

HEMATOPOIETIC FEATURES AND APOPTOSIS IN THE BONE MARROW OF SEVERE *PLASMODIUM FALCIPARUM*-INFECTED PATIENTS: PRELIMINARY STUDY

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Abstract. The mechanism of anemia in severe falciparum malaria is still not completely understood. The purpose of this study was to determine whether apoptosis in the erythroid lineage causes anemia in falciparum malaria. Bone marrow aspirated from 8 severe falciparum malaria patients, 3 normal volunteers and 5 retrospective normal bone marrow smears were investigated. By light microscopic study, 5 of 8 hyperparasitemic patients had hypocellular bone marrows and erythroid hypoplasia, whereas the other 3 patients had normal cellularity. The mean myeloid : erythroid ratio of these 5 patients was significantly ($p \leq 0.05$) higher than normal. Apoptosis of bone marrow nucleated cells (BMNC) could be determined from the exposure of phosphatidylserine (PS) on the cell membrane but not DNA fragmentation (180-250 bp) or ultrastructural morphology. The percentages of apoptotic BMNC and apoptotic erythroid cells in bone marrow from each patient and controls varied from low to high, and were not associated with parasitemia. This study suggests that destruction of erythroid lineage, particularly through apoptosis regulation, cannot solely account for anemia in falciparum malaria.

INTRODUCTION

In acute and severe falciparum malaria, anemia can develop rapidly, cause morbidity and probably mortality, particularly in young children and non-immune adults (Harinasuta and Bunnag, 1988). Anemia is also a major complication of chronic falciparum malaria in endemic areas (Weatherall, 1988). Pathogenesis and causes of anemia in the disease are complicated and still not clearly understood. At schizogony, the infected erythrocytes rupture and release numerous merozoites to undergo the next

asexual cycle. As a consequence of hemolysis, anemia may develop rapidly, particularly in severe cases (Harinasuta and Bunnag, 1988). Prolonged anemia may occur because of several weeks shorter life-span of non-infected red blood cells in falciparum patients after antimalarial treatment, and complete parasite clearance (Looareesuwan *et al*, 1987). During malaria infection, there are changes in components on the erythrocytic membrane, *eg* exposure of phosphatidylserine and an increase in the level of some complement regulatory proteins which contribute to erythrophagocytosis and lysis by complement activation (Waitumbi *et al*, 2000). Some anti-malarial drugs, such as quinine, may lead to immune-mediated hemolysis (Weatherall, 1988). Liver and kidney damage and other disturbances in severe malaria lead to a decrease in the synthesis of erythropoietin, which regu-

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lates erythropoiesis and megakaryopoiesis (Harinasuta and Bunnag, 1988; El Hassan *et al*, 1997). The suppression of erythropoiesis and ineffective erythropoiesis, and the consequences of sequestration in bone marrow sinusoids, are also suggested as important causes of anemia (Wickramasinghe *et al*, 1987). Some cytokines, including tumor necrosis factor α (TNF- α), interferon- γ (IFN- γ), interleukin-6 (IL-6), and interleukin-10 (IL-10) are increased in severe falciparum patients (Clark *et al*, 1989; Kern *et al*, 1989). These cytokines particularly TNF- α and IFN- γ play an important role in erythropoiesis depression (Allen *et al*, 1999) and also apoptosis in erythroid cells, both *in vitro* and in animal models (Dai and Krantz, 1999). Previous studies have demonstrated malaria-associated apoptosis in peripheral lymphocytes (Balde *et al*, 1995; Kemp *et al*, 2002). Recently, significant apoptotic lymphocytes from both uncomplicated *P. falciparum* and *P. vivax* infected patients were observed when the cells were cultured for 24 hours, although there were no differences in those detected *ex vivo*, between healthy donors and patients (Riccio *et al*, 2003). However, no evidence indicates an association between apoptosis in the bone marrow and the development of anemia in falciparum malaria. The present study was designed to determine whether apoptosis in progenitor cells, particularly the erythroid lineage, plays a role in the pathogenesis of anemia in severe falciparum malaria.

