Thioredoxin Reductase is More Effective than Glutathione Reductase in Inducing Protection against *Plasmodium Berghei* Infection.

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Abstract

Thioredoxin reductase and glutathione reductase are important enzymes of redox system that help the malaria parasite to maintain an adequate intracellular redox environment and contribute greatly to the antioxidant capacity of the cell. In the present study humoral immune response directed against thioredoxin reductase and glutathione reductase during *Plasmodium berghei* infection were investigated. Thioredoxin reductase and glutathione reductase induced humoral immune response in Balb/c mice immunized with purified parasite protein using saponin. Pre-challenge sera gave antibody titre of 1:512. IFA showed specificity of antimalarials antibodies towards parasite. Immunization with thioredoxin reductase provides partial protection compared to glutathione reductase in mice to *P. berghei* infection. Immunization with thioredoxin reductase delays the progression of infection as compared to normal controls.

Keywords: thioredoxin reductase, glutathione reductase, malaria, immune response, saponin.

Introduction

Malaria remains a major global health problem with 2.4 billion people living in areas at high risk of infection (Miura *et al*., 2009). In the absence of a potent vaccine and growing drug resistance in parasite the situation is becoming even grimmer. Erythrocytic stages of *Plasmodium* are under enhanced oxidative stress due to

Reactive Oxygen species (ROS) produced by parasites metabolism and host immune system. Oxidative stress is combated by the parasite with antioxidant enzymes like superoxide dismutase (SOD), thioredoxin reductase (TrxR), glutathione reductase (GR). *P. berghei* parasitized host erythrocytes contain higher GR and TrxR activities compared to normal mice erythrocytes (Kapoor and Banyal, 2009).

Plasmodium possesses 2 functional redox systems involving the low molecular weight thiol glutathione (GSH) and thioredoxin (Trx) that are heavily involved in the redox regulation of the cell and contribute greatly to the antioxidant capacity of the cell (Prada *et al*., 1995).

Glutathione has been shown to be the main non-enzymatic antioxidant defense in *Plasmodium* (Atamna and Ginsburg, 1997)) and helps in maintaining the reduced environment of the cytosol. Glutathione reductase (GR; E.C. 1.8.1.7) is an ubiquitous flavoenzyme of disulphide reductase family catalyzing the nicotinamide adenine dinucleotide phosphate reduced (NADPH) dependent reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH), which permits GSH to function as intracellular reducing agent. Thioredoxin system composed of small peptide thioredoxin, thioredoxin reductase (TrxR; E.C. 1.8.1.9) and NADPH as reducing cofactor is involved in maintenance of crucial redox state. TrxR transfers electrons from NADPH to Trx, which itself acts as a reductant for disulphide containing process such as ribonucleotide reductase (Holmgren 1985).

The defense mechanism against malaria requires cooperation among antibodies and cellular immune responses (Bouharoun-Tayoun *et al*., 1995). Evidences support the role of antibodies in the reduction of parasitaemia in malaria patients by passive transfer of immunoglobulins (Plebanski and Hill, 2000). Present study evaluated the humoral immune response against *Plasmodium berghei* thioredoxin reductase and glutathione reductase and the protection induced against *P. berghei* infection

Materials and Methods

Parasite

P. berghei (NK-65) was maintained in 6-8 week old white Swiss mice (Balb/c) by inoculating intraperitoneally (i.p) 1×10^5 *P. berghei*-infected erythrocytes in citrate saline (2:1) from infected to naïve mice. Course of infection was monitored daily by preparing Giemsa stained thin blood smears (Kapoor *et al*., 2008). All experiments were carried out by procedures authorized by the Institutional Animals Ethics Committee (IAEC) of H. P. University, Shimla, vide letter number IAEC/Bio/19-2005.

Cell-free parasite

The citrated blood (2:1) from *P. berghei* infected mice was collected, passed through CF-11 cellulose (Whatman) and the eluate centrifuged at 1,000g for 10 min at 4˚C. Pellet was suspended in equal volume of saponin (0.2%w/v) in phosphate buffer saline (PBS, 0.01M), pH 7.2 and incubated for 30 min at 4˚C with intermittent mixing. The suspension was then centrifuged at 15,000g for 20 min at 4˚C. Haemolysate obtained was aspirated and erythrocyte membranes overlying cell-free

parasite were separated and cell-free *P. berghei* was washed thrice with chilled 0.01M PBS, pH 7.2.

Preparation of homogenate and purification of enzymes

Cell-free parasite was suspended in appropriate volume of 0.01M PBS, pH 7.2 and homogenized in Potter-Elvehjem homogenizer (REMI, Bombay) at 4°C centrifuged at 1,000g for 10 min at 4°C and the supernatant used as parasite homogenate (Kumar and Banyal, 1994). Protein was determined according to Lowry *et al* (1951). The cell-free parasite homogenate was subjected to precipitation with ammonium sulphate (between 0% to 100%). The precipitates of each cut were dissolved in minimum volume of 50mM potassium phosphate buffer pH 7.2 for GR and 10mM Tris-HCl buffer pH 7.5 for TrxR and dialyzed at 4°C using same buffer. The dialyzed samples were separately loaded onto Sephadex G-200 (Sigma) column and elutions in 1.0 ml volume collected at 4°C. Fractions containing enzyme activity were pooled and used as enzyme extract. TrxR activity was determined according to Holmgren and Bjorsnstedt (1995) and GR activity was determined according to modified method of Worthington and Rosemeyer (1974).

