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CORRELATION BETWEEN DESTRUCTION OF MALARIAL PARASITES BY POLYMORPHONUCLEAR LEUCOCYTES AND OXIDATIVE STRESS

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The role of reactive oxygen species (ROS) generated by polymorphonuclear leucocytes (PMNs) in the host response against malaria was investigated. Non-activated human PMNs were added to cultures of *P. falciparum* in microtitre cells. Parasite viability was evaluated by the incorporation of radioactive hypoxanthine. Using PMN/RBC = 1/150 (starting parasitemia was 1%) the incorporation on the second day in culture was only 61% of the control cultures. An effect could be observed already after two hours of incubation (30% reduction at a 1/50 PMN/RBC ratio). A direct contact between the effector and target cells was obligatory for the expression of the damage.

Parasites within G6PD-deficient erythrocytes were more sensitive to the PMNs than normal parasitized erythrocytes. This difference could be attributed to the production of reactive oxygen intermediates in the experimental system, since G6PD-deficient erythrocytes are generally more sensitive to oxidant stress.

Salicylic acid was used as a scavenger and reporter molecule for hydroxyl radical fluxes. It is converted to the corresponding dihydroxybenzoic acid derivatives, which could be detected by HPLC. Uninfected NRBC or parasitized erythrocytes containing young ring forms could trigger the PMNs to produce much less ROS than the mature forms of the parasites. Other factors associated with PMNs may inactivate the parasites, such as phagocytosis, lysosomal enzymes or degradation toxic products of the PMNs. However our results indicate that increased oxidative stress induced by PMNs interfere with the growth of *P. falciparum* and could play a role in human evolution of abnormal erythrocytes.

KEY WORDS: Malaria, oxidative stress, polymorphonuclear leucocytes.

ABBREVIATIONS

CGD	chronic granulomatous disease
DHBA	dihydroxybenzoic acid
G6PD	glucose 6-phosphate dehydrogenase
HPLC	high pressure liquid chromatography
Hx	hypoxanthine
PE	parasitized erythrocytes
PMA	phorbol myristate acetate
PMNs	polymorphonuclear leucocytes

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RBC red blood cells
ROS reactive oxygen species
SOD superoxide dismutase

INTRODUCTION

The role of oxygen radicals in protection against malaria infections is in debate.[1] However there are many findings indicating the importance of leucocyte oxidative burst.[2,3] This study evaluates the specific destructive role of ROS generated by PMNs.

An increase in the number of PMNs in human subjects infected with *P. vivax* or *P.* faciparum has been noted.[4,5] Similar results were found in rodent models.[6,7] In all cases PMNs counts returned to normal after recovery. The chemotactic activity of PMNs obtained during infection was not affected by the general phenomenon of immunosuppression during malaria attack.[8] Moreover it was found that PMNs from infected children are metabolically more active than normal PMNs.[9]

It has been shown that stimulated PMNs can affect *P. falciparum* development *in vitro*.[10,11] However various free radical scavengers and protective enzymes could not alter this deleterious effect. It was also found that PMNs from chronic granulomatous disease (CGD) patients which are defective in their respiratory burst can still inhibit parasite development.[12] It should be also noted that PMNs can phagocytose and destroy various stages of malaria parasites.[13–15] As PMNs are disintegrating after 5–7 hours of *in vitro* incubation, their degradation products may also kill the parasites (imitation of extracellular degranulation) and lead to uninterpretable results.[11] Such experiments should be short in order to enable the discrimination of the specific role of ROS.

In view of these inconsistencies we were aiming to evaluate the idea that ROS induced by PMNs are involved in host defence against malaria.