MATERIALS AND METHODS

Subjects

Eight severe *P. falciparum* infected patients, who had peripheral blood smear examinations, and symptoms and signs consistent with *P. falciparum* infection, were enrolled from the Hospital for Tropical Diseases, Mahidol University. The patients were not infected with HIV or other microorganisms, had no blood clotting disorder or underlying diseases. This study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University. A consent form from each patient was obtained for this study. Controls included three healthy volunteers with

normal complete blood counts and two retrospective normal bone marrow smears.

Specimen and history collection.

One to two milliliters of heparinized bone marrow were aspirated at the posterior iliac crest from each enrolled patient on the day of admission (day 0), while they were comatose (Glasgow Coma Score ≤ 7) (Wilairatana and Looareesuwan, 1995) and from the volunteers. Each aspirated bone marrow was divided into 2 portions, 1 portion (200 μ l) for bone marrow smear preparation, and the other for nucleated hematopoietic cell (BMNC) isolation. The histories of the patients and the clinical examinations on admission were recorded (Table 1).

Morphological studies

The air-dried bone marrow smears on glass slides were fixed with methanol for 2-5 minutes at room temperature (RT). After air-drying for 20-30 minutes, the fixed smear was stained with Wright's-Giemsa stain. The morphology of the hematopoietic cells was investigated under a light microscope. The bone marrow smear was differentially counted (at least 500 cells per slide) into erythroid, granulocytic, agranulocytic, and megakaryocytic series. The myeloid : erythroid ratio (M:E ratio), calculated from the proportion of total myeloid lineage cells and total erythroid cells (1:4), was recorded. The proportion of fat and BM smears were compared to normal, and identified as hypocellular, hypercellular and normal BM (M:E ratio = total myeloid cells/total erythroid cells).

In situ labeling of fragmented DNA (TUNEL)

The air-dried bone marrow smears on Vectabond reagent (Vector Laboratories, CA, USA) coated slides, fixed with methanol and pretreated with 20 μ g/ml of proteinase K were detected for DNA fragmentation of hematopoietic cells using an *in situ* apoptosis detection kit (Trevigen, Gaithersburg, USA) according to the recommendations of the manufacturer. To identify erythroid lineage, all bone marrow smears were further stained for glycophorin A by the streptavidin alkaline phosphatase method using mouse monoclonal anti-human glycophorin A (DAKO, Denmark), as described previously (Dai and Krantz, 1999).

Detection of phosphatidylserine (PS) exposed to outer cell membrane

Nucleated hematopoietic cells (BMNC) were separated from heparinized bone marrow aspirates by gradient centrifugation on Isoprep, (Robbins Scientific corporation, Norway) at 400g, for 30 minutes at 10°C. BMNC (5×10^5) were detected by PS inversion in the cell membrane using an apoptosis detection kit (Pharmingen, San Diego, USA) according to the recommendations of the manufacturer. Briefly, the cell pellet was added to 0.5 ml of binding buffer mixed well by vortex, then incubated with 2 μ l of fluorescein-conjugated annexin V at RT for 12-15 minutes in darkness. The pellet was then added with 0.1 μ g of propidium iodide at RT for 12-15 minutes in darkness. The stained cells were analyzed for the detection of apoptosis and necrosis erythroid cells by flow FACS Sort (Becton Dickinson, CA, USA).

Another 5×10^5 apoptotic erythroid cells were detected using a mouse monoclonal anti-human glycophorin A and an apoptosis kit. Briefly, the pellet was incubated with 0.2 μ g of rhodamine conjugated mouse antihuman glycophorin A at RT for 12-15 minutes in darkness. The cells were washed with 2.5 ml of hemaline (Becton Dickinson Immunocytometry System, CA, USA). The cells were detected for PS inversion in the cell membrane using the apoptosis detection kit mentioned above, but omitting propidium iodide in the final step. The stained cells were analyzed by a flow cytometry FACS Sorter. The erythroid population was gated to detect apoptosis by the binding of FITC conjugated annexin-V to PS on the outer leaflet. Apoptotic ($An^+/Gly A^+$) erythroid cells were bound with rhodamine-conjugated mouse antihuman glycophorin A and FITC conjugated annexin-V but not propidium iodide. Total apoptotic (An^+/PI^-) BMNC were bound to FITC conjugated annexin-V but not propidium iodide. The dead cells, stained with propidium iodide, were excluded from analysis.

