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Ineffective Erythropoiesis in Acute Human P. Falciparum Malaria*

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Summary. An analysis of erythroblast cell kinetics utilizing quantitative ^{14}C-autoradiography has been performed in five cases of acute *Plasmodium falciparum* malaria prior to and, in four patients, 3 or 6 days after the onset of antimalarial therapy. Associated with no or only moderate anemia were changes of erythroblast morphology, a considerable shift in the frequency of red and white blood cell precursors in the bone marrow, and a reduced rate of erythroblast proliferation. There was a marked loss of polychromatic erythroblasts, which was smaller but still detectable during the therapeutic phase. The results provide some quantitative data on the extent of "parenchymal damage" of bone marrow and stress the impact of ineffective erythropoiesis and reduced rate of erythropoietic proliferation on the emergence of anemia in Plasmodium falciparum malaria.

Key words: Malaria – Anemia – Ineffective erythropoiesis – Proliferation kinetics – Quantitative autoradiography

Malaria-induced anemia is considered to be of complex origin [1, 11, 12]. Red cell destruction and phagocytosis [2–4, 14, 27], hypersplenism [13, 22], autoimmune mechanisms [10, 18, 20, 30], inhibition of red cell production and ineffective erythropoiesis [1, 5, 21, 23–26, 28, 29] have been investigated and traced with the occurrence of the anemia of Plasmodium falciparum malaria (PFM; malignant tertian malaria). More recently, Abdalla et al. [1] have pointed out that the causes may be different in the various stages of disease, such as acute, intermediate and chronic PFM. According to these authors dyserythropoiesis and erythroblast destruction is a feature predominantly of the chronic stage in which the anemia was found to be more profound. On the other hand, it may be due to technical reasons that ineffective erythropoiesis is difficult to demonstrate in the more acute stages. The reliability of ferro-

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kinetics as the classical method for this purpose is limited in this situation, since it depends on a number of samples taken over a period of time and thus requires steady conditions of erythropoiesis for roughly one week as a minimum. This is hardly possible since therapy will be instituted in these cases immediately upon diagnosis. Here the more tedious technique of quantitative ^{14}C-autoradiography [6] is superior, as using this method an analysis can be made on only one sample of bone marrow. The present study was performed utilizing this technique in five patients immediately before the onset of antimalarial therapy and repeating it in four patients three or six days after therapy had started. The results show a high degree of ineffective erythropoiesis confined to the polychromatic erythroblast compartment and a clear tendency for normalization after therapy had started.

Material and Methods

Six patients with PFM were randomly selected. They included non-immune Europeans as well as semi-immune persons from malaria-endemic countries. The laboratory investigations were performed immediately before, and on several days after the initiation of therapy. The diagnosis was based on the evaluation of thick blood film preparations stained by Giemsa solution, and of blood smears stained panoptically by the Pappenheim stain.

Hemoglobin concentration, red blood cell and platelet counts were determined by an electronic device (Coulter counter). Reticulocytes were counted after supravitally staining with brilliant cresyl blue solution. GOT, GPT, LDH, and bilirubin were measured by routine laboratory methods[1]. Haptoglobin levels were determined by radial immuno-diffusion (Mancini technique).

After informed consent had been obtained from the patients, bone marrow aspiration was performed. The bone marrow films were analyzed after Pappenheim panoptical staining. An aliquot of the aspirate was provided for cell kinetic studies in five cases prior to therapy and in four cases 3 or 6 days after the onset of therapy. Cells were immediately suspended in TC medium 199 and passed through a glass pore filter in order to obtain a single cell suspension; 5-fluorodeoxyuridine was added to block the pathway of endogenous synthesis of thymidine monophosphate. Deoxycytidine was added to prevent a depletion of the deoxycytidine triphosphate pool following the addition of 10^{-5}M thymidine (TdR). The suspension was incubated with ^{14}C-TdR (specific activity 56 mCi/mmol), and smears were prepared and provided with ^{14}C-standard sources. After autoradiographic processing using AR.10 stripping film the grains were evaluated by an automatic grain counter and converted into values of radioactivity and TdR incorporation per cell by virtue of the standard blackening and the specific activity of the ^{14}C-TdR applied. Under the chosen conditions of in vitro incubation the TdR incorporation rate equals the DNA synthesis rate from which the DNA synthesis time (t_s) in a morphologically defined bone marrow cell compartment can be calculated. Further details of this technique have been described elsewhere [6, 9]. Parallel to ^{14}C-TdR incubation an aliquot of the suspension was incubated with ^{3}H-TdR for the determination of labeling indices in the individual morphological compartments.