MATERIALS AND METHODS

Experimental design

The experimental systems were based on *in vitro* cultures of *P. falciparum* in either normal or in G6PD-deficient erythrocytes incubated with PMNs that were present in the medium throughout the experiment. In one set of experiments the erythrocytes were preincubated with PMNs for 2 h, the PMNs were then removed, and erythrocytes were infected. The uptake of radioactive hypoxanthine (Hx) served as a parameter for parasite development. Each of the experiments was repeated at least three times, and was performed in triplicate cultures. For each individual experiment the deviation did not exceed 7.5% of the mean value (of the triplicates). The figures and tables depict a typical set of results for each experiment. The variability among the results of repeats of the same experiment was higher and could reach even 40–45%. For example, in one experiment, the incorporation of labelled hypoxanthine by control parasites was 1500 cpm during 2 hours. The addition of PMNs, at a ratio of 1/30 PMN/parasitized RBC reduced the incorporation by 55%. In another experiment similarly designed, the incorporation was 2500 cpm and the reduction by PMNs was 41%. This variability could be attributed to the variations inherent to

this biological system. These include PMNs from different donors at various unknown immunological conditions. The parasites are grown in normal erythrocytes donated by various subjects and are used within a time range of 10 days. The time "window width" of the parasites and their exact state in the life cycle could vary among the experiments.

Parasites

Plasmodium falciparum (strain FCR-3) was cultured according to the method of Trager and Jensen.[16] Cultures were synchronized by sorbitol treatment[17] every other day.

Blood

Blood was collected from normal healthy men or from G6PD deficient [Med(−)] individuals and stored at 4°C. The experiments involving normal and G6PD deficient erythrocytes initiated within 24 h after collection of the blood. Each experiment was repeated in blood donated from at least 3 individuals. The activity of G6PD in the deficient blood was determined and was found to be less than 3% of the normal activity.

Separation of PMNs from blood

PMNs were separated from freshly drawn blood by Ficoll-paque (Pharmacia) density gradient centrifugation and dextran sedimentation.[18] Hypotonic lysis was used to remove residual red cells. The purity and viability of the granulocytes was above 98% (based on microscopic observation and trypan blue exclusion).

Treatment

Parasitized erythrocytes (in their advanced forms, late trophozoites and schizonts) were adjusted to 1% parasitemia in red blood cell suspension (5% hematocrit in RPMI 1640) containing PMNs. The cells were dispensed into a 96-well microplate (100 μl/well) and incubated at 37°C using the candle jar method of Trager and Jensen.[16] The supernatant was replaced every day. In some experiments parasitized erythrocytes were concentrated from cultures by the gelatin sedimentation technique[19] before treatment.

Pretreatment

Suspensions of normal or G6PD deficient erythrocytes (5%) were incubated for 2 h with PMNs and phorbolmyristate acetate (PMA, 10^{-7} M; PMN/RBC ratio 1/30). Following the incubation, the PMNs were removed from the cell suspensions by passage through cellulose powder column,[20] the erythrocytes were washed with RPMI 1640 growth medium and subsequently infected by addition of an inoculum of parasitized erythrocytes obtained by gelatin sedimentation. The contamination by non-parasitized erythrocytes in this inoculum was less than 1% of all the erythrocytes.

Treatment of target PE with PMNs located in a separate compartment

Disposable plates including chambers with or without a 0.4 μ filters were used (tissue culture treated pvp free, polycarbonate membrane, Costar). PE were in the bottom while PMNs were in the upper part separated by the filter. As control, we used the same chambers without filters where PE and PMN were mixed in the same volume. These cultures were pulsed during 4 h with Hx.

Parasite development

Incorporation of radioactive Hx was measured according to the method of Golenser, Casuto and Pollack[21] as follows: [^{3}H]-hypoxanthine (18.5 kBq/well, New England Nuclear), was added in 25 μl of medium to the culture. The cells were collected by filtration on glass microfibre filters and radioactivity was counted (by a Mixami, Tri-Carb 4000, Packard). The incorporation of Hx in normal erythrocytes (without parasites) or PMNs alone, did not exceed 3% of the lowest level of uptake by parasitized red blood cells.

