High Oxygen Radical Production Is Associated with Fast Parasite Clearance in Children with *Plasmodium falciparum* Malaria

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It has been hypothesized that reactive oxygen intermediates (ROI) released by leukocytes play a major role in the immune response to many infectious agents. In the present study, the parasitologic and clinical courses of 75 Gabonese children with *Plasmodium falciparum* malaria were compared with the ability of their granulocytes to produce oxygen radicals. The luminol-dependent chemiluminescence in granulocyte suspensions for the children was measured without stimulation and after stimulation with phorbol-12-myristate-13-acetate, N-formyl-methionyl-leucyl-phenylalanine, or tumor necrosis factor. A significant association was found between fast parasite clearance time and high oxygen radical generation in both the unstimulated and stimulated granulocyte preparations. No correlation was found between fever clearance time and ROI generation. These findings suggest that ROI play a pivotal role in the immune response as a first line of defense against *P. falciparum* malaria.

Reactive oxygen intermediates (ROI), such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), hypochlorite (OCL^-), and singlet oxygen ($^1O_2$), that are released by polymorphonuclear leukocytes and mononuclear phagocytes play a major role in the nonspecific immune response against invading microorganisms [1]. Antigens of *Plasmodium falciparum* can activate blood phagocytes to produce ROI during malaria attacks [2] or, as shown in murine malaria [3, 4], during cerebral malaria. This radical production contributes to parasite death and pathology, including hemolysis. However, it is not known how big a role the production of ROI plays in determining the outcome of malaria infection in humans. Malaria-infected children treated with paracetamol as an antipyretic had a prolonged parasite clearance time (PCT), and it was hypothesized that reduced tumor necrosis factor (TNF) and ROI production were responsible [5].

Phagocytes produced ROI after experimental stimulation with different compounds, including the protein kinase C activator phorbol-12-myristate-13-acetate (PMA) [6], the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) [7], and the cytokine TNF [8]. Addition of a chemiluminescent compound, such as luminol, to cells, organelles, or acellular reactions generating ROI results in light emission, which can be quantified in both whole blood or isolated cells [9].

This study was done to determine whether the amount of oxygen radical production in children with *P. falciparum* malaria correlates with PCT and fever clearance time (FCT). To that end, we compared the stimulated and nonstimulated luminol-dependent chemiluminescence in granulocyte suspensions for 75 malaria-infected Gabonese children with the children's parasitologic and clinical courses.

Patients and Methods

The study took place at the Albert Schweitzer Hospital in Lambaréné, Gabon. Girls ($n = 48$) and boys ($n = 27$) between 8 months and 11 years of age (average, 46 months) presenting with *P. falciparum* malaria were enrolled. Exclusion criteria were signs of severe malaria or other acute infection and intake of antimalarial drugs within the preceding week. Children with other chronic diseases or homozygous for hemoglobin S were also excluded.

We obtained venous blood samples from each patient at admission, and then the children were treated with a single dose of sulphadoxine-pyrimethamine [10]. Every 12 h, rectal body temperature was measured, and Giemsa-stained thick blood smears were done to determine parasitemia until parasitologic and clinical cure. The median initial parasitemia was 12,500 parasites/μL (range, 1000–55,000/μL), and the mean ($\pm$SE) hemoglobin count was 106 ($\pm$0.2) g/L. Mean ($\pm$SE) FCT was 56 ($\pm$2.1) h, and mean PCT was 53 ($\pm$3.3) h.

Granulocyte stimulation. We used the density gradient separation method as previously described [11] to extract granulocytes. Equal parts of anticoagulated venous blood and PBS were mixed and added 2:1 to separation fluid (Isopaque ficoll; Sigma, Deisenhofen, Germany). After the separation fluid was centrifuged for 20

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Informed consent was obtained from the parents of the patients. Human experimental guidelines of the Declaration of Helsinki were followed. The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital.

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min at 600 *g,* mononuclear cells appeared as a band at the top and erythrocytes and granulocytes sedimented to the bottom of the tube. To separate granulocytes from erythrocytes, we suspended the cell pellet in PBS and centrifuged it for 7 min at 600 *g*. The supernatant was removed, and the pellet was reconstituted with PBS and 6% dextran solution (10% of the total volume). The supernatant containing the granulocytes was collected after 20 min of sedimentation, and contaminating erythrocytes were lysed by use of 0.17 *M* NH_4Cl. The cells were washed twice in KRPG (Krebs Ringer phosphate glucose) medium and brought to a concentration of 10^6 cells/mL. Four polypropylene tubes containing 1 mL of the cell suspension and 11 μM luminol each were supplemented with PMA (end concentration 600 n*M*), FMLP (250 n*M*), recombinant human TNF (600 n*M*), and KRPG medium, respectively.