Detection of DNA fragmentation by DNA extraction and gel electrophoresis

DNA, separated from $1-3 \times 10^6$ nucleated hematopoietic cells in TRIZOL[®] reagent (Gibco BRL, USA), in 50 μ l of TE buffer was incubated

with RNase 1 μ g/ml at RT for 1 hour, incubated with proteinase K 100 μ g/ml at 37°C overnight, and finally reextracted with phenol, chloroform: isoamyl alcohol and precipitated with ethanol and resuspended in TE buffer. DNA samples were electrophoretically (Bio-Rad Laboratory, CA, USA) separated on 1.8% agarose gel containing 0.4 μ g/ml ethidium bromide at 80 volts for 1 hour (Gavrieli *et al*, 1992). The gel was then visualized by a UV (302 nm) transilluminator and photographed with a Polaroid camera (Fotodyne, USA).

Ultrastructural study

The pellets of BMNC 10^6 cells were fixed with 2.5% glutaraldehyde for at least 24 hours, then processed according to the standard method for TEM study (Rossi, 1997). Briefly, after washing with PBS, the BMNC were postfixed with 1% osmium tetroxide and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate, then observed under a transmission electron microscope (JEOL 1200 EX II Tokyo, Japan).

RESULTS

Light microscopic study

The overall history, clinical data and results of the patients and controls are shown in Table 1. The light microscopic studies, bone marrow differential counts and M:E ratios are shown in Table 2. The differential counts of 5 normal bone marrow smears were in the normal range compared to standard data (Rothstein, 1993). Patients Nos. 2, 3, 5 and 8 (with hyperparasitemia) had higher M:E ratios and hypocellular bone marrows. The numbers of erythroid cells in the bone marrow patients Nos. 2, 3, 5 and 8 were less than those in the normals (erythroid hypoplasia). There were increased numbers of eosinophilic, lymphocytic and plasmocytic progenitor cells in 6, 2, and 3, of 8 patients, respectively, as shown in Table 2. Immature forms in the granulocytic and lymphocytic series were also found in the patient group.

Examination of exposed phosphatidylserine on bone marrow cell membrane

The apoptotic (An^+/PI^-) BMNC and ($An^+/Gly A^+$) erythroid cells in the patients and the control

Table 1
The individual histories, clinical manifestations and results of patients on the day of bone marrow aspiration.