Determination of hydroxyl radical by HPLC

Non-infected or parasitized erythrocytes (from the same donor) were incubated for 2 h at 37°C in 3 ml, RPMI 1640 containing 10% erythrocyte suspension (normal or PE with 50% parasitemia of advanced stages) and 10 mM salicyclic acid. At the end of the incubation period the suspensions were centrifuged and the supernatant and pellet were separated. Equal volume of supernatant and 5% TCA were mixed. The pellet was frozen and thawed and its volume was completed to 1 ml with 450 μl saline and 500 μl of 5% TCA. The resulting products were centrifuged at 12,000 g and filtered through 0.2 μ filter before the application to the HPLC. A LiChrospher 100 RP-18, 5 μM, 25 cm × 4 mm (E. Merck) column was used in order to separate the hydroxylation products of the salicylic acid. The mobile phase was prepared with deionized water and filtered before use through a 0.45 μ filter (Millipore). The flow rate of the mobile phase was 1.2 ml/min. The mobile phase contained 0.03 M citric acid, 0.03 M acetic acid, 1% (vol) methanol and 0.28 g/l sodium azide. The mobile phase was titrated with solid NaOH to pH 3.0 and then with solid CH_3COONa to a final pH of 3.6. The biological samples were compared to two standards 2,5- and 2,3-dihydroxybenzoic acid (1.6 ng of each) using an electrochemical detector with potential of +0.8 V vs Ag/AgCl electrode.

RESULTS

A. Establishment of the experimental system

The basic experimental system includes parasitized erythrocytes (PE) incubated with PMNs. Parasite development was evaluated by [^{3}H]-hypoxanthine (Hx) incorporation. Table I shows that the PMNs reduce Hx incorporation in a dose response manner. For each PMN/RBC ratio the effect is more pronounced in the second day.

PMNs undergo a progressive disintegration in culture within 32 hours. This may lead to release of intracellular proteolytic enzymes, which in turn, could affect parasite

TABLE I
The effect of PMNs on hypoxanthine incorporation by parasitized erythrocytes*

Ratio PMN/RBC	Percent** inhibition 0–24 h	Percent inhibition 24–48 h
1/50	31	82
1/150	22	61
1/450	1	17

*Starting parasitemia 1% at the trophozoite stage.

**Hx incorporation in the absence of PMNs was about 14,500 CPM and 11,000 CPM in the first and second day respectively, and served as control values for the evaluation of the degree of inhibition. Uninfected RBC or PMNs did not incorporate more than 1% of the control PE cultures.

growth. In order to evaluate this destructive effect of disintegrated PMNs on Hx incorporation, we compared the effect of intact and viable PMNs with that of PMN-extracts, both incubated with PE for 24 h (Figure 1). The effect of the extracts was higher than that of the intact PMNs. This led us to examine the duration of the experiment. PMNs were incubated together with PE for various periods. After 1 h there was a substantial effect. However, the radioactive counts were low. Also, the Hx incorporation was probably more influenced during the first hour by conditioning of the cells to the culture. This led us to choose a basic incubation period of 2–4 h.

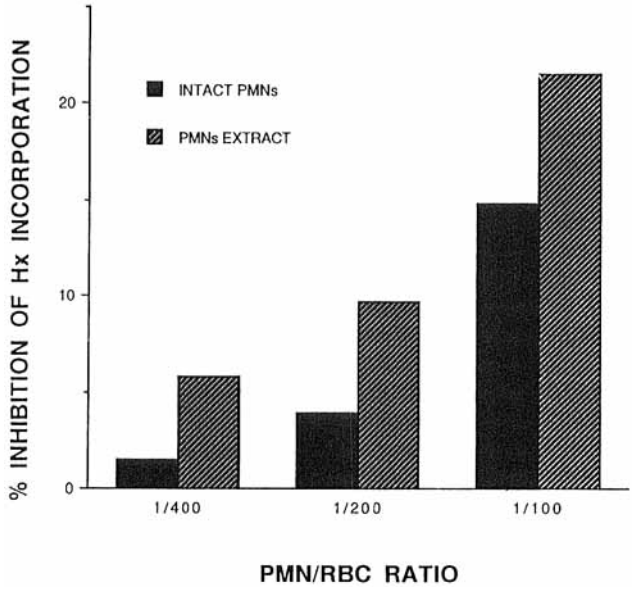

FIGURE 1 The effect of PMNs extract on parasite development. PMNs were extracted by 3× freeze-thaw process in liquid nitrogen and 37°C. The intact PMNs or their extracts were added to parasitized erythrocytes. Hx incorporation was measured during 24 hours pulse.

