inhibitor of erythropoiesis was not TNF-α, IL-1β, or IFN-γ (49, 50). Therefore, the malarial anemia factor(s) remained unknown.

Stevenson and colleagues have recently published a series of studies demonstrating the role of IL-12 in malarial anemia in murine models (29, 30, 31, 39). IL-12 is an immunomodulatory cytokine involved in various aspects of the regulation of cellular and humoral immunity (47). Moreover, IL-12 confers protection against various bacterial, viral, and parasitic infections (21). Sam and Stevenson first demonstrated that B6 mice, which were resistant to *P. chabaudi* AS infection, had higher levels of IL-12 during infection than the susceptible A/J mice (39). Mohan and Stevenson then showed that IL-12 levels in these mice correlate with the extent of anemia and that A/J mice are defective in IL-12 production during the early course of *P. chabaudi* infection (30). Injection of A/J mice with IL-12 during early stages of *P. chabaudi* infection resulted in a sig-

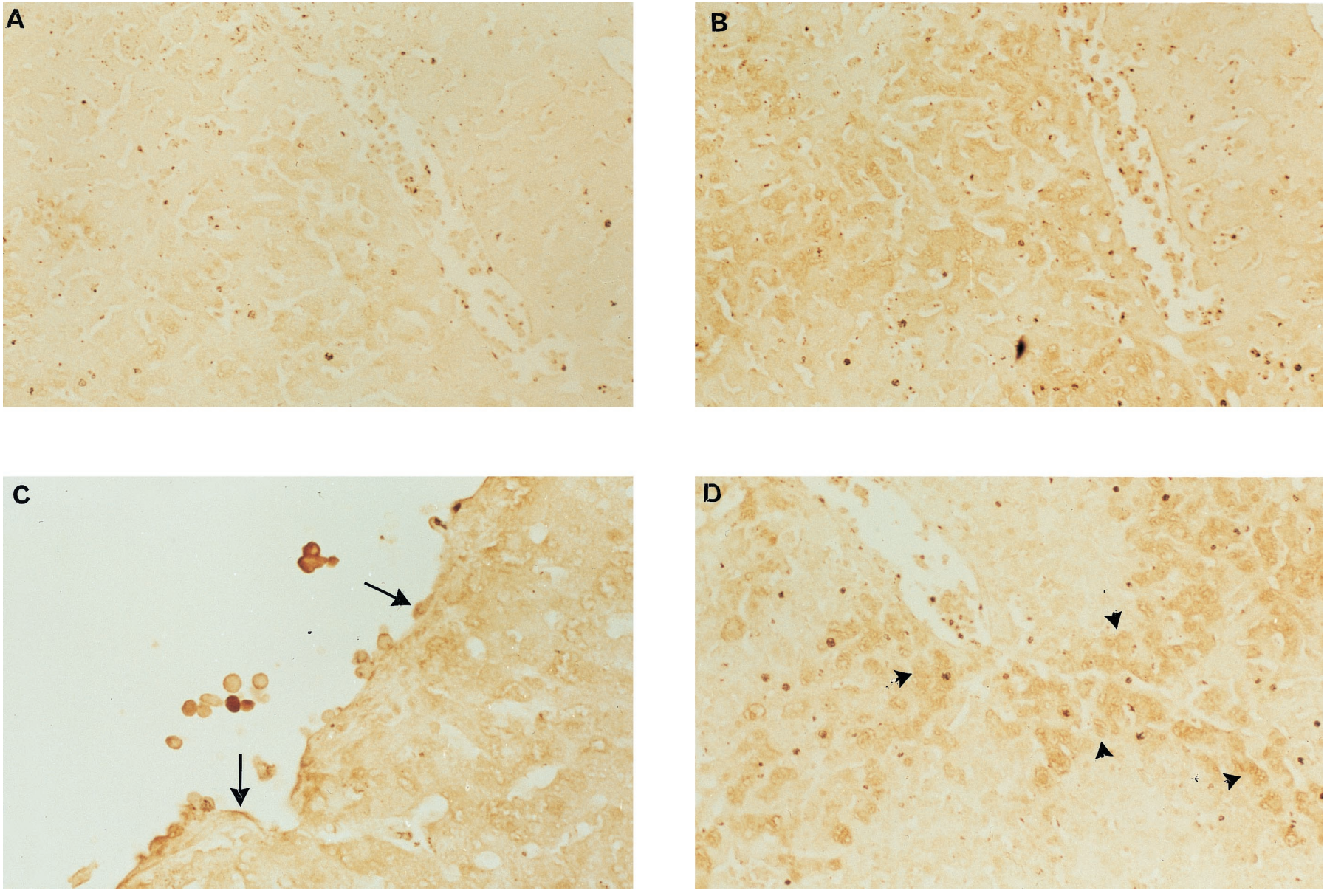

FIG. 6. MIF liver immunohistochemistry of *P. chabaudi*-infected mice with acute disease (43% parasitemia). Panels A and B are serial sections: panel A is stained with preimmune serum, while panel B is stained with MIF antiserum (×20). Panel C shows immunoreactive inflammatory cells and endothelium (arrows) within the lumen of blood vessels (×40), while panel D illustrates MIF immunoreactivity in hepatocytes surrounding blood vessels (arrowheads) (×20).

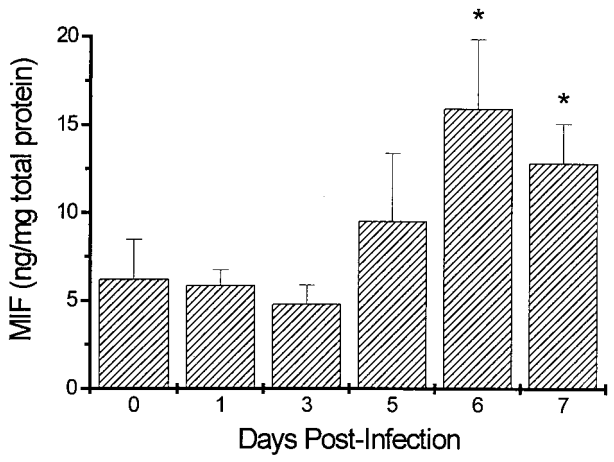

FIG. 7. MIF levels in bone marrow lysates from *P. chabaudi*-infected mice. Bone marrow lysates were prepared from the femurs of mice at various times postinfection as described in Materials and Methods. Protein concentrations were determined with the MicroBCA Kit. Quantification of MIF was performed by using an MIF-specific sandwich ELISA. Data are expressed as nanograms of MIF per milligrams of protein. Data shown are the means ± the SEM of two different experiments performed in duplicate. Values that are statistically different ($P < 0.05$) from day 0 (uninfected) are denoted by an asterisk.

