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Red Cell Disorders

Hemozoin- and 4-hydroxynonenal-mediated inhibition of erythropoiesis. Possible role in malarial dyserythropoiesis and anemia

Malarial anemia involves destruction of parasitized and non-parasitized red blood cells and dyserythropoiesis. Malarial pigment, hemozoin (HZ), is possibly implicated in dyserythropoiesis. We show that supernatants of HZ and HZ-fed-monocytes, and 4-hydroxynonenal generated by them, inhibited progenitor growth.

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Malarial anemia involves destruction of parasitized and non-parasitized red blood cells (RBC), and dyserythropoiesis.[1-3] Dyserythropoiesis is common and severe, but poorly understood.[1-3] Malarial pigment, hemozoin, (HZ) is possibly implicated in dyserythropoiesis. First, HZ-containing trophozoites and schizonts as well as HZ and HZ-containing macrophages are abundantly present in the bone-marrow of malaria patients.[2] Secondly, HZ and HZ-laden monocyte/macrophages generate inhibitory lipid-peroxidation derivatives.[4,5] Lastly, HZ-laden phagocytes produce potentially inhibitory cytokines.[6]

We show here that supernatants of HZ and HZ-fed monocytes inhibit erythroid-progenitor growth. Inhibition was reproduced by 4-hydroxynonenal (4-HNE), a final product of lipid-peroxidation generated by HZ and present in supernatants.[5] These observations indicate that toxic molecules generated by HZ or HZ-fed monocytes may play a role in malaria dyserythropoiesis.

Trophozoites were isolated from *P. falciparum* cultures (FCR-3 strain, mycoplasma-free) and native HZ prepared as described elsewhere[4,5] and supernatants of HZ/delipidized-HZ were obtained.[4] Anti-D IgG-opsonized RBC, serum-opsonized trophozoites, HZ, or latex beads were fed to adherent human monocytes.[4] After removal of non-ingested meals, monocytes were reincubated at 37°C for a further 6 h before the conditioned medium was harvested, centrifuged, filtered, and added to the progenitor culture medium (see below and legend to Table 1 for details).

BFU-E/CFU-E colonies were obtained from low-density cord or bone-marrow cells cultured as previously described.[7,8] When indicated, 0.1 mL HZ/monocyte-supernatant was added to the progenitor culture medium. Colonies were counted after 14 days (see legend to Table 1 for details). 4-HNE (Biomol, Berlin, Germany) was supplemented to the progenitor culture medium at 0.07-7.0 μM (final concentration). In some experiments, 4-HNE was supplemented with liposomes (12.6 μM phosphatidylcholine, 3.6 vM stearylamine and 1.8 μM cholesterol, final concentrations) and quantified in conditioned supernatants (see legend to Table 2 for details).[5] As shown in Table 1, HZ-supernatants inhibited BFU-E growth by 38±11% (p=0.006; n=16) when supernatant generated by HZ equivalent to 12.5 trophozoites/progenitor was added to the cultures. This compares to the ratio between progenitors and *nursing* macrophages in the erythroblastic islets. Supernatants of

Table 1. Inhibitory effect on erythroid progenitor growth of supernatant of native HZ and supernatants of monocytes fed with serum-opsonized HZ, anti-D IgG-opsonized RBC or serum-opsonized Latex.

Supernatant of	*BFU-E Percent inhibition*	*Significance of difference*
Native HZ	38±11 (n=16)	p<0.006, HZ vs control
Delipidized HZ	13±2.2 (n=9)	p<0.001, HZ vs delipidized HZ; 0.17, delipidized HZ vs control
Monocytes fed HZ	48±8.2 (n=10)	p<0.001 HZ vs control; p<0.005 HZ vs RBC; p<0.006 HZ vs latex
Monocytes fed RBC	2.1±0.4 (n=9)	p>0.5 RBC vs control
Monocytes fed Latex	11.2±2.3 (n=7)	p>0.5 Latex vs control

HZ was prepared from the trophozoite-enriched cultures (>95% trophozoites) by osmotic shock and 4 washes with ice-cold hypotonic phosphate buffer (10 mM potassium phosphate, 1 mM EDTA, 1 mM mannitol, pH 7.2). After the last wash, HZ was suspended in sterile PBS (approx. 20% wt/vol), flushed with nitrogen and frozen at -20°C under nitrogen. Before phagocytosis, HZ was opsonized with an equal volume of fresh human serum for 30 min at 37°C.[4] Frozen HZ suspension (see above) was thawed, homogenized by 10 passages through a fine syringe needle and further diluted with PBS. HZ was quantified according its heme content by measuring heme luminescence. Three hundred micrograms of HZ, corresponding to 50×10⁶ trophozoites were added to 3.7 mL PBS, pH 7.4 and incubated at 37°C for 6 h under agitation. At the end of incubation, HZ-conditioned supernatant was obtained by brief centrifugation (2 min at 12,000g) with an Eppendorf Microfuge, filtered through a sterile filter with a pore diameter of 0.1 μm to exclude debris, and added to the progenitor culture medium (see below). In some experiments, HZ supernatant was obtained from delipidized HZ, prepared as indicated.[4] BFU-E colonies from 10⁵ non-adherent, low density human bone-marrow cells were cultured without or with supernatants or RBC lysate equivalent to 12.5 trophozoites or RBCs per bone-marrow cell. Adherent human monocytes were fed ad libitum with: native HZ prepared from mycoplasma-free trophozoites; Anti-D IgG-opsonized RBC; serum-opsonized Latex beads (2.5% solids, diameter 0.105 μM, Sigma). Monocyte supernatant (0.1 mL) was obtained 24 h after start of phagocytosis of the respective meals and added to the progenitor culture medium. On average, each bone-marrow cell was supplemented with the supernatant produced by 0.2 monocytes. Control bone-marrow cells received 0.1mL medium. Triplicate dishes were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air and colonies were counted after 14 days. Data are expressed as percentage inhibition of growth compared to controls (mean±SD, n=10). Statistical comparisons were by Student's t test on paired samples. The mean (±SD) number of control BFU-E colonies was 123±32 (n=10).