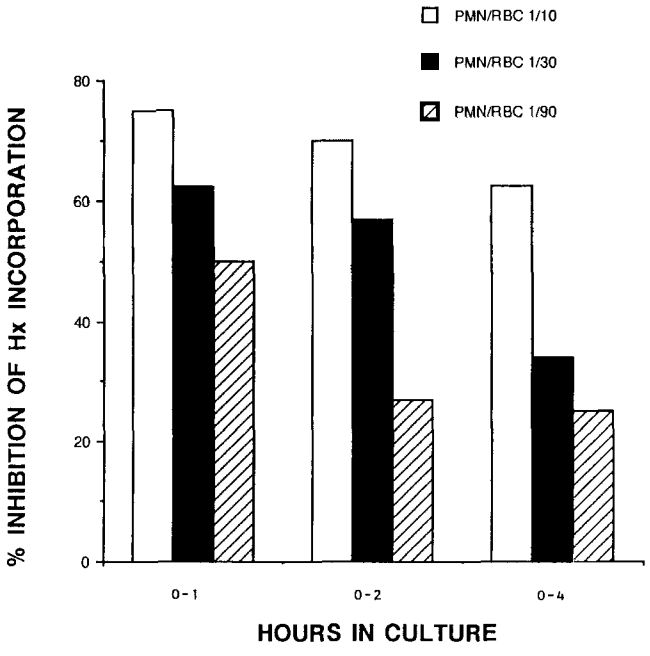

FIGURE 2 The effect of PMNs on parasite development during short term exposures. PMNs at various proportions were incubated with parasitized erythrocytes (10% parasitemia of advanced forms) for pulses of 1, 2 or 4 hours. Hx was added at the onset of the experiment.

Longer incubation periods were ruled out as the PMNs start to disintegrate after 5–7 h (Figure 2). Table II depicts the results of 6 different experiments in which PMNs were incubated for 4 h with erythrocytes parasitized with advanced forms of *P. falciparum* at PMN/RBC ratio of 1/30 and 1/90. The results show that there is a variability in Hx incorporation in control and experimental cultures and in the effect as expressed by the percent inhibition. However, for each single experiment the results were statistically significant.

B. The dependency of PMN effect on parasite developmental stage and type of host cell

(*a*) *Pretreatment of non-infected erythrocytes.* Normal of glucose-6-phosphate dehydrogenase (G6PD) deficient erythrocytes were pretreated with PMNs (PMN/RBC ratio 1/30) in the presence of PMA, for 2 h. The PMNs were separated from the erythrocytes on cellulose powder column. Subsequently, an inoculum of untreated parasitized erythrocytes was added to yield 1% parasitemia. The erythrocyte suspensions were divided into microplates and Hx incorporation was measured in two consecutive pulses of 15 and 21 hours. Figure 3 shows the similar but marginal effect of PMNs within the first 15 h on Hx incorporation by *P. falciparum* grown in either in G6PD(+) or G6PD(−) erythrocytes. In contrast, during the following 21 h (15–36 h after inoculation) the effect of PMNs on parasites within both types of RBCs becomes marked, but more pronounced in the G6PD(−). *Treatment* of PE when compared to the *pretreatment* of non-infected RBCs with the same PMNs and PMN/RBC ratio yielded a much higher effect.