Measurement and calculation of chemiluminescence. Chemiluminescence was measured in kRLU (kilo relative light units) by use of a luminometer (Lumat LB 9501-0; Berthold, Wildbad, Germany). Measurements were done every 2 min up to 10 min and then every 5 min until ROI production decreased. Integrals of the chemiluminescence were calculated at 30-min intervals (stimulation with PMA and FMLP) or at 70-min intervals (stimulation with TNF).

Calculation of FCT and PCT. PCT and FCT were calculated as time from admission to time of first negative thick blood smear and first rectal body temperature <37.5°C, respectively.

Statistical analysis. Spearman's rank correlation was used to compare continuous variables. Patients were divided into subgroups according to whether their PCTs or FCTs were above or below the mean (PCT, 53 h; FCT, 56 h), and the Mann-Whitney *U* test was performed. We used the Wilcoxon signed rank test to compare paired data. $P < .05$ was considered statistically significant.

Results

In FMLP- and PMA-stimulated suspensions, chemiluminescence increased over 30 min from a basal level of 177 kRLU to 834 ($P < .001$) and 3474 ($P < .001$) kRLU, respectively. When TNF was used as a stimulus, chemiluminescence increased from 362 to 566 kRLU in 70 min ($P < .001$).

In nonstimulated suspensions of separated granulocytes and in TNF-stimulated suspensions, significantly higher chemiluminescence was measured in children with fast PCTs. After stimulation with FMLP, granulocytes from these children also showed increased chemiluminescence compared with granulocytes from children who had slower PCTs; however, the difference was not significant ($P = .07$). Both subgroups showed almost no difference in chemiluminescence after stimulation with PMA (table 1). Furthermore, we found a significant reciprocal correlation between PCT and unstimulated chemiluminescence of granulocytes ($\rho = -0.31$; $P = .02$) and between PCT and stimulation with FMLP ($\rho = -0.26$; $P = .03$) and TNF ($\rho = -0.28$; $P = .02$). No correlation could be detected after stimulation with PMA. There was also no connection between FCT and chemiluminescence.

Table 1. Chemiluminescence (in kilo reactive light units) in stimulated and unstimulated granulocyte suspensions for malaria-infected children with different parasite clearance times (PCT).

Medium or stimulant	PCT >53 h	PCT <53 h	P[a]
Medium[b]	113 (86)	250 (151)	.02
FMLP[b]	710 (460)	848 (567)	.07
PMA[b]	3420 (1217)	3556 (1851)	.39
Medium[c]	232 (158)	552 (359)	.02
TNF[c]	378 (276)	944 (558)	.01

NOTE. Data are median (median absolute deviation). FMLP = N-formyl-methionyl-leucyl-phenylalanine; PMA = phorbol-12-myristate-13-acetate; TNF = tumor necrosis factor.

[a] Mann-Whitney *U* test.

[b] 30-min interval.

[c] 70-min interval.

Discussion

In this study, we examined the association between oxygen radical production and PCT or FCT in children with *P. falciparum* malaria. Chemiluminescence was determined in pure granulocyte preparations for the patients. Increased chemiluminescence was measured after stimulation with PMA, FMLP, and TNF, with the highest values after stimulation with PMA. This is in accordance with previous findings in which the protein kinase C activator PMA, the chemotactic peptide FMLP, and the cytokine TNF were shown to activate the respiratory burst of granulocytes [6–8].

Of interest, we found an inverse association between PCT and oxygen radical production before and after stimulation with FMLP and TNF. No correlation between FCT and ROI production was found.

High-capacity release of TNF after stimulation of leukocytes from patients with *P. falciparum* malaria has been correlated with shorter FCT and PCT [12]. Since TNF is known to activate the respiratory burst and degranulate neutrophils [13], our results suggest that enhanced oxygen radical release is the underlying mechanism of the previous findings of our group. However, although it was shown that human phagocytes activated by *P. falciparum* antigens release ROI [2] and these may contribute to both pathology and parasite death in malaria [3], it remains uncertain to what extent oxygen-dependent mechanisms participate in phagocyte-mediated killing of malaria parasites.

Polymorphonuclear leukocytes from patients with chronic granulomatous disease, which lack the capacity to produce oxygen radicals, also inhibited growth of *P. falciparum* after stimulation with TNF. Therefore, it was suggested that oxygen-independent mechanisms are involved in parasite destruction [14]. On the other hand, scavengers of oxygen radicals, such as superoxide dismutase, catalase, and vitamin E, could abrogate fatty acid–enhanced neutrophil-mediated killing of *P. falciparum* in vitro [15]. Nevertheless, it is possible that the faster parasite clearance we observed is partly due to oxygen-independent mechanisms, while the simultaneous elevated

chemiluminescence indicates a greater degree of leukocyte activation.

In conclusion, our data support the thesis that ROI generation by granulocytes plays a pivotal role as a first-line antiparasitic defense in *P. falciparum* malaria.

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