nificant increase in hematocrit, BFU-E, and spleen cellularity (31). Finally, a combination of low-dose IL-12 and chloroquine rescued susceptible A/J mice from lethal *P. chabaudi* AS infection, demonstrating the possibility of using immunotherapies to treat malarial anemia (29).

We have previously identified novel macrophage factors induced after ingestion of the malaria-specific product hemozoin, such as the pyrogenic chemokines MIP-1α and MIP-1β (42). In the present study, we demonstrated that MIF is also released from murine macrophages after the ingestion of *P. chabaudi*-infected erythrocytes or malarial pigment (hemozoin). MIF is known to function as a physiological counterregulator of glucocorticoid action within the immune system, since it overrides the inhibitory effects of glucocorticoids on the immune response (summarized in reference 25). The immune regulatory properties of MIF are significant within the context of a response against an infectious organism. However, we discovered another function of MIF which could be relevant within the context of malarial anemia: the suppression of Epo-dependent erythroid (BFU-E) and multipotential (CFU-GEMM) progenitor cells in vitro. Since MIF fits the published criteria for the putative host factor inhibitor of erythropoiesis, we hypothesized that MIF was produced by macrophages in response to malarial infection and could be a factor involved in severe anemia. Interestingly, MIF also suppressed the growth of granulocyte-macrophage (CFU-GM) progenitors. Neutrophil development and differentiation appears to be altered during malarial infection (24). MIF production could play a role in this phenomenon via CFU-GM suppression.

