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RESEARCH ARTICLE

ROLE OF POLYMORPHO NUCLEAR LEUKOCYTES (PMNS) IN MALARIAL INFECTION AND THEIR CORRELATION WITH APOPTOSIS

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ABSTRACT

Neutrophil from the malarial (*Plasmodium yoelii)* infection was isolated by Ficoll-Hypaque and Dextran sedimentation method, the viability of neutrophil was assessed by trypan blue to be 95-98%. The viable neutrophil follows apoptosis and modulates the myeloperoxidase (MPO) activity. Our data suggesting that, the malarial infection inhibits MPO activity of neutrophil. During *P. yoelii* infection, the induction of apoptosis was found in neutrophils using FACS. This data suggesting that this apoptosis may be initiated by leukocyte hemozoin coming after phagocytic ingestion of parasite-libratedhemozon (HZ) in peripheral blood.

INTRODUCTION

Malaria remains one of the leading causes of morbidity and mortality in tropics. It has been estimated to represent 2.3% of the overall global disease burden and 9% in Africa, ranking third among major infectious disease threats. Major reason for this devastating situation is the emergence of drug resistance to classical and affordable antimalarial drug.The neutrophil is only one arm of the immune system, which includes other leukocytes, lymphocytes and molecular components such as complement, antibodies, acute phase protein and cytokines these cellular and molecular component of immunity constitute a coordinated and sophisticated network that has evolved in order to maximize the survival of the host against the range of pathogens it encounters daily.Neutrophils produce first line of defense against infection by mean of phagocytosis. They developed in bone marrow by the process called as haematopoiesis, 55-60% cells produced by bone marrow are neutrophils. The mature neutrophil has diameter of 10-12 µm, neutrophil has a single multilobed nucleus and a dense granular cytoplasm. Granules are membrane bound organelles, which synthesized at promyelocyte stage of neutrophil development (Edwards 1994). The cytoplasm of mature neutrophil contains very few distinguishable mitochondria. These cells do not utilize mitochondrial respiration or oxidative phosphorylation to fulfill their energetic requirements rather; they derived their energy from glycolysis, which is oxygen independent process. Thus neutrophil can function efficiently at low oxygen tensions that may be present with in inflamed tissues (Zhinhong Hu and Mohammed 2004; Edwards 1994).

PMNs constitutes 40%-60% and 20%-40% of total leukocytes population in human and mice blood respectively and found in concentration 3-5 x 10^6 cells/ml blood. While the number increases dramatically in case of infection. They have relatively short half-life in circulation, estimated about 8-24 hrs. But this may extend up to several days if the cells leave the circulation and enters into the tissues. PMNs recognize their target with the help of compliments receptors present on plasma membrane. Here the plasma membrane and cytoplasmic granules play an important role; the plasma membrane contains an unusual enzyme NADPH oxidase which is capable of generating a series of a reactive oxygen intermediates/species (ROI/ROS), which has broad-spectrum antimicrobial property. During infection cytotoxic process activated and delivered to the pathogen (Fossati2003).

The cytoplasmic granules also play an important role in pathogen killing. They have range of protein with cytotoxic property like myeloperoxidase (MPO); proteases; hydrolytic enzyme; highly specialized Bactericidal/permeability inducing protein (BPI) Protein that affects the permeability of microbial targets and help in eradication of pathogen (Fossati2003). This present study is showing the comparative myeloperoxidase study on infected an uninfected mice neutrophil. The apoptosis

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in neutrophil during malarial infection is also evaluated by using FACS.

MATERIALS AND METHODS

Parasite and experimental host

Swiss albino mice weighted 18-25 gms were infected with Plasmodium *yoelii*, intraperitonialy($1x10^7$ infected erythrocytes/ml) (Martin 1995).Parasitemia counts were monitored time to time by microscopic examination of slides stained with Giemsa Stain. Blood samples were collected at high level of parasitemia 50-60% in sterile ACD (Acid citrate dextrose) (sodium citrate 70 mM, dextrose 136mM and citric acid 35mM) solution for further studies (Chatterjee, 1980).