Variety (normal value)	Cerebral malaria patients (n=8)											Control (n=3)		
	No1	No2	No3	No4	No5	No6	No7	No8	No1	No2	No3			
Sex ^a	F	M	M	F	M	M	M	M	M	M	M	M		
Age (years)	14	17	21	16	29	52	25	22	39	35	25	25		
Duration of illness before admission (days)	3	4	3	4	UK	1	3	3	-	-	-	-		
Fever (°C)	39.7	39	36.6	39.5	39	37.1	37	37.8	37	37	37	37		
Jaundice	+	-	+	-	+	+	+	+	-	-	-	-		
Glascow coma score	6	5	6	5	5	5	5	4	4	4	4	4		
Duration of CM (days)	3	2	2	1	1	2	1	4	-	-	-	-		
Parasitemia/ μ l	1	203,600	350	5,200	201,400	821,500	196,500	139,920	-	-	-	-		
Parasite clearance time (hr)	18	45	35	24	46	64	47	56	-	-	-	-		
Hematological findings														
Hb (mg/dl) (M=13.5-18.0 g/dl, F=11.5-16.5 g/dl)	9.1	12.5	15.3	8	10.4	9.8	12.1	8.1	ND	ND	ND	ND		
Hematocrit (Hct) (M=40-54%, F=37-47%)	26	38	46	24	33	29	35	25	-	-	-	-		
Reticulocyte count (0.5-2%)	ND	1	0.5	0.7	0.2	0.4	0.3	6.4	-	-	-	-		
Direct bilirubin (0.3-0.52 mg/dl)	1.56	0.4	6.05	0.29	3	0.8	2.2	10.9	-	-	-	-		
Total bilirubin (0.10-1.2 mg/dl)	3.91	1.8	1.3	1.22	7.8	4.2	4.5	14	-	-	-	-		
Liver function														
SGOT (7-40 U/l)	155	130	140	43	100	70	57	264	ND	ND	ND	ND		
SGPT (7-40 U/l)	52	81	55	27	58	30	28	330	ND	ND	ND	ND		
Renal function														
creatinine (0.6-1.4 mg/dl)														
BUN (5-19 mg/dl)	48.8	26.7	12.7	9	44	33.8	54.1	58.2	-	-	-	-		
Total protein (6.3-8 g/dl)	106	7.2	7.05	6.05	48	48	38	60	-	-	-	-		
Albumin (3.5-5.0 g/dl)	2.6	4.5	4.1	3.5	2.3	2.8	2.5	2.5	-	-	-	-		
EPO (2.6-34)	74.9	14.8	3.8	35.9	125	>200	151	59	8.2	3.8	4.5	4.5		
Bone marrow findings														
M:E (1.5-3.3)	2.66	7.04	5.77	1.99	4.25	2.66	1.86	6.19	3.17	1.5	1.5	1.5		
% apoptosis in BMNC	41.96	13.65	23.81	22.36	21.6	ND	37.96	4.38	62.62	56.23	56.23	56.23		
	(-43)	(-38)	(-30)	(-17)	(-53)	(-45)	(-29)	(-10)	(-46)	(-37)	(-37)	(-37)		
% apoptosis in erythroid lineage	54.88	12.81	24.72	34.66	45.54	9.84	57.31	8.4	53.49	74.55	74.55	74.55		
	(-25)	(-14)	(-53)	(-29)	(-60)	(-47)	(-43)	(-9)	(-48)	(-46)	(-46)	(-46)		

Note: ^asex M= male, F= female; ND= not done, + = positive; determined from deficiency in red blood cells, hemoglobin, or total volume; In males: RBC <4.5 x 10¹²/l, Hb <13.5 g/dl, Hct <40.5%. In females: RBC <3.83 x 10¹²/l, Hb <11.5 g/dl, Hct <34.5% (Hoffbrand and Pettit, 1993); CM=cerebral malaria, SGOT=serum glutamic oxaloacetic transferase, SGPT=serum glutamic aspartic transferase, BUN=blood urea nitrogen, EPO=erythropoietin, M:E ratio = myeloid:erythroid lineage ratio.

Table 2
Differential counts and myeloid:erythroid (M:E) ratios of bone marrow smears in severe falciparum patients and normal volunteers.

Cell types	Percentages for differential bone marrow smear counting												
	Cerebral malaria patients					Normal volunteers							
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 1	No. 2	No. 3	No. 4	No. 5
Erythroid	21.48	9.51	7.26	9.8	5.62	1.12	4.7	6.27	4.58	4	3.01	1.76	2.17
Myeloblast	1.76	2.07	2.35	1.2	1.75	0.99	0.98	0.76	0.8	2.2	0.94	1.37	0.98
Neutrophilic	54.1	72.87	40.59	32.2	41.56	35.21	43.84	57.9	40.28	48.6	31.16	55.49	52.56
Eosinophilic	21.48	9.51	7.26	9.8	5.62	1.12	4.7	6.27	4.58	4	3.01	1.76	2.17
Basophilic, mast cell	0	0	0.2	0	0	0.2	0	0	0	0	0	0	0
Lymphocyte	11.91	19.17	32.35	19.6	23.3	29.84	13.11	21.71	19.84	20.04	23.83	7.06	9.84
Plasma cell	0.78	29.34	1.96	3.6	3.88	0.99	2.74	0	0.8	1.6	0.56	1.57	1.38
Monocyte	0.2	0.35	0.78	1.4	0.19	0.2	0	0	0.2	1.6	1.3	0	0.39
Macrophage	0	0	1.76	0	0	3.36	0.98	0	0	0	0	0	0
Megakaryocyte	0	0	0.59	0	0.19	0	0	0	0	0.2	0	0.2	0.2
M:E ratio	2.66	7.04	5.77	1.99	4.25	2.66	1.86	6.19	1.91	3.17	1.5	1.95	1.97