Relative cell production rates in the bone marrow were calculated by counting the number of labeled cells in a morphological compartment per 1,000 nucleated bone marrow cells and dividing this figure by t_s of the compartment. In normal erythropoiesis the ratio of relative cell production rates amounted to 0.88:2.0:4.77 for proerythroblasts: basophilic erythroblasts: polychromatic erythroblasts [6]. This was interpreted to mean that one proerythroblast divides into two basophilic and each of these into two polychromatic erythroblasts. Some of the polychromatic erythroblasts pass a second cell cycle before entering the maturation compartment of orthochromatic erythroblasts. The validity and limitations of this interpretation based on a

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sequential model of erythropoiesis have been amply investigated [6, 17]. While a precise indication of the number of erythroblast divisions cannot be given due to the assumptions inherent in the sequential model, gross deviations from the normal ratio have been shown to be meaningful and to conform to established criteria of deranged erythropoiesis, especially in the case of ineffective erythropoiesis. If, as an example, in an established case of dyserythropoiesis a ratio of relative cell production rates of 1:1:1 were found instead of 0.88:2.0:4.77, this could be taken to mean that approximately 50% of the cell divisions are missing in the basophilic compartment, and somewhat more than 50% are lacking at the polychromatic stage. The calculation of cell loss is therefore as follows: r is assumed to be the quotient of normal cell production P of compartment a over b ($r = P_a/P_b$). The fractional cell loss, L_b, in compartment b is derived from the pathological production rates p_a and p_b in compartments a and b according to $L_b = 1\text{-}r \cdot p_b/p_a$. This calculation implies that there is no cell loss in normal erythropoiesis. In previous studies literature has been reviewed indicating some minor amount of normally occurring ineffective erythropoiesis [6, 7] which, however, can be disregarded for the purpose of this study, which sets out to find gross deviations from the normal state.

Treatment was initiated in all patients immediately after the diagnosis was established by administering chloroquinediphosphate at standard dosages (1.5–1.8 g within 3 days). The drug was administered either by the oral route, or parenterally in the case of severe malaria infection.

Results

The laboratory data are summarized in Table 1. According to the hemoglobin values, all but one patients are to be ranked among the acute cases as categorized by Abdalla et al. [1], who determined mean hemoglobin values of 9.4 g/dl for the acute and 5.1 g/dl for the intermediate group at presentation. Conversely, all but one of our patients are to be allocated to Abdalla's intermediate group if parasitemia is taken as the criterion. Regardless of these inconsistencies with the categorization as proposed by Abdalla et al. [1], it appears justified to regard our group of patients as belonging to the acute rather than chronic forms. This conforms to the individual case histories, since there was a delay of only 4–12 days between the onset of clinical signs of malaria and presentation at the hospital. Thus, malaria infection dated back between 10 and 20 days. Table 1 further shows that only one patient (T.J.) was severely anemic at the time of presentation and that in all but one patients the E/G ratio was already considerably shifted.

In all cases, although to a variable extent, morphological equivalents of dyserythropoiesis were present (Fig. 1). These include nuclear fragments, internuclear bridges, multinuclearity and irregular nuclear shapes, karyorrhexis and, finally, erythroblastophagocytosis (Fig. 2).