Isolation of peripheral leukocytes from control and Infected blood by ficoll-hypaque and dextran sedimentation method

Control and infected blood was collected from control and infected mice using anticoagulant (2% sodium citrate) to isolate neutrophils. Cells were separated by using Ficoll-Hypaque and Dextran sedimentation method described by Iwai (Iwai, 1994). Briefly, 16 ml of control and infected blood was applied for centrifugation at 150g for 15 min to separate plasma layer. Pellet was dissolved in 2 volume of 0.9% saline of pH 7.4 and mixed gently. Sample was loaded on 3.5 ml Ficoll- hypaque of density (1.077) and centrifuged at 350 g for 40 mins at room temp. Pellet was resuspended in 4ml dextran and allowed to settle for 60 mins at room temp. The supernatant was collected and washed three times with 0.9% saline at 150 g for 10 min. at room temp. Pellet was mixed with 3 fold ice-cold NH_4Cl and kept it for 7-8 mins on 4^0C, with discontinuous shaking. The color of pellet was turned from red to black showing bursting of erythrocytes then centrifuged at 400 g for 5 mins at 4^0C than pellet was resuspended into NH_4Cl with 0.5% albumin and incubated on ice for 15 mins and again Centrifuged at 400 g for 5 mins at 4^0C, supernatant was aspirated with pipette. Pellet was dissolved in 200µl of 1x PBS. 10µl of sample was taken for viability measurement and 10µl for cell counting. Observed the viability, by mixing the equal volume of cell suspension with Trypan Blue (1:1 ratio).Percentage of viable cells was calculated by the formula-

$$\% \text{ of viable cells} = \frac{(\text{no of viable cells})}{(\text{no of viable cells} + \text{no of dead cells})} X\,100$$

Estimation of myeloperoxidase activity of control and infected peripheral leukocytes

MPO activity of control and infected peripheral leukocytes were estimated by methods described earlier (Giovanni et al.). Briefly, control and infected peripheral leukocytes were incubated with 1ml of Guaiacol PO_4 mixture (50 mm PO_4 buffer and 20 mm guaiacol). The reactions were initiated by addition of freshly prepared 20mM H_2O_2. The time dependent changes in OD_{460} were recorded up to 120 sec at 15 sec intervals. The ΔOD / min were also calculated.

Protein Estimation and estimation of Specific Activity

The protein concentration were calculated by using Bradford method (Bradford, 1976) using BSA as standard. The specific activity of control and infected sample were calculated as ΔOD/min/mg protein.

Detection of Apoptosis in control and infected PMNs by Fluorescence activated cell-sorting (FACS)

Infected and control PMNs were purified by previously described ficoll – hypaque and dextran sedimentation method. The sediment pellet (10^5-10^6 cells) were dissolved in 1ml of fluorescein-conjugated annexin v (50mM Tris-HCl pH 7.5 containing 100mM NaCl) and incubate with binding buffer (100mM HEPES/NaOH, pH 7.5). Propidium iodine solution (10mM potassium phosphate buffer containing 150mM NaCl, pH 7.5) was added to the cell suspension, to a final conc. of 1.0µg/ml propidium iodine. After that cells were analyzed by flow cytometer (Walrend S, 2003).

RESULTS AND DISCUSSION

Peripheral leukocytes were isolated from subcellular fractionation of blood after a high percentage of parasitemia (~50% after 5 days of infection) (Fig-1). The blood sample without infection served as control.