Extensive pigment and PRBC deposition is seen in the spleen and bone marrow, organs capable of erythropoietic expansion during intense erythropoietic challenge. The bone marrow of patients with multiple malarial episodes appears black due to the accumulation of malarial pigment. Yap and Stevenson reported pigment-laden macrophages adjacent to developing erythroblasts in the red pulp of the spleen (50). Ultrastructural analysis of the bone marrow of severely anemic children demonstrated the presence of macrophages containing ingested PRBC and malarial pigment (2, 3, 17). Pigment (hemozoin) and PRBC sequestration within the spleen and bone marrow could result in localized MIF production and subsequent inhibition of erythropoiesis. Consistent with this model, we detected increased MIF protein within the spleen and bone marrow of *P. chabaudi*-infected mice at peak levels of parasitemia. We interpreted the biphasic production of MIF by cultured spleen cells by noting that the initial release of MIF occurs at the onset of PRBC sequestration within the spleen (day 5 postinfection) and that the second peak occurs at the time of extensive pigment deposition within the spleen (days 8 to 9 postinfection). Of note is the fact that macrophages secrete small amounts of MIF even after the ingestion of uninfected erythrocytes. Facer and Brown demonstrated that Gambian children with acute *P. falciparum* infection and who were severely anemic showed monocyte phagocytosis of uninfected erythrocytes (C. A. Facer and J. Brown, Letter, Lancet **i**:897–898, 1981). This could serve as an additional stimulus and source of MIF production.

Immunoreactive MIF is found in the livers of normal mice, localized to hepatocytes and endothelial cells surrounding sinusoids or venules (4). During the course of malarial infection, liver immunoreactivity increased severalfold in these areas and was also detected in Küpffer cells and inflammatory cells. This pathology is reminiscent of that observed during systemic LPS administration (4), and it may reflect a generalized macrophage-based proinflammatory response. This is unlikely to contribute directly to malarial anemia except as an additional source of circulating MIF. However, a similarity between LPS-induced pathologies and malarial pathogenesis has been previously reported (14).

The present data support the hypothesis that a host factor(s) capable of suppressing erythropoiesis underlies the pathogenesis of malarial anemia. MIF fits several of the criteria previously established. Yap and Stevenson showed maximal inhibitory activity at peak levels of parasitemia (49) and production within the bone marrow and spleen (50). Likewise, we found the highest levels of MIF production by spleen cells and bone marrow, as well as circulating MIF, during the last 4 days of disease, at the time of peak parasitemia. It is at these high levels of parasitemia that erythropoietin increases up to 100-fold in *Plasmodium*-infected mice (43). Therefore, significant amounts of MIF are produced at the time of erythropoietin production, which could potentially counteract its proerythropoietic function.

Yap and Stevenson determined some of the biophysical characteristics of the putative soluble host-derived inhibitor of erythropoiesis (50). Using membrane ultrafiltration, they determined the activity band had a molecular mass of more than 10 kDa; MIF has a molecular mass of 12.5 kDa and exists as a 37-kDa trimer (5). The activity precipitated at 50 to 70% ammonium sulfate saturation and eluted in the void volume of a Sephadex G-25 column, as does MIF. Partial inactivation of activity was obtained by heat treatment at 95°C but not at 56°C; similar treatment has comparable results with MIF (5).

MIF could synergize with TNF-α and/or IL-12, compounding the pathogenesis of anemia. Hemozoin, PRBC, and other parasite products induce macrophage TNF-α production, and TNF-α has been shown to induce erythrophagocytosis and dyserythropoiesis in the bone marrow of mice suffering from low-density infection with *P. vinckei* (13) as well as suppression of erythropoiesis (10, 27). High levels of MIF induce macrophage TNF-α secretion and synergize with IFN-γ to promote macrophage NO production (9). Several microorganisms and microbial products induce macrophage secretion of IL-12 and MIF. Therefore, MIF could be a major factor in the induction of bone marrow ultrastructural changes, act locally to amplify

macrophage proinflammatory responses, and synergize with other cytokines to enhance phagocyte-mediated damage.

Disease severity, susceptibility to severe anemia, and other aspects of malarial pathophysiology could each derive from the response of host macrophages to various parasite-specific products. The outcome of such an interaction can have important consequences for disease progression, morbidity, and mortality, in addition to presenting possible avenues for therapeutic interventions. One possibility is derived from the "antitoxin" vaccine proposed by Playfair et al. (35). Antibodies raised against a parasite product or "toxin" may suppress the pathogenicity of the disease without requiring eradication of the infecting organism. Similarly, antibodies against a parasite-induced host pathogenic factor may ameliorate a specific clinical manifestation without eradicating the disease. Neutralizing anti-MIF antibody treatment has been used successfully in animal models to suppress the lethality and pathology associated with LPS-induced septic shock, glomerulonephritis, and arthritis (summarized in reference 25). We are currently investigating the effects of anti-MIF in murine malaria. Identification of the host factor(s) inducing erythropoiesis suppression is critical in understanding the pathophysiology of malarial anemia and in developing potential intervention routes.

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