Five cases were studied by means of quantitative ^{14}C-autoradiography prior to the administration of antimalarial therapy. Three of them were reinvestigated 6 days after therapy had been started, and another patient (E.G.) was studied 3 days after institution of treatment. The four patients treated were evaluated as one group. Prior to treatment (Table 2) all erythroblast compartments showed a significant reduction of the labeling index and a significant prolongation of the DNA synthesis time. In the group under treatment these changes were still present, although to a lesser extent and in most cases with no further statistical significance.

The relative rates of cell production (Table 3) show a highly significant deficit ($p < 0.001$) in the compartment of polychromatic erythroblasts of the pretreatment

Table 1. Laboratory findings of patients studied for erythroblast kinetics

Patients	K. H.		H. R.		B. M.		T. J.	H. G.	E. G.	
Days of treatment	0	6	0	6	0	6	0	0	0	3
Hemoglobin g/dl	10.0	9.1	11.6	12.6	15.2	13.5	7.6	14.7	10.9	7.8
Reticulocytes $\times 10^3/\mu l$	170	280	10	40	10	40	110	20	70	10
LDH U/l	283	220	700	213	574	238	1112	932	653	945
SGOT U/l	25	16	75	22	101	77	41	164	27	36
SGPT U/l	29	23	67	34	109	140	38	190	20	30
Bilirubin:										
total mg/dl	0.8	0.8	1.2	0.6	4.0	1.6	3.3	3.6	11.2	16.9
indirect mg/dl					1.1		1.9		2.1	6.9
Haptoglobin mg/dl	220	36			532	300[a]	<10	30	<10	<10
Parasitized erythrocytes %	3.9	0	*	0	7.2	0	3.3	4.6	11.6	2.7
Hemoglobin in serum mg/dl		4.5			8.85	2.8	20.0	4.2	3.75	7.25
E/G ratio	1:0.9	1:1.1	1:1.8	1:1.8	1:2.4	1:0.9	1:0.7	1:3.3	1:1.6	1:1.2