Table 2. Inhibitory effect of 4-HNE on erythroid progenitor growth.

4-HNE concentration (μM, final concentration)	*0.07*	*0.7*	*7*
	Percent inhibition		
BFU-E (cord blood, n=3)	7±5 *p*=0.07	13±6.6 *p*=0.02	20±7.1 *p*=0.008
BFU-E (cord blood, n=2) + liposomes	5.3/0	30/21	100/93
BFU-E (bone-marrow)	6±0.1 n=3; *p*=0.01	19±1.7 n=5; *p*=0.01	30±2.2 n=4; *p*=0.01
CFU-E (cord blood, n=3)	5±2.9 *p*=0.04	25±10.7 *p*=0.017	38±13.6 *p*=0.009

4-HNE (final concentration, 0.07, 0.7 and 7.0 μM) was added as a single dose to plated precursors isolated from cord blood or bone-marrow immediately after their plating. Triplicate dishes were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air. Colonies were counted after 14 days. In some experiments, 4-HNE was supplemented with liposomes. Liposome stock suspension was prepared by sonicating, at 400 W for 5×10 s, a suspension containing 63 mM phosphatidylcholine from egg yolk, 18 mM stearylamine and 9 mM cholesterol (all Sigma) in PBS. Liposome stock suspension was further diluted and 4-HNE (0.07-7 μM, final concentration) was added to liposomes that contained 12.6 μM phosphatidylcholine, 3.6 μM stearylamine and 1.8 μM cholesterol (final concentrations). Data are expressed as percentage inhibition of growth compared to controls±SD. Statistical comparisons were made by Student's t test on paired samples. The significance of differences refers to the difference between treated samples and untreated controls.

delipidized HZ was significantly less effective. Supernatants of HZ-fed monocytes inhibited BFU-E growth by 48±8.2% ($p<0.001$; n=10), whereas supernatants of latex-fed or RBC-fed monocytes had no effect. In these experiments, each progenitor was incubated with the supernatant produced by 0.2 variously-fed monocytes.

HZ supernatants contained 0.7 μM 4-HNE, while ultracentrifugation at 200,000g for 10 h led to the isolation of a vesicle-containing pellet fraction enriched 4 to 10-fold with 4-HNE compared with non-centrifuged supernatants. Growth of BFU-E and CFU-E was inhibited dose-dependently at 4-HNE 0.07-7 μM (final concentration), and completely at 7 μM (final concentration) 4-HNE added with liposomes (Table 2).

Native HZ and trophozoites/schizonts, the natural meals of monocyte/macrophages during malaria attacks, contain large amounts of polyunsaturated fatty acids (PUFA).[4] These generate stable hydroxy-derivatives (HETEs, HODEs) and 4-HNE, found in high concentrations in isolated HZ and HZ-fed monocytes.[4,5] It is likely that 4-HNE produced and shed by isolated HZ, trophozoites, or HZ-fed macrophages could reach blood precursors differentiating in their proximity, concentrate in their membranes, and interfere with cellular development. Indeed, we show here that low-micromolar concentrations of 4-HNE added to erythroid cultures were capable of significantly inhibiting the growth of the erythroid cultures. The 4-HNE concentrations applied were in the same range as that found in supernatants of HZ (0.7-7 μM) and HZ-fed monocytes (approximately 20 μM 4-HNE, assuming a cell volume of 1000/fL, respectively, and intra/extra-cellular equilibrium).[5] Low-micromolar concentrations of 4-HNE are known to be cytotoxic, to inhibit DNA/RNA-synthesis and to induce apoptosis in several cell-types.[9]

Macrophages fed with HZ produce increased amounts of potentially inhibitory cytokines.[6] However, data in the literature appear to exclude that tumor necrosis factor-α, γ-interferon or interleukin-1β are host-derived inhibitors of erythropoiesis in malaria models.[10] The present data indicate that inhibition of erythroid growth is likely to be due to shedding of inhibitory molecules. One of those molecules was identified as 4-HNE, which reproduced the inhibition dose-dependently. Other still unidentified inhibitors produced by HZ and HZ-fed phagocytes may additionally contribute to dyserythropoiesis in malaria.

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Key words: malarial anemia, 4-hydroxynonenal, hemozoin, monocytes, phagocytosis.

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