TABLE II
Reproducibility of the effect of PMNs on hypoxanthine incorporation by parasitized erythrocytes

Experiment	Control cpm ± s.d.	Ratio PMN/RBC	Experimental cpm ± s.d.	Percent inhibition
1	13850 ± 530	1/90	11560 ± 420	13
		1/30	9920 ± 70	29
2	18570 ± 440	1/90	17180 ± 390	10
		1/30	14200 ± 630	25
3	8970 ± 320	1/90	7520 ± 280	17
		1/30	6330 ± 620	30
4	17330 ± 140	1/90	15490 ± 220	11
		1/30	14480 ± 170	17
5	13470 ± 200	1/90	11280 ± 70	17
		1/30	10580 ± 220	21
6	26280 ± 330	1/90	21160 ± 600	20
		1/30	17550 ± 350	33

(*b*) *Treatment of PE.* Figure 4 demonstrates that young stages are equally sensitive to PMNs if grown in either normal or in G6PD deficient erythrocytes. However, advanced stages are more sensitive to PMNs when grown within G6PD deficient erythrocytes (at low PMN/RBC ratios). At PMN/RBC = 1/10 there is often increased PMN aggregation which accelerates PMN disintegration and increases

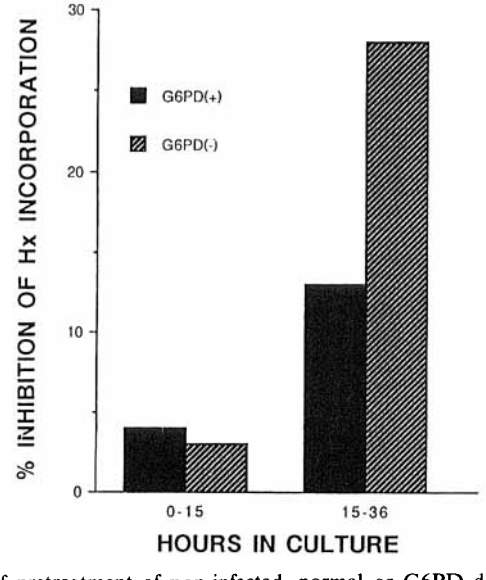

FIGURE 3 The effect of pretreatment of non-infected, normal or G6PD deficient erythrocytes with PMNs on parasite development. The erythrocytes were pretreated for two hours after which the PMNs were removed by filtration through a cellulose powder column. The erythrocytes were washed and an innoculum of untreated parasitized erythrocytes was added. Hx was added either in 0 or 15 h and the cultures were harvested in 15 or 36 h, respectively.

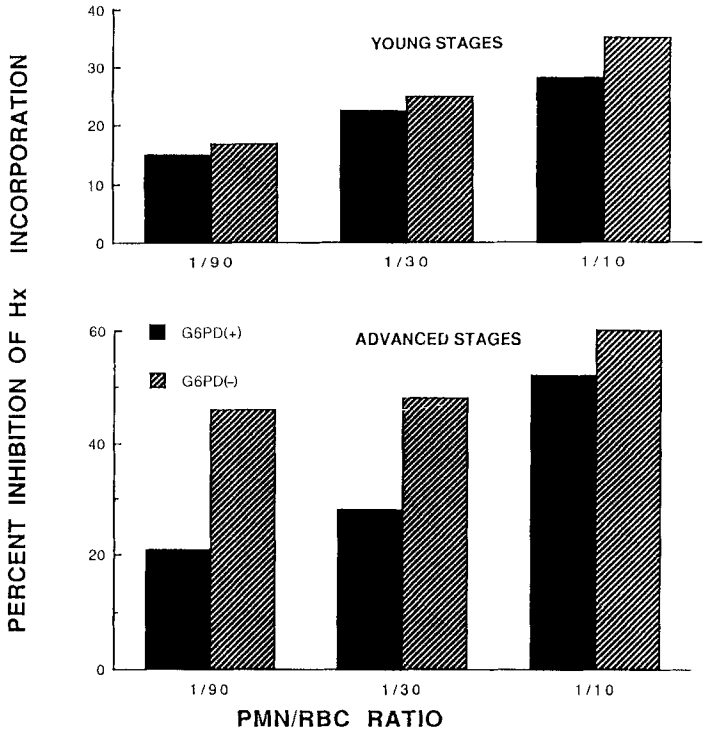

FIGURE 4 The effect of PMNs on young or advanced stages of *P. falciparum* within normal or G6PD deficient erythrocytes. PMNs at various proportions were incubated with normal or G6PD deficient erythrocytes containing young or advanced stages (ring forms or trophozoites) for 4 h. Parasitemia was 10% in both stages.

parasiticidal effects which are not associated with oxidant stress. Thus, at this ratio there is no difference in sensitivity.