groups were detected by flow cytometry (Fig 1). In our study, we could not demonstrate An⁺/PI⁻ or An⁺/PI⁺ erythroid cells because of the limitations of our FACS to differentiate between PE and PI fluorescence. However, the numbers of total An⁺ BMNC were not significantly different from An⁺/PI⁻ BMNC (data not shown) and the numbers of An⁺ BMNC were associated with those in the erythroid population ($r^2=0.667$, $p=0.007$). Therefore, we assume the number of necrotic erythroid cells was not interfering the real numbers of apoptotic erythroid cells. The percentages of apoptotic BMNC and erythroid cells varied from low to high, as shown in Table 1.

DNA fragmentation

By gel electrophoresis, there was no DNA ladder or DNA fragments demonstrated (180-200 bps) in either the patients or the controls. The DNA size was larger than 1,500 bps. DNA fragmentation could not be found using the TUNEL kit to localize the fragment ends on the bone marrow smears of either the patients or the controls, although we could demonstrate it clearly in positive controls (data not shown).

DISCUSSION

In this study the morphological features of bone marrow cells from severe malaria patients corresponded with those observed in previous studies (Srichaikul *et al*, 1967; Abdalla *et al*, 1980) including hypocellular bone marrow, increases in eosinophilic and lymphocytic lineage cells. The total numbers of myeloid progenitor cells changed slightly but were still in the normal range. Therefore, the higher M:E ratio in four of eight patients was the consequence of erythroid hypoplasia.

We found that detection of apoptotic cells in bone marrow could be performed efficiently by determination of PS exposure using flow cytometry, but not DNA fragmentation using either the *in situ* TUNEL method or gel electrophoresis. DNA fragmentation (180-200 bp) is reported as an obvious characteristic of the apoptotic process at the end stage (Gavrieli *et al*, 1992). The lack of DNA fragmentation was in agreement with the finding that DNA laddering