Immunization of mice

Two separate groups of 10 mice each were immunized intraperitoneally with 50µg of purified protein TrxR and GR respectively mixed with 30µg of saponin in PBS (0.01M, pH 7.2) as adjuvant. Control group of 5 mice received only the adjuvant in PBS. Two booster doses were given on day 14 and 28 post-immunization. Pre-immune sera were collected before immunization. Blood samples were also collected on day 14 and 28 post-immunization and sera were analysed by enzyme-linked immunosorbent assay (ELISA).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was carried out to evaluate the antimalarial antibody titre in the sera collected in 96-well plates according to Banyal and Inselburg (1985) using horseradish peroxidases goat antimouse immunoglobulin (Genei, Bangalore)]. Reference malaria positive and negative sera were used with each plate.

Immunofluorescence assay (IFA)

IFA was performed according to Collins and Skinner (1972) using fluorescein isothiocyanate conjugated goat anti-mouse IgG (FITC, Genei). The slides were examined under UV light and phase contrast using fluorescence microscope (Leica, Germany).
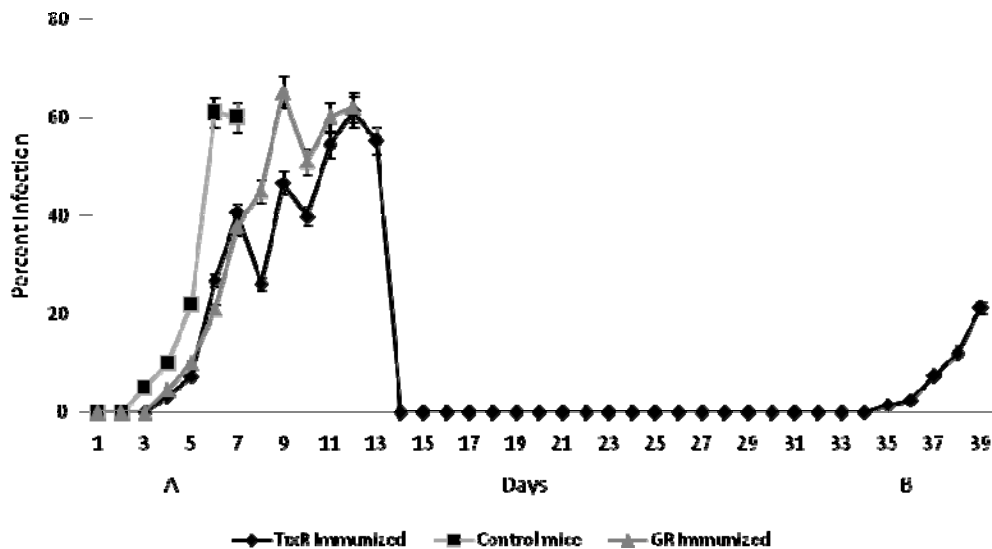
Challenge of immunized mice

The protective efficacy of TrxR and GR was studied by parasite challenge to TrxR and GR immunized mice with 1×10^5 *P. berghei*-infected erythrocytes on day 35-post immunization. Along with experimentals the control mice were also challenged with 1×10^5 *P. berghei*-infected erythrocytes. Course of parasitaemia was monitored by daily smear examination.

Results

Thioredoxin reductase and glutathione reductase were purified form *P. berghei* and SDS-PAGE analysis of *P. berghei* TrxR and GR resulted in single band of 22 kDa and 25 kDa respectively.

Sera of mice immunized with purified *P. berghei* TrxR and GR showed presence of antibodies as determined by ELISA. Pooled pre-challenge sera of mice immunized with purified parasite TrxR gave antibody titre of 1:512 on day 35 also antibody titre of 1:512 was recorded the same day before challenging the mice in GR immunized mice. No antibody titre was observed in the sera of the control mice. Post challenge serum of TrxR immunized mouse gave a high antibody titre of 1:1024. Reference malaria positive and negative serum used with experimental sera gave positive and no reaction respectively.

Ten mice immunized with 50 µg of purified TrxR and 30 µg of saponin in PBS as adjuvant upon challenge with 1×10^5 *P. berghei*-infected erythrocytes on day 35 post-immunization showed parasites in their blood by day 4-post challenge. Three of the mice died by day 8 post challenge with maximum parasitaemia of 70%. Five mice showed migration of the parasite into reticulocytes of which 2 mice died by day 12 with 60% and 55.2% infection respectively. The other three mice died on day 14 with a maximum parasitaemia of 52.5%. Only two mice survived the parasite challenge. In these mice maximum parasitaemia observed was 3% and 7% respectively and by day 8 the mice were cleared off the parasite. The immunized mice surviving challenge were re-challenged on day 67 post-immunization (32 days after first challenge). Upon re-challenge the mice died with parasite showing a patent period of 5 days (Fig. 1)


Figure 1: Course of parasitaemia in control mice and mice immunized with purified TrxR, GR and challenged, (A) with 1×10^5 *P. berghei*-infected erythrocytes and subsequently re-challenged (B) with *P. berghei* infected erythrocytes on day 32. Bar represents data of ten mice with SD.