C. *Mechanistic aspects*

(*a*) *Production of ·OH radicals in parasitized erythrocytes.* The conversion of added salicylate to dihydroxybenzoates (DHBA) was employed as a quantitative indicator for the produced fluxes of ·OH radicals. Table III shows that the level of DHBA in parasitized cells is higher than that in the non-infected cells (basal level). The incubation of non-infected RBC with PMNs increased DHBAs production by 3.4–4.3 fold. However, when PE were present, the increase was about 10 times, indicating an increased stimulation of PMNs, by PE.

(*b*) *"Contact inhibition".* Superoxide dismutase (SOD, 50 μg/ml) and/or catalase (90 μg/ml) could not alter the inhibitory effect induced by PMNs. This is in accord with Halliwell's suggestion[22] that enzyme protection should not necessarily be expected when the effector cell and its target are in tight contact.

TABLE III
The production of 2,3- and 2,5-dihydroxybenzoates (DHBA) in parasitized erythrocytes incubated with PMNs

	PMNs	2,3-DHBA ng/ml	R*	2,5-DHBA ng/ml	R
Non-parasitized	–	31	1.0	7	1.0
	+	134	4.3	24	3.4
Parasitized	–	36	1.2	18	2.6
	+	364	11.7	182	26.0

PMN/RBC at a ratio = 1/50 were employed as indicated in Methods.
*R is the ratio between DHBA level under given conditions and that in the non-infected cells in the absence of PMNs. PMNs alone produce less than 0.3 ng/ml 2,3 or 2,5-DHBA.

We have designed experiments in which PE were kept separated from the PMNs by a 0.4 μ filter (1 mm distance). Identical chambers without the filters, where tight contact between PE and PMNs is possible, were used as control. For comparison, the PMNs were also stimulated by PMA. The results are demonstrated in Figure 5. PMA-stimulated PMNs were more active than non-stimulated PMNs. Both reduced Hx incorporation by PE, in a dose response manner. Separation between PE and PMN (whether PMA-stimulated or non-stimulated) completely reduced PMN effect, proving the close association needed for the expression of the damage.

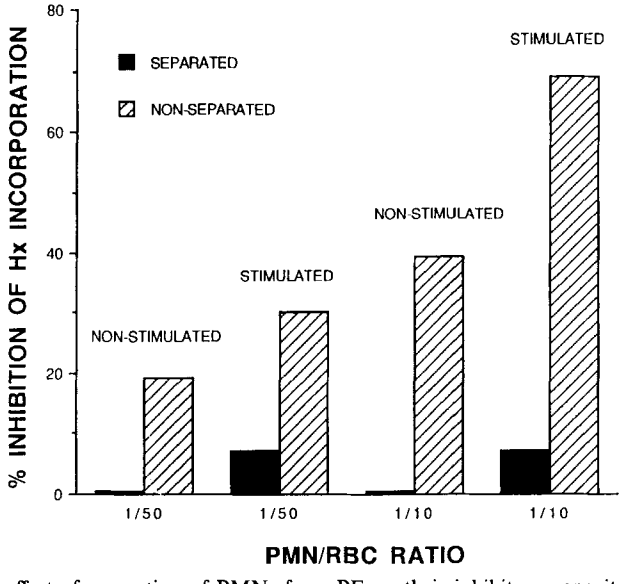

FIGURE 5 The effect of separation of PMNs from PE on their inhibitory capacity. In some cultures the parasitized erythrocytes (80% parasitemia, advanced stages) were separated from the PMNs by a 0.4 μ filter and the PMNs were stimulated by 10^{-7} M phorbal myristate acetate. The duration of the experiment was 4 h.