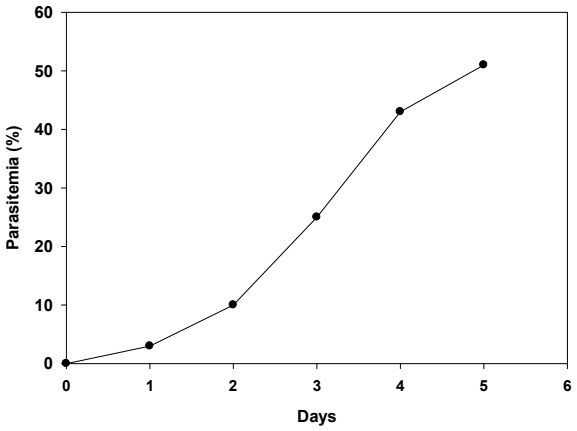


Fig-1 (a) Time dependent parasitemia curve

Myeloperoxidase (MPO) enzyme is synthesized early in the development of the neutrophils, at the promyelocyte stage and packed into azurophilic granules. It is glycosylated, arginine rich and extremely cationic protein. MPO is used as marker enzyme for phagocytic activity. During phagocytosis of microorganism, maximal rates of $O_2^-$and H_2O_2 production occurs, MPO utilizethe ROI and converted into HOClduring respiratory burst, which is actually responsible for eradication of range of microorganism (Gianluea, 2003; Edwards Steven W. 1994). In our study, infected sample shows lower MPO activity then control. The change of OD (ΔO.D/min) is found 0.053 and 0.099 in case of infected and control respectively (Fig-2). These data suggesting that *Plasmodium yoelii*infection leads to alter MPO activity.

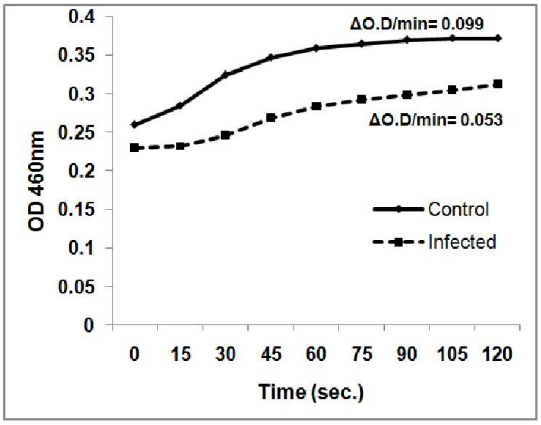


Fig. 2. Comparative study of MPO activity in control and infected PMNs

Specific Activity of enzyme defined as "Number of enzyme unitspermilligram of total protein". The specific activity was measured to compare the concentration of MPO enzyme in control and infected neutrophil, to observe that the changes occurred after infection. During purification its value increased and become maximal and constant, when enzyme is pure. Higher value of specific activity shows that enzyme is pure, but as the specific activity increases the amount of total protein will decrease in solution. It can be calculated and expressed asΔOD/min/mg protein (William *et al.,* 1983). The specific activity of control and infected PMNs summarized as follows found (Table-1 and Fig-3). The MPO activity of infected PMNswas found significantly lower than control which suggesting that the infection alters the MPO activity.

Table 1. Specific activity of control and infected samples

Samples	Specific activity(ΔOD/min/mg protein)
Control	2.2/min/mg protein
Infected	1.4/min/mg protein

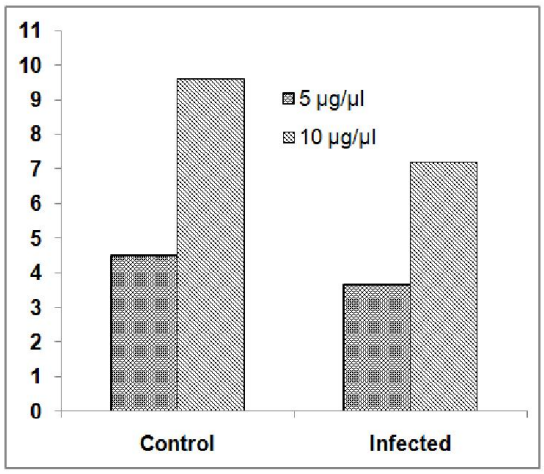
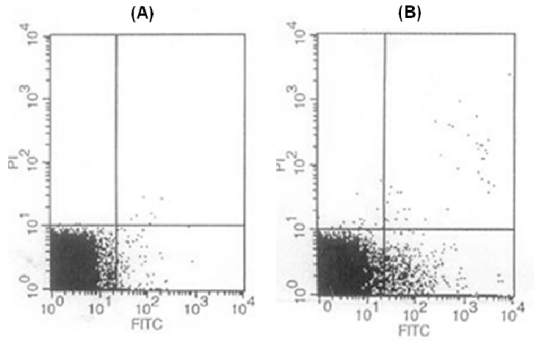