[a] M. Bechterew

* Thick film showed only few parasites

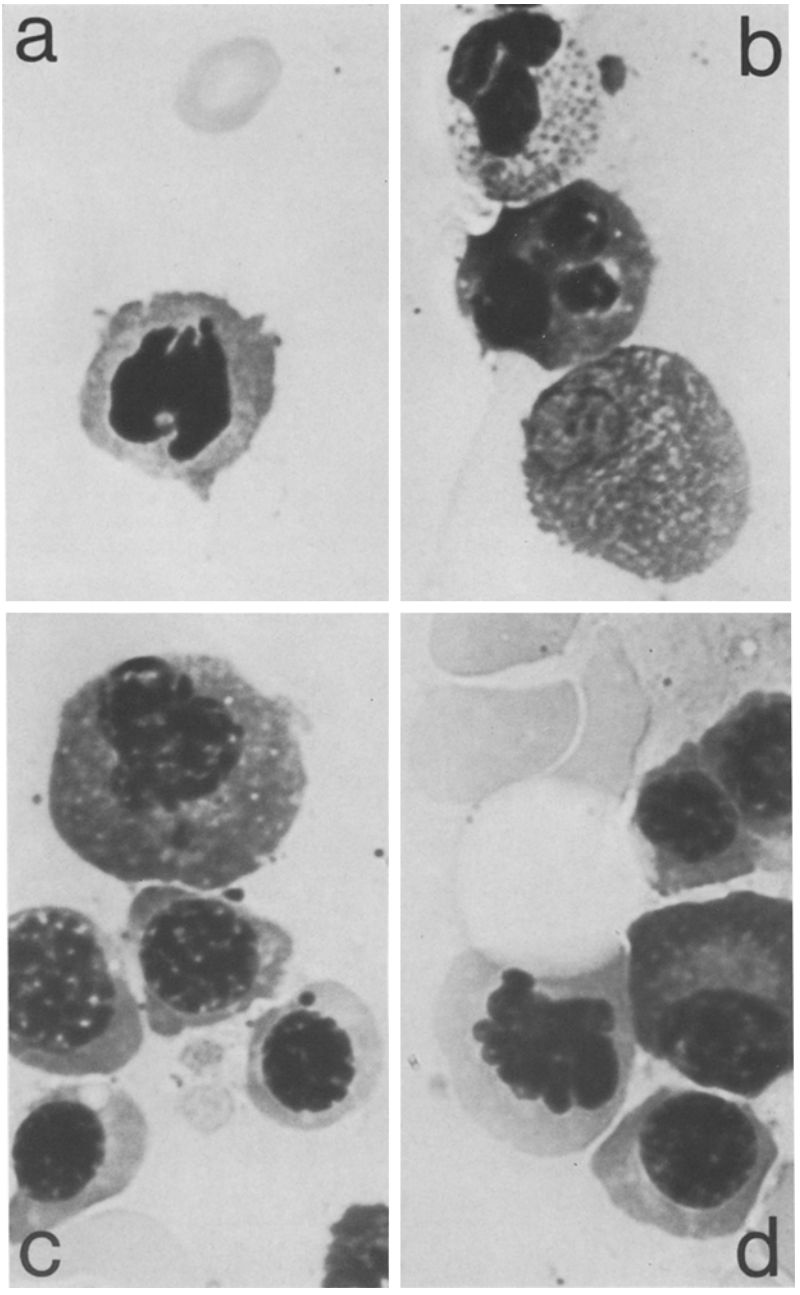

Fig. 1. Dyserythropoietic features in the bone marrow of patients with Plasmodium falciparum malaria. **a** Karyorrhexis; **b, c** and **d** different types of nuclear fragmentation

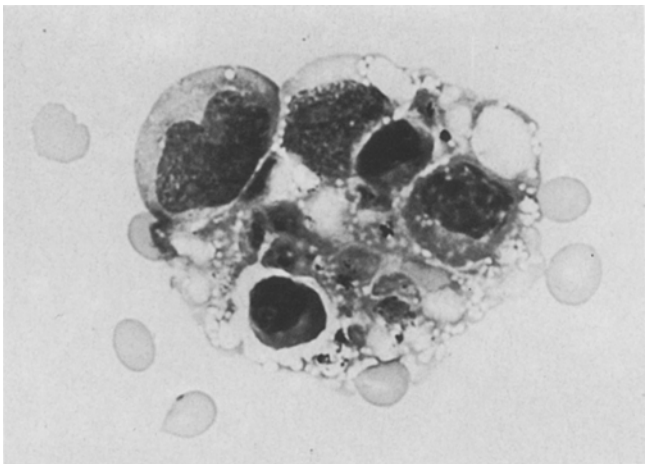

Fig. 2. Erythroblastophagocytosis and erythrophagocytosis in a bone marrow macrophage in Plasmodium falciparum malaria. The macrophage shows 3 erythroblasts and several erythrocytes some of which appear to be parasitized. The dark dots in the macrophage correspond to malaria pigment

Table 2. Labeling indices (Li) and duration of DNA synthesis (t_s) in erythroblast compartments prior to and after onset of therapy. Normal values were taken from Dörmer [6]. A statistical analysis was performed between the normal values and the data from malaria patients. Abbrevation: n.s., not significant. Mean values are complemented by standard deviations