Ten mice were immunized with 50 µg of purified *P. berghei* GR and 30 µg of saponin. Upon challenge with live parasite none of the mice survived the challenge. Purified GR immunized mice showed parasite in their blood by day 3 post challenge. Five mice died on day 9 with 69% infection and of the remaining 5 mice, 3 died on day 10 with 51% parasitaemia and 2 died on day 12 with 62% parasitaemia respectively (Fig. 1).

The placebo control mice (5) received only saponin in PBS, pH 7.2 during immunization. All mice upon challenge with parasite showed patency two days after post challenge. Three of the mice died by day 5 post challenge and showed a typical *P. berghei* infection pattern. The remaining 2 mice showed migration of the parasite to reticulocytes and hence, their patent period increased to 7 days after which these animals also died (Fig. 1).

The specificity of the antigen-antibody reaction was analysed by IFA. Negative control using normal mouse serum did not show any fluorescence and only red counter stained reaction was seen under UV light whereas, reference positive serum showed bright fluorescence on whole of the parasite. Purified protein immunized mouse sera showed specificity of parasite for antibody raised as fluorescing structure corresponded to parasite when seen under phase contrast microscope (Fig. 2).

A   B
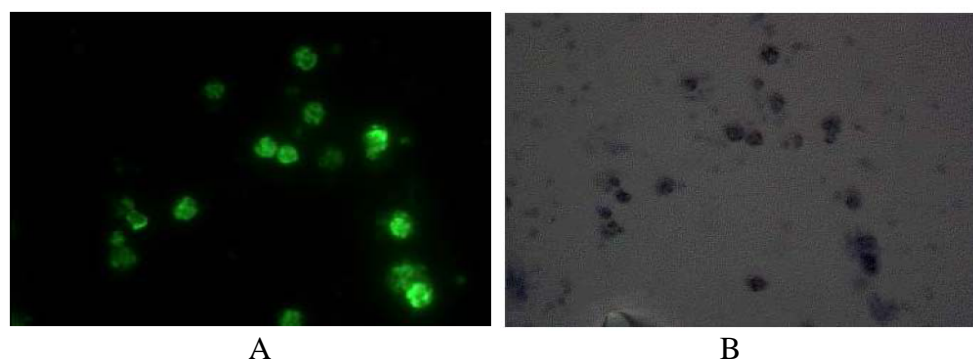
Figure 2: IFA reaction using serum of mice immunized with purified thioredoxin reductase. A. As seen under UV light (x1000) B. As seen under phase contrast (x1000).

Discussion

Immunity to malaria is complex and still incompletely understood. Present study was carried to evaluate the humoral immune response directed against *P. berghei* thioredoxin reductase and glutathione reductase using saponin as an adjuvant. Adjuvants play a major role in eliciting protective response in mice and the choice of adjuvant is important in potentiating the immune response besides determining which effector arm of immune system is stimulated. Many adjuvants like Freunds complete (FCA) and incomplete (FIA) adjuvants have been used in animals but they produce severe side effects and hence are not suitable for human use. Aluminium salts used in various vaccines are generally regarded as safe, however recent study revealed that

aluminium adjuvants at levels comparable to those administered to Gulf war veterans can cause motor neuron death (Petrik *et al*., 2007). Saponin used as adjuvant exhibited significant humoral response and partially protected TrxR immunized animals when challenged with live parasites.

P. berghei TrxR immunized mice exhibited partial protection as compared to GR immunized mice when challenged with 1 x 10^5 *P. berghei* infected erythrocytes. The sera obtained from immunized mice showed antibody titre of 1:512 in pre-challenge sera, which increased (1:1024) in TrxR immunized animals that survived the challenge. The increase in antibody level may be due to the activation of memory cells, which proliferate and release antiparasitic antibodies in the blood. The placebo control sera did not show any demonstrable antiparasitic antibodies. A delay in the onset of infection was observed in TrxR and GR immunized mice than the control mice and immunized mice died much later than the control mice. Perhaps the amount of antibody in GR immunized mice was not sufficient to destroy the parasite and parasitized erythrocytes *in vivo* following a challenge infection with *P. berghei*.

Other enzymes and cysteine proteases like falcipain-1 and aspartic proteases (plasmepsins) of malaria parasite have also been found to protect against infection (Kumar *et al*., 2007) suggesting that intracellular enzymes of *Plasmodium* may act as targets for antimalarial, chemotherapeutic or immunogenic. The work reported here suggests that TrxR is more immunogenic than GR and generates a humoral immune response. It would be of interest to investigate further the role of TrxR against multiple vaccine candidate antigens. Also the finding can contribute to a better understanding of the role of TrxR in immunity to malaria and may provide new insight into the development of malaria control strategies especially, vaccine.

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