Fig 3. Protein Estimation of control and infected samples

Previous reports demonstrated that engulfment of hemozoin (HZ) by monocytes or leukocytes induces apoptosis and there is a definitive mechanism of engulfment of HZ by peripheral WBCs (Evelin *et al.,* 1996). Intra leukocytes HZ may alter leucocytes function by affecting key enzyme MPO. The reports suggest that HZ can exhibit pro-oxidant activity. The inhibition of MPO activity in infected leukocytes indicates the inhibition of MPO activity may lead to the accumulation of ROI like H_2O_2. H_2O_2 is known for stimulating apoptosis in many cells and offers oxidative stress, therefore supported the view that increase level of H_2O_2 accumulated within WBCs after MPO inactivation may produce apoptosis in infected WBCs (Walrend *et al.,* 2003). FACS is a sophisticated flow sorting technique that measure the fluorescence emitted by individually labeled cells in a mixed cell population. The instrument used an argon-laser beam to excite fluorescently labeled cells beings passed in single file through a laminar flow chamber.Area of application of flow cytometry is in the identification and quantitation of apoptotic or necrotic cells; recognition is based on the presence of a particular biochemical or molecular marker that is characteristic for either apoptosis or necrosis. The apoptosis associated changes in cell size and granulity were detected by analysis of laser light scattered by the cell in forward and side direction. Some of the methods rely on the apoptosis associated changes in the distribution of plasma membrane phospholipids (Alvarez *et al.,* 2000).



Quad	Events		% Total	
	Control	Infected	Control	Infected
UL	4	18	0.01	0.04
UR	8	37	0.02	0.07
LL	49882	48900	99.76	97.80
LR	106	1045	0.21	2.09

(C)

Fig. 4. FACS analysis; Dot plot of control (A) and infected peripheral leukocytes (B), table (C) indicating the statistical analysis showing that the total events in each quads

The FACS data is showing that control has large number of cell on LL quad while in LR quad has very low in comparison to infected (Fig-4, A and B). The total event of infected in LR was found ~10 times higher than control. That means infected peripheral leukocytes are prone to apoptosis (Fig-4 C). These data suggesting that, *Plasmodium yoelii*infected may lead apoptosis in peripheral leukocytes. During apoptosis this asymmetry is broken and phpophotidylserine becomes exposed on the outside of plasma membrane. The anticoagulant protein annexin V- FITC binds with high affinity to negatively charged. Thus fluorescence tagged- annexin V has found as a marker of apoptosis cells. Therefore by staining cells with a

combination of fluorescence-conjugated annexin V and propidium iodide, it is possible to detect unaffected, non-apoptotic cells; (Annexin V negative/Pi negative) early apoptotic cells (annexin v positive/PI negative), and late apoptotic or necrotic cells (PI positive) by flow or laser scanning cytometry (Walrend 2003).Live, non-apoptotic cells have low green (fluorescein) fluorescence and little or no red PI fluorescence. Cells undergoing apoptosis stain green but continue to be negative for PI staining; such cells often have low forward and high side scatter.Longer incubation time with fluorescence dye should avoid, because it allows cells to take stain, which still maintain membrane integrity. Due to the fragility of cells undergoing programmed are deaths, rapid methods that maintain cells as close possible to their natural state might be expected to provide the most reliable results.

Conclusion

From the comparative myeloperoxidase study in infected an uninfected mice neutrophil it has been concluded that myeloperoxidase, which is a marker enzyme for neutrophil phagocytic activity is reduces during infection. Result obtained indicated that malarial infection inhibits the MPO activity of neutrophil purified from infected blood. Interestingly, malaria infection also induces apoptosis in neutrophil of infected blood as evidence from FACS using PI and Annexin V FITC binding. Data obtained by FACS analysis of infected and uninfected neutrophil indicated that infected neutrophil undergoes rapid apoptosis in comparison to uninfected neutrophils, which may be due to engulfment of malarial pigment, hemozoin (HZ). The further work is under way to understand the exact mechanism of induction of apoptosis under infection.

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