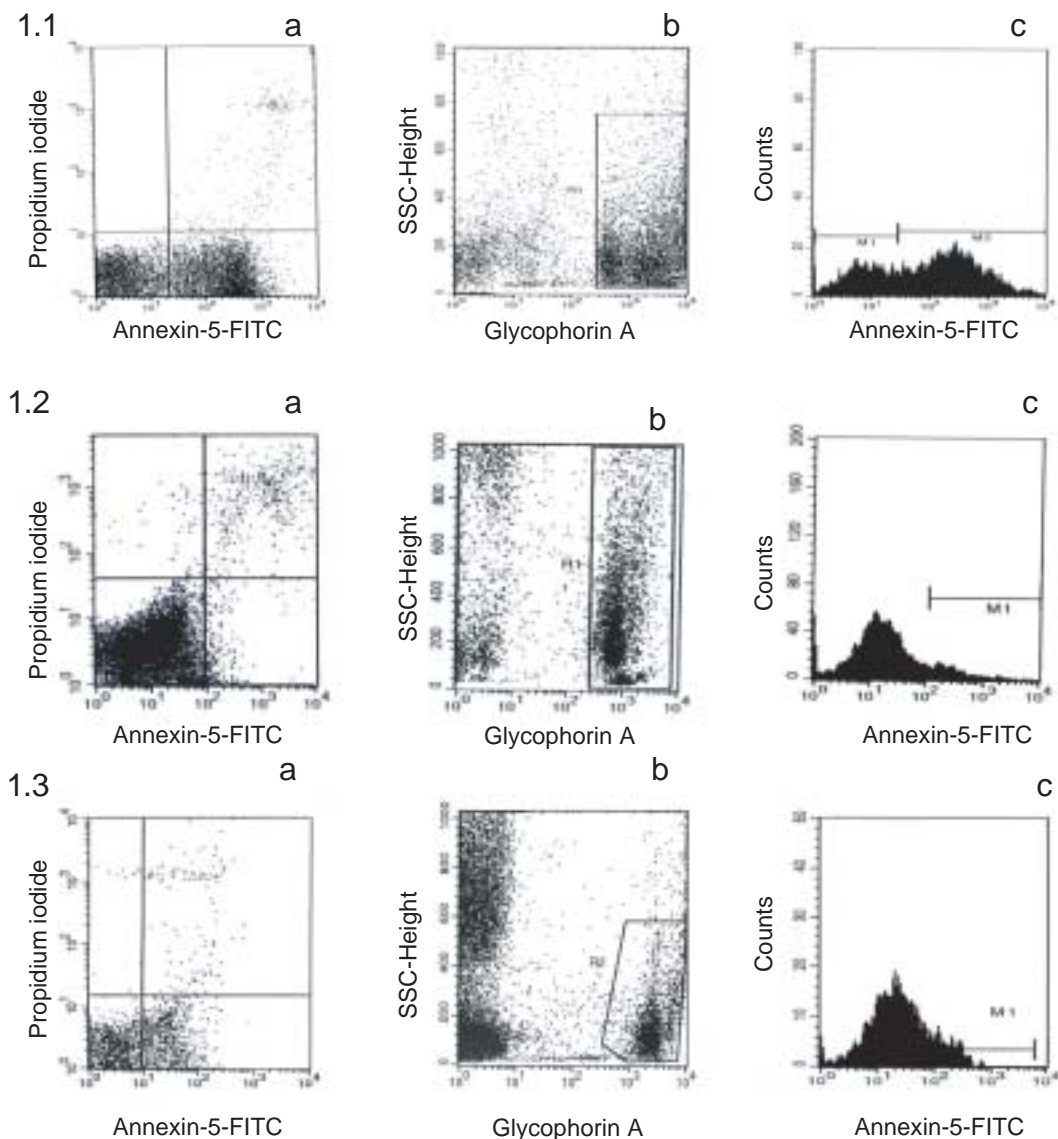


Fig 1—Phosphatidylserine (PS) exposure on cell surface of BMNC and erythroid cells detected by FACS. The BMNC of normal volunteer No.3 (1.1) and coma falciparum malaria patients (1.2 and 1.3) were surfaced stained for PS with annexin-FITC (a and c) and glycophorin A with anti-glycophorin A-PE (b and c) and stained for necrotic cells with propidium iodide (a). The quadrant markers were set using unlabeled cells to facilitate recognition of positive and negative cell population. The apoptotic (An+/PI-) BMNC were located in lower right quadrant that show negative PI and annexin V positive staining (a). The apoptotic (An+/Gly+) erythroid cells were gated in areas of annexin V and glycophorin A and positive cell population (c).

was a process step, which is not required for normal apoptosis (Oberhammer *et al*, 1993; Samaha *et al*, 1998; Gooch and Yee, 1999). For example, only some specific strains of the MCF-7 breast cancer cell line could undergo DNA fragmentation at the end of apoptosis (Gooch and

Yee, 1999). Our present findings were confirmed by ultrastructural investigation of the BMNC that showed only condensation of apoptotic cells. However, failure in examining DNA fragmentation may be because the apoptotic bodies from DNA fragmentation were engulfed immediately

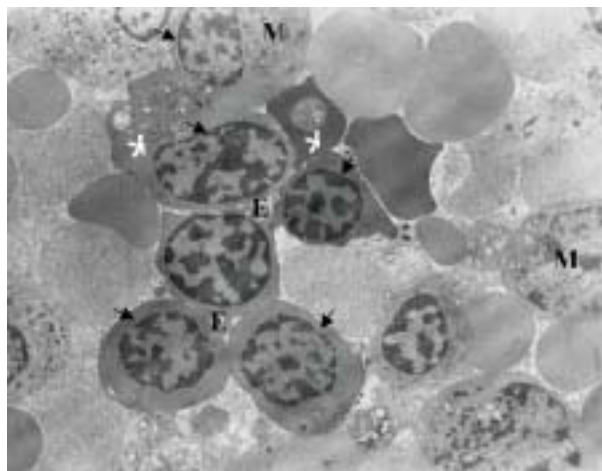


Fig 2—Ultramicrograph of BMNC from severe falciparum malaria patient No. 2: both myeloid and erythroid progenitor cells show only nuclear condensation (arrow) but not DNA fragmentation or apoptotic bodies (x 2,000 magnification). M= myeloid progenitor cells, E = erythroid progenitor cells and IRBC = infected RBC (*).

by resident macrophages (Kerr *et al*, 1994).