	Cases	Proerythroblasts		Basophilic erythroblasts		Polychromatic erythroblasts	
		Li	t_s (h)	Li	t_s (h)	Li	t_s (h)
Normal (n = 5)		0.74 ±0.04	9.4 ±1.9	0.68 ±0.05	11.0 ±2.3	0.49 ±0.03	17.0 ±3.4
Pretreatment	K. H.	0.64	12.6	0.58	15.1	0.45	24.0
	H. R.	0.63	11.5	0.51	16.1	0.47	22.4
	B. M.	0.64	10.8	0.60	13.5	0.31	24.6
	H. G.	0.76	12.2	0.50	15.3	0.20	19.5
	T. J.	0.68	15.3	0.58	22.7	0.32	28.3
		0.67 ±0.05	12.5 ±1.5	0.55 ±0.04	16.5 ±3.2	0.35 ±0.10	23.8 ±2.9
p (*t*-test)		<0.05	<0.05	<0.002	<0.02	<0.02	<0.01
After onset of therapy	K. H.	0.59	10.9	0.55	17.3	0.32	28.2
	H. R.	0.62	10.4	0.60	15.6	0.41	24.5
	B. M.	0.67	13.7	0.62	14.3	0.36	19.6
	E. G.	0.84	8.5	0.78	11.1	0.47	13.5
		0.68 ±0.10	10.9 ±1.9	0.64 ±0.09	14.6 ±2.3	0.39 ±0.06	21.5 ±5.5
p (*t*-test)		n.s.	n.s.	n.s.	<0.05	<0.02	n.s.

Table 3. Ratio of realtive rates of cell production in the erythroblast compartments. The values are normalized to give a figure of 2.0 for the compartment of basophilic erythroblasts. Cell loss is calculated as the per cent deficit of cell production in the compartment of polychromatic erythroblasts. Normal values were taken from Dörmer [6]. Mean values are complemented by standard deviations

	Cases	Proerythro-blasts	Basoph. erythr.	Polychromat. erythrobl.	Cell loss %
Normal (n = 5)		0.88 ± 0.21	2.0	4.77 ± 0.82	0
Pretreatment	K. H.	0.88	2.0	2.27	52
	H. R.	1.1	2.0	3.3	31
B. M.	B. M.	1.1	2.0	2.1	56
	H. G.	0.9	2.0	1.7	64
	T. J.	1.1	2.0	2.2	54
		1.02 ± 0.1	2.0	2.31 ± 0.53	51.4 ± 11.0
After onset of therapy	K. H.	0.93	2.0	2.4	50
	H. R.	1.50	2.0	3.8	20
	B. M.	0.80	2.0	2.6	45
	E. G.	1.10	2.0	3.4	29
		1.08 ± 0.26	2.0	3.05 ± 0.57	36.0 ± 12.1

group. The deficit in this compartment decreases after therapy has been started. The calculated loss of polychromatic erythroblasts amounts to 51.4% before, and 36% after administration of antimalarial therapy. It is worth noting that in no instance is there a change in the ratio of relative cell production rates between proerythroblasts and basophilic erythroblasts.

Discussion

In our cases of acute PFM infection significant changes in the bone marrow have been observed whereas profound anemia was present only in one patient. These changes include erythroblast morphology, cell distribution (E/G ratio), and erythroblast kinetics. The labeling index was reduced and the duration of DNA synthesis prolonged. Both these factors indicate a reduction in the erythropoietic cell flux equivalent to an accumulation of cells. As far as the changed E/G ratio reflects erythroblast hyperplasia as described by others [5, 16], this change must be attributed at least in part to the accumulation of erythroid cells and is not equivalent to an increase in erythroblast production.

The ratios of relative cell production rates in the different erythroblast compartments show a high deficit or loss at the stage of polychromatic erythroblasts. Several mechanisms may conceivably account for this phenomenon:

1. It may be argued that after malarial infection erythropoiesis is no longer in a steady state. This view, however, is not consistent with the completely unchanged ratio between proerythroblasts and basophilic erythroblasts. In all of the cases studied

one to two weeks had elapsed between the first symptoms of malaria and the admission to hospital. The conditions of the present group of patients were therefore by no means comparable to those seen after acute loss of blood. In fact, two patients had no anemia at all upon presentation and showed the same deficit of polychromatic erythroblast production.