TABLE IV
The effect of cytochalasin B on the inhibitory activity of PMNs

Cytochalasin	PMNs	CPM	Percent inhibition
−	−	2280	0
−	+	1640	28
+	−	1990	13
+	+	1440	36

PMNs were incubated with cytochalasin B for 5 minutes after which parasitized erythrocytes were added. PMN/RBC at a ratio 1/90 were incubated for two hours. Hx incorporation (added immediately) serve for the evaluation of the degree of inhibition.

(*c*) *Discrimination between phagocytic effect and the results of the respiratory burst.* PMNs were incubated with cytochalasin B for 5 minutes (initial cytochalasin concentration was 5 μg/ml) after which parasitized erythrocytes at the trophozoite stage (20% parasitemia) were added (PMN/RBC ratio was 1/90 in a final volume of 1 ml). The cultures were harvested after two hours pulse. At this cytochalasin concentration there is total inhibition of phagocytosis and 13% inhibition of Hx incorporation. The inhibition of incorporation by both PMNs and cytochalasin B—36%, is close to the theoretical cumulative value of the combination of the separate effects of PMN and cytochalasin B (Table IV). This result suggests that a substantial damaging effect is originating from the respiratory burst.

DISCUSSION

The role of PMNs in eliminating malaria parasites has been well documented.[11] It has also been suggested that the PMNs activity is augmented by complement, antibodies and tumor necrosis factor.[23] Several modes of action by which PMNs can eliminate the parasite have been proposed: phagocytosis, ROS production, and the release of lysosomal components. Despite PMNs known potential to release ROS, the significance of this function in destruction of plasmodia has not yet been established. Moreover, various antioxidants could not reverse the inhibitory activity of neutrophils.[12] All other activities of PMNs are also augmented during respiratory burst[9,23] and PMNs defective in their oxidative metabolism could still inhibit the *in vitro* growth of *P. falciparum*.[12] This inhibition is not evidence against an oxygen-dependent mechanism, it only shows that other mechanisms may be also involved.

This study confirms previous results which demonstrate a neutrophil-mediated protective response in malaria. The experimental system is only partially standardized: throughout the series of experiments we used the same strain of *P. falciparum*, which was grown in the same human plasma and batch of RPMI-1640. It was impossible to repeatedly use the erythrocytes or the PMNs of the same donor. Another factor that could interfere with reproducibility was that the erythrocytes could not always be used on the same day following their donation and that the developmental stage of the parasite could not exactly be matched to previous experiments (i.e. late trophozoites could be mixed with early schizonts in one

experiment, while in another one, there were only trophozoites). The effect of PMNs on parasite development varied from person to person. Even the activity of PMNs donated from the same person on different days was not identical. However, in all cases, PMNs caused a profound significant reduction in the incorporation of Hx into *P. falciparum* grown in culture.

We were aiming at the evaluation of the discrete role of oxidant stress in inhibiting parasite development. The effect of PMNs in *P. falciparum* was already seen within 1 hour. However, due to low Hx counts during the first hour in culture, it was preferable to extend the experiments to 2–4 hours. It is possible that the parasites overcome some of the initial interference, however, most of the effect is not reversible during this period. Longer periods of incubation could lead to artifacts due to cytotoxicity associated with PMNs degradation products, or reflect proteolytic activity of the PMNs. Varani *et al.*[24] found that target endothelial cells are killed by PMNs during the first 4 hours, mainly by oxygen products. If the time course for exposure of the target cells is extended to 18 hours progressive injury is mediated by PMN proteases. It is possible that in the system of Varani *et al.*, as well as in ours, and as suggested by Weiss,[25] the ROS immediately damage the parasite but also modify the parasitized erythrocyte to render it more sensitive to a subsequent effect of PMNs lysosomal products. It is unlikely that the PMNs reduced the viability of the parasites by competing with them for nutrients because of the short duration of the experiment and due to the small amount of PMNs needed to inflict the damage: in some experiments, two hours incubation of PMNs with parasitized erythrocytes at a ratio of 1/30 was sufficient to reduce hypoxanthine incorporation by 40 percent.