Apoptosis has been suggested as the active physiological form of programmed cell death that played an important role in development, regulation and maintenance of adult tissue homeostasis, including hematopoiesis. Under anemic conditions some factors may regulate erythropoiesis in bone marrow cells to maintain an optimal number of erythroid cells. As shown in Table 1, the apoptotic cells in BMNC and erythroid lineages, parameters for indicating anemia (eg M:E ratio, Hb, Hct, reticulocyte count) and levels of erythropoietin that stimulate erythropoiesis in each patient and the control, could not be interpreted in the same way to explain the role of bone marrow cell apoptosis, particularly in the erythroid lineage in falciparum anemia. These factors could not be clarified in our study. Severe falciparum patient No. 1 had a high number of apoptotic erythroid cells and a high level of EPO, but the M:E ratio was normal. This should indicate that the erythropoiesis and destruction of the erythroid cells is balanced but there may be other factors involved in the mechanism of anemia in patient No. 1. Patient Nos. 5 and 7 had high percentages of apoptotic

erythroid cells, high M:E ratios, high levels of EPO, and low percentages of reticulocyte counts. It is possible that these patients might have had anemia by ineffective erythropoiesis and increased destruction of the erythroid line by apoptosis, whereas patient Nos. 2, 3, and 4, with increased M:E ratios showed anemia, had low percentages of apoptotic erythroid cells, normal reticulocyte counts, and approximately normal EPO. This may indicate that the patient Nos. 2, 3, and 4 might have normal effective erythropoiesis, although they had anemia. Patients Nos. 6 and 8 had very low destruction of erythroid cells by apoptosis and high levels of EPO, showing effective stimulation of erythropoiesis but unsuccessful erythropoiesis in patient No. 6 (reticulocyte count slightly lower than normal).

Anemia in severe malaria can develop from different etiologies, such as: 1) ineffective erythropoiesis and dyserythropoiesis in the bone marrow (Srichaikul *et al*, 1969; Abdalla *et al*, 1980; Wickramasinghe *et al*, 1987); 2) an increase in erythroid progenitor cell destruction (Srichaikul *et al*, 1969). In this study, the percentage of apoptotic cells in the erythroid lineage of severe malaria was lower than the normals ($p > 0.05$); 3) hemolysis in peripheral blood (Phillips and Pasvol, 1992), all patients in our study had increased levels of indirect bilirubin; 4) rupture of parasitized red blood cells (Phillips and Pasvol, 1992); 5) a decrease in levels of erythropoietin (Zamai *et al*, 2000); 6) increased cytokines regulating erythropoiesis and other growth factors, such as TNF- α (Brown *et al*, 1999; Papadaki *et al*, 2002), IFN- γ (Dai and Krantz, 1999), IL-3, IL-10, stem cell factors (SCF) and erythropoietin (Weber-Nordt *et al*, 1996); and 7) the apoptotic process can be controlled by endogenous factors such as c-myc and p53 (Grand *et al*, 1995) or Fas ligand (Kern *et al*, 2000). However, our findings cannot confirm this suggestion.

In conclusion, this study demonstrated: 1) the appropriate method to determine apoptosis in bone marrow cells is the detection of PS exposure but not DNA fragmentation; 2) apoptosis in erythroid cell lines might not be the main cause, but related to a negative feed back

mechanism of erythropoiesis in anemic conditions during falciparum malaria. However, it is difficult to explain dynamic findings from only static information, from the small sample size of the bone marrow aspirates. Further studies of *in vitro* cultures of progenitor cells stimulated by *P. falciparum* or *ex vivo* studies with larger sample sizes and time to observe the bone marrow aspirates are needed to gain a better understanding of apoptosis regulating anemia in acute falciparum malaria.

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