2. The deficit in the polychromatic erythroblast production rate may be due to skipping of this stage of erythropoietic differentiation. If this were true, 50% of the basophilic erythroblasts would attain the appearance of orthochromatic erythroblasts immediately after mitosis. We should then find a large number of cells presenting the nuclear morphology of basophilic erythroblasts but associated with a degree of hemoglobinization typical of polychromatic erythroblasts. Alternatively, a large number of orthochromatic erythroblasts exhibiting a degree of hemoglobinization typical of early polychromatic erythroblasts should be expected. Neither of these cell types has been observed on a large scale in any of the cases studied.

3. Polychromatic erythroblasts may undergo intramedullary cell death. This explanation tallies with the morphological features of dyserythropoiesis observed in the present study which have also been described by others at the light and electron microscopic level [1], and of erythroblastophagocytosis (Fig. 2). Further, it is in accordance with in vivo and in vitro ferrokinetic studies in human falciparum malaria [5, 24, 25, 29]. The latter explanation is also in keeping with our own observation and that of others [1, 15, 19, 26] suggesting an inadequacy of the reticulocyte response. Even six days after therapy has been started erythroblast kinetics have not yet reverted to the normal rate and there is still indication of cell loss. This easily explains why in some instances reticulocytosis shows an unexpected delay in response to antimalarial therapy (M. Dietrich, personal observation).

The cell-kinetic status of erythropoiesis in the cases studied is comparable to some extent with the observations made in thalassemia major [7] and acute myeloid leukemia [9]. In thalassemia major the rate of erythroblast proliferation is also reduced; however, these cases show intramedullary cell death from basophilic erythroblasts onward and the surviving proliferative erythroblast fraction is in the order of 20%. Erythropoiesis in acute leukemia, on the other hand, does not show a reduced rate of proliferation. However, the percentage of cell loss in the proliferative pool is also in the order of 80%. The question may be raised whether the cell-kinetic changes observed in PFM are specifically due to the action of the parasites which have never been observed within the erythroblasts. While there is no observation supporting this view, comparable but less extensive changes found in other types of infectious diseases [8] favor the assumption of an indirect effect on erythroblasts in malarial infections, which awaits further characterization. With regard to lesions of other organs in PFM, such as the liver, the quantitative data of erythroblast destruction presented herein would suggest the qualification of bone marrow involvement in this type of infection as a "parenchymal damage".

In a recent analysis of the erythroblast cell cycle distribution in PFM Wickramasinghe et al. [28] have observed a consistent increase in the G2 fraction which was more pronounced in the chronic than in two acute cases and associated in the chronic cases with the appearance of some U-cells. These are cells in the S-phase not incorporating ^{3}H-TdR. The increase in the G2 fraction was explained by a delay in the proliferative activity which is in agreement with the present kinetic study. Regar-

ding the occurrence of U-cells in the chronic cases, the authors concluded in accordance with a previous study of the same group [1] that the anemia in chronic malaria is primarily caused by ineffective erythropoiesis whereas in acute malaria it is predominantly caused by peripheral hemolysis. Our data, however, point to the significance of ineffective erythropoiesis in the acute PFM also. In four of the six cases no or only a moderate anemia was associated with a high degree of intramedullary cell death. Ineffective erythropoiesis was severe enough to be considered a significant factor causing the anemia with ongoing disease. Since it was shown by Abdalla et al. [1] that morphological indications of dyserythropoiesis increase from the acute towards the chronic forms, it is easily conceivable that intramedullary cell death is even more pronounced in chronic malaria.

This study has only dealt with the events in the bone marrow of acute PFM infections and does not consider the contribution of hemolysis to the actual degree of anemia. A quantification of the various factors, namely ineffective erythropoiesis, red cell destruction, reduction of proliferative activity and increase in erythroid cell mass, with regard to their contribution to the emergence of the anemia, is therefore impossible. This contribution may vary with the nutritional state of the patients, their immune status, the phase of the disease and the degree of parasite infestation.

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