While phagocytosis was prevented by cytochalasin B,[26] PMNs still interfered with parasite development. Cytochalasin would not prevent the respiratory burst accompanied with extracellular release of ROS. Another indication for the involvement of ROS in our system is the increased effect of the PMNs on parasites developing in G6PD-deficient erythrocytes, since G6PD-deficient erythrocytes are more sensitive to oxidant stress.[27,28]

The PMNs influenced parasite development by virtue of a damage which had been exerted prior to the invasion (i.e., *pretreatment* of non-infected erythrocytes) or by virtue of changes induced by *treatment* of the parasitized erythrocytes. In both cases G6PD-deficient erythrocytes were associated with increased interference with parasite development. Pretreatment of the erythrocytes could change the fate of the parasites by both affecting the invasion and the preceding development while treatment could damage the intracellular development. Thus, components of the immune system could play a selective role in evolution, providing an advantage to this otherwise deleterious type of erythrocyte. It was suggested that the oxidative stress is responsible for retention of the genetic features of both erythrocytes and effector cells—by selecting various traits of erythrocytes associated with increased sensitivity to oxidant stress[29–31] and by shaping the ROS-producing cytotoxic cells of the immune system[32] during evolution. Our results which demonstrate the increased effect of PMNs in *P. falciparum* developing in G6PD deficient erythrocytes, support the two parts of this hypothesis.

Catalase and SOD could not prevent the effect of PMNs. It has been suggested[22] that this does not necessarily exclude ROS involvement. We found that a tight contact is needed between the PMNs and the PE, in order to inflict or initiate the injury. Protective enzymes may operate less efficiently in such a microenvironment. Another possibility is that following the contact between the effector and the target cells, ·OH

radicals are produced within the parasitized erythrocyte (especially when the parasite is well developed and digestion of hemoglobin probably releases redox-active iron containing structures[33,34]). It is unlikely that the protective enzymes can penetrate the parasitized erythrocyte and prevent internal radical production.

An additional substantiation for radical involvement is the generation of the highly reactive hydroxyl radicals. There is a general agreement that this radical is among the most reactive and deleterious ROS.

It has been speculated whether PMNs are capable of forming hydroxyl radicals.[35] However, an ESR study shows ·OH production through Haber Weiss or Fenton type reactions, requiring trace iron either inside or outside the cell.[36] Indications for the progressive increase in redox-active iron during the growth of *P. falciparum* have been recently found in our laboratory.[33,34] Thus, the iron may catalyze the production of the ·OH radical in the plasmodial system. The increased level of hydroxylation of salicylate in a system containing PMNs activated by parasitized erythrocytes, suggests that ·OH radicals produced by the Fenton reaction are involved in plasmodial killing. However, there is a variety of other cytocidal molecules which could participate in eliminating malaria parasites and have not yet been investigated in this context. Likewise, neutrophils also contain the enzyme myeloperoxidase, some of which is released during activation. Myeloperoxidase catalyzes an H_2O_2-dependent oxidation to halide ions to produce OCl^-. The hypochlorite ion exists in equilibrium with its protonated form (HOCl). Both are powerful oxidizing agents that can attack a wide range of biomolecules.[37]

It should be noted that phagocytic cells including neutrophils are capable of producing nitric oxide.[38] The nitric oxide and related molecules, the nitric oxide radical (NO·) and its oxidized forms nitrite and nitrate (termed reactive nitrogen intermediates) have anitplasmodial activity.[39] We present circumstantial evidences that there is a correlation between the *in vitro* destruction of *P. falciparum* by the PMNs and the oxidative stress. Other factors (which may interact with the ROS) could also be involved.

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